

Cardiac ryanodine receptor phosphorylation by CaM Kinase II: keeping the balance right

Susan Currie

Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, Scotland

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1. ABSTRACT

Phosphorylation of the cardiac ryanodine receptor (RyR₂) is a key mechanism regulating sarcoplasmic reticulum (SR) Ca²⁺ release. Differences in opinion have arisen over the importance assigned to specific phosphorylation sites on RyR₂, over the kinase (s) suggested to directly phosphorylate RyR₂ and surrounding the possibility that altered phosphorylation of RyR₂ is associated with contractile dysfunction observed in heart failure. Ca²⁺/calmodulin dependent protein kinase II (CaMKII) can phosphorylate RyR₂ and modulate its activity. This phosphorylation positively modulates cardiac inotropic function but in extreme situations such as heart failure, elevated CaMKII activity can adversely increase Ca²⁺ release from the SR and lead to arrhythmogenesis. Although other kinases can phosphorylate RyR₂, most notably cAMP-dependent protein kinase (PKA), evidence for a key role of CaMKII in mediating RyR₂-dependent Ca²⁺ release is emerging. Future challenges include (i) fully identifying mechanisms of CaMKII interaction with the RyR₂ complex and (ii) given the ubiquitous expression of CaMKII, developing *selective* strategies to modulate RyR₂-targeted CaMKII activity and allow improved understanding of its role in normal and diseased heart.

2. INTRODUCTION

Sarcoplasmic reticulum (SR) Ca²⁺ release plays an essential role in mediating cardiac myocyte contraction. Release from the SR Ca²⁺ store occurs in response to plasma membrane depolarization and influx of extracellular Ca²⁺ via voltage-gated Ca²⁺ channels. This Ca²⁺-induced Ca²⁺ release (CICR) from the SR is mediated by activation and opening of ryanodine receptors (RyRs) located in the SR membrane that are functionally coupled to the channels in the plasma membrane (1). The resulting increase in cytosolic (Ca²⁺) is accompanied by myofilament activation and contraction during systole. For the cell to relax during diastole, cytosolic (Ca²⁺) must be lowered. This is accomplished (i) by inactivation of RyR₂ and (ii) by mechanisms that remove Ca²⁺ from the cytosol. These mechanisms include re-uptake of Ca²⁺ into the SR via the sarco-endoplasmic reticulum Ca²⁺ATPase (SERCA) and removal of Ca²⁺ from the cell via the Na⁺/Ca²⁺ exchanger (NCX) (2). The contribution of each of these removal processes varies between species with SERCA contributing ~70% to removal of cytosolic Ca²⁺ in human and rabbit and ~90% in rat and mouse. This is a highly simplistic view of the excitation-contraction coupling (ECC) process which has been reviewed in detail elsewhere (3). The key point to

note is that in order for the heart to function normally, a balance must exist between the mechanisms that lead to increased cytosolic (Ca^{2+}) during systole and those that remove it, leading to a decrease in cytosolic (Ca^{2+}) during diastole. This balance is subject to tight modulation by an array of physical and chemical mediators that function both locally, at the level of the individual Ca^{2+} handling proteins themselves and globally, at the level of the whole cell.

RyR₂, primarily responsible for the intracellular Ca^{2+} release that triggers cell contraction, is a transmembrane protein of 565kDa that exists as a homotetramer of ~2.2MDa and forms a functional Ca^{2+} release channel (1). Each RyR₂ monomer has a large N-terminal domain that protrudes into the cytoplasm. Only a small percentage (~10%) of the protein spans the SR membrane and is in contact with the SR lumen. The large cytosolic portion of the channel provides immense scope for interaction and regulation processes to occur and it is now recognized that RyR₂ exists as a large multi-protein complex. RyR₂ molecules can associate with other RyR₂s via an interaction site found in the cytosolic domain. This results in formation of 2D arrays and increases the sensitivity to ligand activation (4). In addition to self-interaction, evidence from various laboratories suggests physical interactions exist between RyR₂ and small molecules as well as proteins such as enzymes, scaffolding proteins and Ca^{2+} binding proteins (5-8). Most of these interactions occur with the large N-terminal cytoplasmic face of RyR₂ and serve a critical role in modulation of channel open probability and activation (9). FKBP12.6 (Calstabin), PKA (protein kinase A), CaMKII (Ca^{2+} /calmodulin-dependent protein kinase II), phosphatases (PPI and PP2A), calmodulin, sorcin and homer have all been shown to interact with and modulate RyR₂ activity through stabilization and/or modulation of phosphorylation (5,7,9-12). Recent work suggests that additional interactions, occurring at the SR luminal face of RyR₂ with the Ca^{2+} binding protein calsequestrin (CSQ) and the junctional SR transmembrane proteins, triadin and junctin, also appear to serve a role in regulation of SR Ca^{2+} release (13). What is apparent is that disruption of the protein-protein interactions that form part of this multi-protein complex either at the cytoplasmic or luminal face, leads to abnormal Ca^{2+} release and a predisposition to diastolic Ca^{2+} leak and subsequent arrhythmia (5,13-15). This has revealed a key mechanistic defect in excitation-contraction coupling (ECC) that can act as a trigger for ventricular arrhythmia and hence is an area ripe for the development of targeted anti-arrhythmic therapies.

Phosphorylation of the RyR₂ complex has been identified as a key mechanism that can alter RyR₂ activity and diastolic SR Ca^{2+} release (15,16). Since the publication of a study in 2000 suggesting that hyperphosphorylation of RyR₂ at Ser 2808 by PKA in both failing human and dog hearts lead to dysregulation of channel function (5), there has been a surge of interest in this area of research. Since 2000, numerous studies have attempted to reproduce and build upon the

initial study (17-24). Notably, there is also considerable evidence to refute the original work. Arguments against the PKA hyperphosphorylation hypothesis are based on various observations. These include the demonstration that PKA phosphorylation of RyR₂ does not affect SR Ca^{2+} release (measured as Ca^{2+} sparks) (25) and that it does not cause dissociation of the stabilizing protein FKBP12.6 (26), the major paradigm of the 2000 study. Experiments in dog, rat and mouse suggest hyperphosphorylation of RyR₂ at Ser 2808 does *not* exist and is *not* a marker for heart failure (27-30). It appears now that Ser 2808 may be a pan kinase substrate which ties in with early work on RyR₂ phosphorylation mentioned in sections 3.1 and 3.2. Interestingly, a separate site for PKA phosphorylation of RyR₂ has recently been identified at Ser 2030 but there is currently no evidence for hyperphosphorylation at this site (28,30). Finally, and of more relevance for this review, evidence is mounting that phosphorylation-mediated alterations in RyR₂ activity and SR Ca^{2+} leak in both normal and failing hearts, may be largely due to RyR₂ phosphorylation by Ca^{2+} /calmodulin dependent protein kinase II (CaMKII) (31-33). This, combined with the controversy surrounding the role of PKA-mediated phosphorylation of RyR₂, particularly in modulation of diastolic Ca^{2+} leak in heart failure, highlights the need for a detailed understanding of exactly what CaMKII does to RyR₂ activity. Is modulation of this interaction something that will be important therapeutically?

CaMKII is an ubiquitously expressed enzyme and is encoded by four genes in mammals (α , β , γ , and δ) (34). In heart CaMKII δ is the predominant isoform and expression is further specialized by the existence of selectively expressed splice variants of the δ isoform (δ_B and δ_C) that exist in the nucleus and cytosol of cardiac myocytes respectively (35). CaMKII activation is highly sensitive to changes in cellular (Ca^{2+}) and, as such, it makes an ideal candidate for 'sensing' and reacting to localized fluxes of Ca^{2+} in cardiac myocytes. This sensitivity can be further increased by autophosphorylation of the kinase at Thr287 (36) and so CaMKII has been referred to as a 'molecular switch', capable of rapidly responding to changes in (Ca^{2+})_i within microenvironments of the cell and coordinating localized phosphorylation of Ca^{2+} handling proteins. Importantly, CaMKII expression and activity have been shown to be increased in various animal models of cardiac hypertrophy and heart failure as well as in human heart failure (37). Given the large number of candidate substrates for CaMKII in cardiac myocytes (and these are only what have been identified to date), the implications of this increased activity are far-reaching and the impact on cardiac Ca^{2+} handling is huge. The possibility that CaMKII acts as a key mediator of ECC and excitation-transcription coupling (ETC) in normal and diseased heart has been reviewed elsewhere (38). This review will focus specifically on CaMKII-mediated phosphorylation of RyR₂, examining the mode of interaction and the functional consequences in normal and diseased heart.

3. CAMKII-MEDIATED DIRECT PHOSPHORYLATION OF RYR₂

3.1. Early evidence for an interaction

In 1984 a study was published which provided the first evidence for CaMKII-mediated phosphorylation of a high molecular weight protein (>200kDa) in cardiac junctional SR vesicles (39). These vesicles were ryanodine-sensitive and the authors stated that work was in progress to clarify the role of these high molecular weight proteins in SR Ca²⁺ uptake and release. By the late 1980's these high molecular weight proteins had been identified as ryanodine receptors, so called because of the tight binding of the alkaloid ryanodine to them (40,41). The group who originally published the work suggesting the existence of CaMKII-mediated phosphorylation of these proteins, confirmed in 1989 that the ryanodine receptor, the SR Ca²⁺ release channel and the high molecular weight proteins were identical (42). Around the same time a study was published suggesting that CaMKII could directly phosphorylate RyR in skeletal muscle (43). Evidence for PKA activity associated with junctional SR was presented but it was noted that the intrinsic activity was much lower than that of CaMKII. The authors noted selective association of CaMKII with junctional SR and 'close proximity' to RyR. They concluded that this phosphorylation system may be important in the modulation of Ca²⁺ release. These results were strengthened when a separate group showed that addition of calmodulin to isolated bovine SR was sufficient to phosphorylate RyR₂ via an endogenous CaMKII (44). Very little phosphorylation was achieved with addition of cAMP or cGMP. A close physical association of CaMKII and RyR₂ was observed since CaMKII activity persisted in purified RyR₂ preparations even after treatment with high salt and detergent. The possibility of a physical interaction between the two proteins was suggested. To date there is no evidence that an anchoring protein (similar to A-kinase anchoring protein (AKAP)) is involved in this interaction. The anchoring protein α -KAP has been demonstrated to localize CaMKII to SERCA 2 and RyR in skeletal muscle (45) but this has yet to be identified in cardiac muscle.

3.2. Phosphorylation at Serine 2808

Molecular cloning of cDNA encoding RyR₂ of rabbit cardiac SR revealed a great deal about potential modulatory binding sites. An ATP binding domain was identified at residues 2619-2652 and the first documentation of a potential phosphorylation site at residue 2809 (this equates to Ser 2808 in human). The authors of this study also identified potential calmodulin binding sites at residues 2775-2807, 2877-2898 and 2998-3016. They therefore suggested that the modulatory binding domain of RyR₂ resided between residues 2619 and 3016 (46). Parallel studies by another group performed on isolated junctional SR vesicles or partially purified RyRs fused with planar bilayers confirmed the proposal that Ser 2809 was a phosphorylation site for CaMKII on the receptor (47). Using exogenously applied CaMKII to the latter of the two preparations, channel activity was increased markedly when compared to activity measured in the presence of ATP and calmodulin but in the absence of CaMKII. It was

suggested that CaMKII-mediated phosphorylation at this single site was sufficient to alter RyR₂ function. However, there were issues with this study that could not be addressed. The artificial nature of the preparations used meant that the contribution of endogenous CaMKII was not studied. The authors were only able to measure increased activity when CaMKII was applied exogenously. They could therefore speculate little about the intrinsic relationship between the kinase and the receptor. They acknowledged that there were 16 putative phosphorylation sites for CaMKII throughout RyR₂ and admitted that it did seem remarkable that only one site (Ser 2809) was phosphorylated upon interaction with CaMKII. Although limited in the technical approach, this study was important in attributing a functional role to CaMKII-mediated effects on SR Ca²⁺ release via a direct effect on Ser 2809 of RyR₂.

Following this study further work confirmed Ser 2809 as a site for CaMKII-mediated phosphorylation (48,49) but again highlighted the potential that other sites of phosphorylation may be involved. It was also suggested that endogenous CaMKII could only phosphorylate one Ser 2809 in the homotetramer of RyR₂ which lead to closure of channels whereas exogenously applied CaMKII was capable of phosphorylating all four and causing activation. These discrepancies in function could have been due to endogenous enzyme being partially stripped during preparation of the vesicles used in this study, however this was not confirmed (49). Again, the limitations of the technical approaches may have lead to some misinterpretations about the role of exogenous and endogenous enzyme as well as adding to the building controversy of whether CaMKII phosphorylation activated or inhibited RyR₂. A separate study using swine SR vesicles and planar bilayer reconstitution experiments suggested that CaMKII decreased both (³H) ryanodine binding and the open probability (P_o) of RyR₂ (50) and that this could be reversed by treatment with exogenous acid phosphatase. The CaMKII-mediated inhibition was Ca²⁺-dependent, suggesting the CaMKII-mediated effect may be to modulate RyR₂ opening and closure in response to changes in local (Ca²⁺). This study really highlighted the potential for phosphorylation and dephosphorylation in the dynamic regulation of SR Ca²⁺ release but added to the speculation about whether CaMKII activated or inhibited RyR₂. Importantly, this was one of the first studies to speculate that CaMKII modulation of RyR₂ activity might not necessarily be via direct phosphorylation of the release channel itself. The suggestion was made that the phosphorylation-dephosphorylation effect might be directed at a protein or proteins closely associated with RyR₂. Given the evidence that was to be presented in future years supporting the existence of an RyR₂ multi-protein complex, this suggestion makes complete sense, although at the time there was no concrete evidence to support this theory. The authors reconciled differences in the ultimate effects of CaMKII between this study and previous work through differences in the ionic conditions of recording solutions used for single channel opening measurements as well as differences in species used for preparations (swine versus canine). Upon reviewing these studies the possibility is highlighted that, depending on experimental/

environmental conditions, CaMKII could potentially either activate or inhibit RyR₂. Differences in experimental approach could serve to demonstrate that one discrete functional effect of CaMKII on RyR₂ may not exist.

A pivotal point in the Ser 2809 story came with publication of a study in 2000 which proposed a number of novel features surrounding RyR₂ phosphorylation at Ser 2808 (human)/2809 (canine) (5). PKA phosphorylation to stoichiometry at Ser 2809 was suggested. Previously only minimal PKA phosphorylation at this site had been observed (47). RyR₂ was shown to exist as a macromolecular complex consisting of the Ca²⁺ release channel with various 'regulatory' proteins physically associated. These included PKA, FKBP12.6 (a channel stabilizing protein), PP1 and PP2A (phosphatases) and mAKAP (an anchoring protein for PKA). Interestingly, the presence of CaMKII as part of this complex was not investigated in this study. In cardiac homogenates prepared from failing human hearts and from dog hearts with pacing-induced failure, RyR₂ was shown to be hyperphosphorylated at Ser 2808/2809 and this was shown to be due to PKA. Using co-immunoprecipitation to demonstrate interaction of RyR₂ and FKBP 12.6, it was shown that phosphorylation of immunoprecipitated protein with PKA caused the RyR₂-FKBP12.6 interaction to be abolished. This was not observed after phosphorylation with either CaMKII or PKC, however whether optimal amounts of either of these kinases were used, and whether sufficient time for optimal phosphorylation was allowed, was not clarified. The authors suggested that PKA hyperphosphorylation lead to defective channel functioning through loss of the stabilizing influence of FKBP12.6 leading to increased Ca²⁺ sensitivity for activation and increased P_o, combined with impaired RyR-RyR interaction and co-operativity. The possible effects or role of CaMKII was largely ignored in this study which was surprising, given the body of evidence that had been produced by that point suggesting CaMKII modulation of RyR₂ activity was mediated via Ser 2809.

The production of phosphorylation site-specific antibodies directed against Ser 2809 (phosphorylated and dephosphorylated) allowed a detailed study of phosphorylation at this site by both PKA and CaMKII (51). For the first time, a good comparison was made, at least for the scope that each kinase had for RyR₂ phosphorylation at this site. This work established that both CaMKII and PKA could phosphorylate Ser 2809 to full stoichiometry. It also showed that CaMKII could phosphorylate RyR₂ to a far greater extent (five times more) than PKA. This meant CaMKII could potentially achieve stoichiometric phosphorylation of at least four additional sites to Ser 2809. However, since the amount of RyR₂ was not quantified in this study, it should be recognized that the number of sites could be subject to over-estimation. That said, the capacity for phosphorylation of RyR₂ by CaMKII (not just at Ser 2809) as this work showed, raised questions about the 2000 study. If Ser 2809 was hyperphosphorylated in heart failure and both PKA and CaMKII were equally capable of contributing to this phosphorylation and were both activated in conditions of elevated β adrenergic stimulation

(such as heart failure), why (as the 2000 study suggested) should PKA be solely responsible for mediating this effect?

Further evidence was provided to support the PKA hyperphosphorylation at Ser 2809 theory (17). The development of mice where Ser 2808 could not be phosphorylated was used to explore the role of this site in mediating cardiac function. When these mice were exposed to myocardial infarction, they showed better cardiac function than wild type mice (18) suggesting potential importance of this site in mediating progressive cardiac dysfunction. However, subsequent work by another group using genetic ablation of Ser 2808 in mice did not support these conclusions, suggesting that in genetically altered mice only modest effects were observed on channel activity with cell function almost unaltered (29). Importantly, there was little protection during chronic stress and diastolic Ca²⁺ responses were unaffected. This would not have been expected had this site played a key role in defective SR Ca²⁺ leak in heart failure. Results from the latter study were strengthened by further *in vivo* and isolated heart work using knock in mice where Ser 2809 was replaced with alanine and could not be phosphorylated. This work demonstrated that isoproterenol-mediated effects in genetically modified mice and wild type mice were no different (27). Recognising that genetic modification of Ser 2809 may have effects other than those specific to RyR₂, this group examined key factors controlling ECC such as the expression levels of other SR/Sarcolemmal Ca²⁺ handling proteins, characteristics of L-type Ca²⁺ current, SR Ca²⁺ release and ECC gain. None of these parameters were significantly different between knock-in and wild type mice. Not only did these results question the original hypothesis from the 2000 paper, suggesting that hyperphosphorylation of this site explained dysfunctional responses to increased β -adrenergic drive, they also questioned the relative importance of this phosphorylation site to RyR₂ function. It remains to be explained how these two research groups have come to such different conclusions regarding the role of Ser 2808. However, the possibility must be considered that inconsistencies between experimental models of cardiac dysfunction in the two groups may exist, leading to different interpretations at least for the role of this site in cardiac dysfunction. The interesting point for this review is that regardless of CaMKII- or PKA-mediated phosphorylation, if ablation of this site presents no real problems to normal cardiac function (as suggested by the latter study), yet evidence suggests the existence of phosphorylation-driven functional effects on RyR₂, the probability exists that other phosphorylation sites on the receptor may be more functionally relevant.

3.3. Phosphorylation at Ser 2814

To specifically investigate the role of CaMKII in cardiac myocyte Ca²⁺ handling, transgenic mice overexpressing CaMKII δ_C were studied. These mice developed dilated cardiac myopathy (52) and there were effects on ECC that suggested acute activation of RyR₂. SR Ca²⁺ content was lower in transgenic mice and diastolic Ca²⁺ spark frequency and twitch fractional release were increased with prolongation of spark duration (53). There

was a ~4-fold increase in SR Ca^{2+} leak and the authors hypothesized that this was modulated by CaMKII-mediated phosphorylation of RyR₂ before the onset of heart failure. Although the 2000 study had suggested a role for PKA and phosphatases in increased RyR₂ phosphorylation and activation in heart failure (5), these mice did not show any changes in either PKA or phosphatases associated with RyR₂. The key mediator here was CaMKII. The possibility that the effects of CaMKII on RyR₂ were direct was suggested by the fact that KN-93 (a CaMKII inhibitor) inhibited the enhancement in spark duration and frequency. This evidence highlighted the concept that CaMKII-mediated phosphorylation of RyR₂ caused significant effects on channel function, yet the key site (s) of phosphorylation were still not clarified.

An important step forward came with the publication of work in 2004 where site-directed mutagenesis of full length recombinant RyR₂ identified a CaMKII phosphorylation site at Ser 2815 (Ser 2814 in human) separate from the previously identified 2809 (54). This study suggested that mutation of Ser 2809 only prevented PKA phosphorylation of RyR₂ whereas mutation of Ser 2815 only prevented phosphorylation by CaMKII, and strengthened the idea of separate phosphorylation sites on RyR₂ for each kinase. Phosphorylation site specific antibodies were also used against both sites to show that both serines were independently phosphorylated by PKA and CaMKII. Since Ser 2815 is only six amino acids away from the originally identified site (Ser 2809), the authors noted the possibility that mistakes could have been made in previous studies due to the use of insufficiently sensitive techniques. This could have led to the observation that PKA and CaMKII phosphorylated the same site (Ser 2809) (40,41) however this theory remains unconfirmed. The 2004 study suggested that CaMKII was also part of the RyR₂ multi-protein complex confirming proposals from other studies (39,44,49). This result was particularly interesting since the same group had largely ignored the possible contribution of CaMKII to RyR₂ activity in earlier work (5). The publication of the 2004 study, particularly by this group, showed recognition that RyR₂ phosphorylation was more complex than had initially been suggested (5). This work proposed that CaMKII phosphorylation at Ser 2815 did not lead to dissociation of FKBP12.6 and incorporated 1.5 moles of phosphate per mole of RyR₂ monomer. Previous studies had suggested CaMKII phosphorylation stoichiometry of anything from 0.2 to 4.0 moles of phosphate per mole of RyR monomer (44,51) however differences in the timing of phosphorylation as well as the modes of assessment could have accounted for these differences. Most of the studies to date, including this one, support the idea that there is at least one other CaMKII site (aside from Ser 2815) that can be phosphorylated in native RyR₂. Again, this has yet to be confirmed.

What does phosphorylation at 2815 do and under what circumstances is it evident? The 2004 study suggested that this was a phosphorylation mechanism that worked in parallel with PKA mediated phosphorylation of 2809. Using channels isolated from flash frozen paced and non-paced hearts, this study suggested that CaMKII mediated

phosphorylation of RyR₂ promoted increased Ca^{2+} sensitivity of the channel at increased heart rates. This was not seen in failing hearts and the authors suggested this could lead to the loss of the force-frequency relationship in heart failure (55). It was proposed that this CaMKII-mediated phosphorylation accompanied phosphorylation mediated by PKA at Ser 2809 and that it could possibly act to prevent excessive dissociation of FKBP12.6 from the channel (caused by PKA-mediated phosphorylation), preventing the phenomenon of uncoupled gating. This study was carefully performed and timed to add to the growing speculation that PKA was not solely responsible for phosphorylation-mediated alterations in RyR₂ activity. It introduced the concept that CaMKII could mediate the force-frequency relationship via phosphorylation-mediated effects on RyR₂. The emphasis on PKA modulation of RyR₂ channel activity was now changing to encompass CaMKII.

Subsequent studies examining Ser 2815 in arrhythmogenic rabbit hearts (31) and in isolated rabbit cardiac myocytes where CaMKII had been overexpressed using adenovirus (56) showed that under both situations, chronic and acute, Ser 2815 phosphorylation was increased. This correlated with increased association of autophosphorylated (active) CaMKII with the channel complex in the chronic situation (31) and increased SR Ca^{2+} leak and decreased SR Ca^{2+} load in both chronic and acute situations. Importantly, in the chronic situation, SR Ca^{2+} leak was reduced by CaMKII inhibition but not by PKA inhibition suggesting CaMKII-dependent phosphorylation may be more important in contributing to increased leak and potentially increased arrhythmogenesis and contractile dysfunction (this contradicts the previous proposals that PKA dependent phosphorylation was most important (5) or equally important (54) in contributing to defective RyR₂ activity). Interestingly, in the acute scenario, where increased CaMKII expression also correlated with increased SR leak and decreased load, there was no evidence for contractile dysfunction. This is suggestive of a possible biphasic effect of CaMKII *in situations* where kinase activity is elevated. Acute elevation, such as in early hypertrophic responses or short-term responses to β -adrenergic stimulation may have beneficial physiological effects, enhancing cardiac Ca^{2+} cycling to meet the demands of the acute stimulus. In chronic conditions where prolonged kinase elevation is observed, numerous effects on Ca^{2+} handling are more likely. Prolonged RyR₂ sensitisation is likely to also be accompanied by SERCA stimulation and increased SR Ca^{2+} content. Consistently increased diastolic leak would be more likely to deplete Ca^{2+} stores and lead to defective contractile activity.

Increasing evidence has been presented for CaMKII dependent increased RyR₂ activity during β -adrenergic stimulation (27, 51). Dose-dependent effects of isoproterenol leading to increased Ser 2815 phosphorylation were demonstrated in a recent study (57) which contradicted previous work suggesting this was not seen (54). Although earlier work had shown that isoproterenol increased SR Ca^{2+} release (58,59), there was

a lack of discrimination between whether the effect was due to PKA or CaMKII-dependent phosphorylation of RyR₂. Increasing evidence suggests that CaMKII activation is observed and this may be dependent (57) or independent (33) of preceding PKA activation. Further work is necessary to fully understand the pathways involved in eliciting the positive inotropic effect of β -agonists. The possibility that increased systolic Ser 2815 phosphorylation occurs, leading to increased systolic SR Ca²⁺ release highlights a positive action for CaMKII in cardiac contractile activity over the detrimental effects exerted on increased diastolic leak in conditions of cardiac dysfunction. This again emphasizes the possibility of CaMKII acting on RyR₂ in a somewhat heterogeneous manner. In acute stimulatory situations, acting to increase systolic SR Ca²⁺ release to meet the demands of the inotropic stimulus and in long-term chronic situations, acting to deplete the SR of Ca²⁺ during diastole leading to arrhythmogenesis. Under both situations the contributions of the Ca²⁺ signal entering the cell via the L-type Ca²⁺ channel as well as the SR Ca²⁺ load are important and will influence release. Importantly, both of these are also regulated by CaMKII (60) therefore an understanding of how CaMKII is targeted to its substrates, particularly during β -adrenergic stimulation, is vital.

The targeting of CaMKII in cardiac myocytes and regulation of SR Ca²⁺ release is further complicated by the fact that different splice variants of CaMKII δ exist in the heart (35). Until recently it was not known whether there was preferential targeting and, as a result, distinct functional roles for each splice variant. Transgenic mice expressing either the cytoplasmic CaMKII δ_C or the nuclear CaMKII δ_B were used to investigate the relative involvement of each splice variant on cardiac myocyte function (61). Specifically, phosphorylation and regulation of SR Ca²⁺ handling proteins and nuclear HDAC phosphorylation and regulation were monitored. Only transgenic mice expressing the δ_C variant showed increased levels of Ser 2815 phosphorylation. This was not observed in transgenic mice expressing the nuclear targeted CaMKII δ_B and was in line with the cytoplasmic variant of CaMKII being responsible for phosphorylation-mediated effects on RyR₂. This has highlighted the fact that nuclear and cytoplasmic CaMKII variants have distinct roles on Ca²⁺ handling and gene expression. Recent work suggesting that RyR₂ is expressed in the nucleus (62) raises the question of whether nuclear CaMKII may be involved in the regulation of these receptors. This has not been investigated. Indeed little is known about the roles of these receptors that exist in the interior of the cell away from L-type Ca²⁺ channels (63). It will be of interest in future work to establish whether these receptors are subject to similar modulation by CaMKII δ_C or whether the nuclear variant may play a role. It has previously been shown that the cardiac nuclear IP₃R is subject to modulation by CaMKII and it was shown using co-immunoprecipitation experiments that it physically interacts with CaMKII δ_B (64).

As well as differential targeting of CaMKII in the heart, there is now evidence for alternative (multiple)

pathways of activation of the kinase. In addition to the classical Ca²⁺/calmodulin mode of activation, a novel cAMP-dependent route (independent of PKA activation) has been proposed. This is particularly interesting for the current review since this pathway of activation has been linked to Ser 2815 phosphorylation. This highly novel route for Ser 2815 phosphorylation has been proposed whereby the cAMP binding protein Epac modulates RyR₂ activity in a PKA-independent manner and via CaMKII (65). The selective Epac activator 8-CPT, caused increased Ca²⁺ spark frequency which was preserved in the presence of PKA inhibition but inhibited in the presence of the CaMKII inhibitor KN-93. The authors suggested that this novel pathway of CaMKII activation could serve as a link for PKA-independent CaMKII-mediated phosphorylation of RyR₂ during β -adrenergic stimulation described previously (33). One problem with this interpretation that has been highlighted is that 8-CPT is highly selective for Epac and will differ from physiological β -receptor activation (65). However, the study does identify another mode of activation of CaMKII and the novel feature of PKA-independent cAMP signaling which could have far-reaching consequences.

To date, evidence that Ser 2815 is a phosphorylation site exclusive to CaMKII is strong. Evidence for CaMKII-mediated phosphorylation of Ser 2809 is less clear and definitely less exclusive, with evidence to suggest that both CaMKII and PKA can result in phosphorylation. The relative role of either of these sites in altering RyR₂ Ca²⁺ sensitivity and SR Ca²⁺ leak in normal and diseased hearts has yet to be fully established. Future work in this area will be particularly important in the quest to design targeted therapeutic intervention strategies aimed at modulating this interaction, as discussed in section 7.

3.4. Other potential CaMKII sites on RyR₂

Early studies examining the possibility that CaMKII could phosphorylate RyR₂ were suggestive of numerous possible phosphorylation sites (up to 16) on the channel (46-48). More recently it has been suggested that in addition to Ser 2809 and 2815 there may be at least a further two additional sites that can be phosphorylated by CaMKII (50,53). This was predicted after stoichiometric phosphorylation by exogenous CaMKII. Greater phosphorylation of the receptor by CaMKII than with PKA was consistently observed and it seems likely that other CaMKII phosphorylation sites on RyR₂ do exist and have yet to be identified. Potential phosphorylation sites are outlined in Figure 1.

The possibility that only one phosphorylation site (i.e. Ser 2815) or even two (Ser 2809/Ser 2815) mediate the entire channel response to CaMKII appears highly unlikely. This is said for two reasons, (i) the fact that so many other potential CaMKII phosphorylation sites exist on the receptor and (ii) the fact that so many other potential CaMKII substrates exist that are part of the greater RyR₂ multi-protein complex. Taking previous observations about RyR₂-specific CaMKII phosphorylation into account, one recent study made the astounding observation that $\sim 10^{10}$ patterns of CaMKII phosphorylation could be possible for a

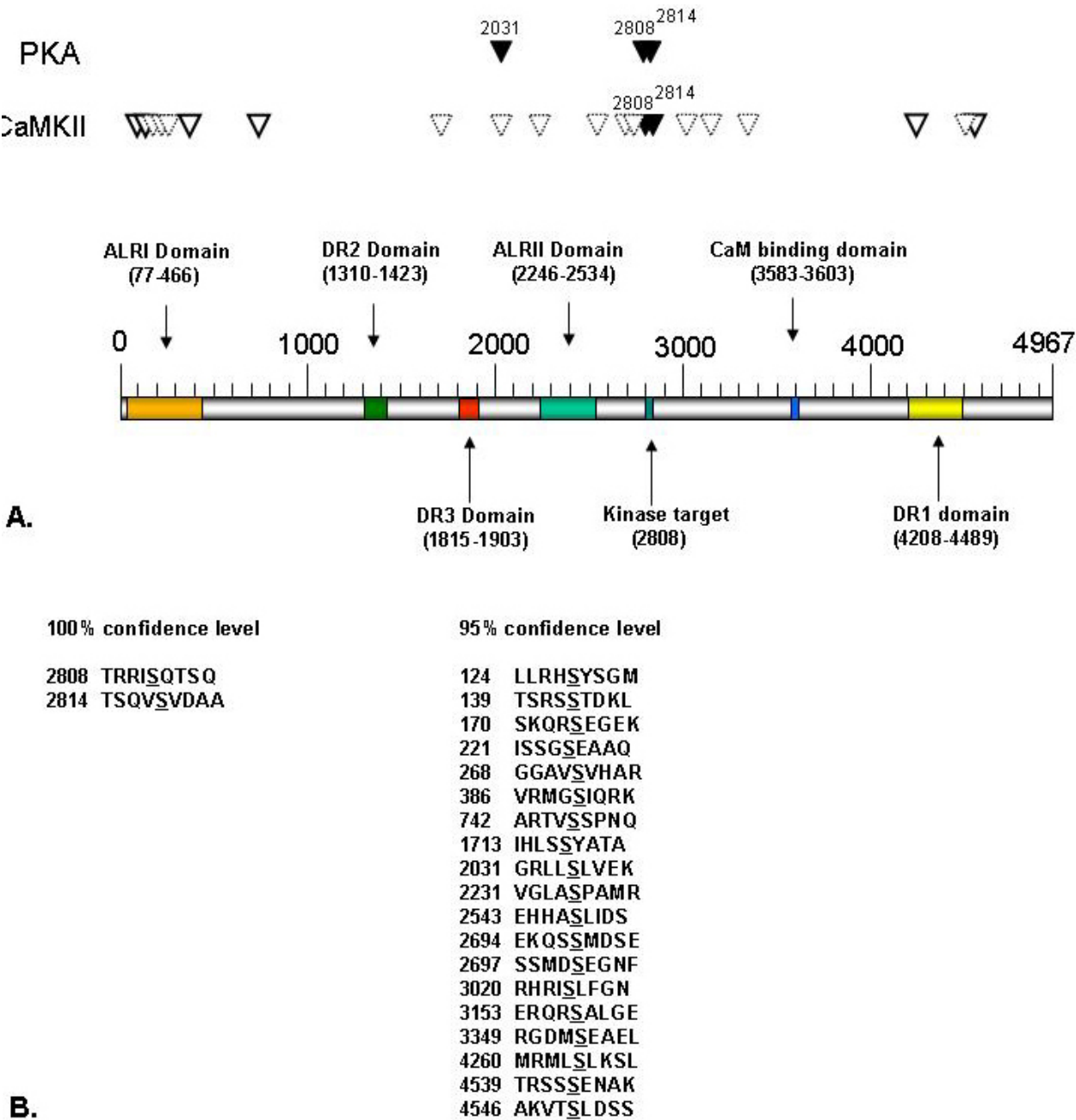


Figure 1. Predicted epitopes for RyR₂ phosphorylation by CaMKII. Schematic diagram outlining phosphorylation sites for CaMKII and PKA that have been established by experimental means (black triangles) or predicted using hidden Markov models using 95% confidence levels. Using Markov models, epitopes predicted to be substrates for CaMKII and PKA are indicated by clear triangles with a solid line. Additional epitopes predicted to be substrates for CaMKII are indicated by clear triangles with a dotted line (KinasePhos, <http://kinasephos.mbc.nctu.edu.tw/>). These sites are placed parallel to a linear representation of the functional domains within RyR₂. ALR I and II domains represent regions for arrhythmia-linked mutations, DR represent areas of sequence diversity among different isoforms of RyR and CaM is the calmodulin binding domain on RyR₂. B. Predicted epitopes for CaMKII were selected based on either 100% confidence levels which highlighted the two sites already determined experimentally or 95% confidence levels which highlighted an additional 19 sites. This data is adapted from (91) and used with permission.

tetrameric RyR₂ (66). This could be increased hugely when considering Ca²⁺ release units that are composed of up to 200 RyRs (67). If we then consider the additional

possibility of indirect channel regulation via CaMKII-mediated phosphorylation of components of the RyR₂ complex, as opposed to, or as well as, the receptor itself,

the potential for complexity of CaMKII-mediated phosphorylation in functional regulation is beyond comprehension.

4. INDIRECT CaMKII MEDIATED EFFECTS ON THE RyR₂ COMPLEX

There is strong evidence to suggest that the Ca²⁺ release channel protein does not exist in isolation. The tetrameric protein has been shown to physically associate with a number of regulatory proteins as well as structural components on both the large cytosolic part of the receptor as well as on the region exposed to the SR lumen (5,6, 68,69). This naturally has implications for functional plasticity, especially in enzymatic regulation of the complex. The possibility exists that CaMKII (and indeed other kinases or phosphatases) associated with the receptor may, in addition to direct phosphorylation of RyR₂, phosphorylate other components of the greater channel complex. This may or may not, depending on the substrate involved, have an additive functional effect to direct channel phosphorylation. Only limited work has been done to relate effects of CaMKII phosphorylation of components of the RyR₂ multi-protein complex with effects on RyR₂ activity. There is evidence however, that this 'indirect' mode of regulation exists.

4.1. Sorcin

Recently, the possibility that CaMKII could modulate RyR₂ activity via phosphorylation of sorcin was proposed (11). Sorcin (or soluble resistance related calcium binding protein) is a Ca²⁺ binding protein belonging to the penta-EF hand family of proteins (70,71). It is a soluble protein but upon binding of Ca²⁺, can translocate to the cell membrane (71). The physical location of sorcin within the cell is important since it has been found to associate with and modulate several cardiac membrane proteins including RyR₂ (71-77). The effects of sorcin on channel function have been investigated in some detail. In RyR₂ reconstituted lipid bilayer studies, sorcin inhibited channel activity in a reversible and concentration-dependent manner (74). Similarly, a reduction in spontaneous Ca²⁺ sparks after sorcin treatment of saponin-permeabilised mouse cardiac myocytes has been reported (78). Adenoviral-mediated overexpression of sorcin in rabbit cardiac myocytes did not cause any difference in caffeine-mediated SR Ca²⁺ release but spontaneous spark frequency, duration, width and amplitude were decreased in sorcin-overexpressing cells (79). It has been suggested that sorcin may play an important role in inactivation of RyR₂ to prevent excessive SR Ca²⁺ leak. This is an additional inhibitory route to that played by FKBP12.6. and it has been suggested that inhibition by sorcin and stabilization by FKBP12.6 may counteract the explosive nature of CICR in the heart (80).

That sorcin is subject to phosphorylation has been noted previously (73). Phosphorylation of sorcin by PKA was reported in cardiac microsomes and effects on RyR₂ activity measured by (³H) ryanodine binding. Phosphorylation relieved the inhibitory effect on RyR₂ however since PKA was not removed from the

'phosphorylated sorcin' mixture, it was difficult to reconcile whether the effects on channel activity were due to phosphorylated sorcin or to direct effects of residual PKA on the channel itself. It was noted in this study that phosphorylation of sorcin by PKA did not completely reverse the inhibitory effects of sorcin (there was still some residual inhibition when compared to channel activity in control conditions, where no sorcin was added). This could be due to residual phosphatase activity in the microsomal preparations limiting phosphorylation levels or it may be that PKA phosphorylation is not capable of fully reversing the inhibitory effects of sorcin on RyR₂ activity.

Recent work investigating CaMKII-dependent phosphorylation of sorcin has examined effects of phosphorylated sorcin on (³H) ryanodine binding in isolated cardiac myocytes (11). Notably phosphorylation was performed using the δ_C variant of CaMKII. Binding was measured after CaMKII had been removed (via chromatographic separation columns) from the phosphorylation mixture (to remove any direct effects of CaMKII on RyR₂) and was also performed in the presence of non-hydrolysable ATP to prevent effects of intracellular phosphatases. Under these conditions CaMKII was capable of higher levels of phosphorylation of sorcin than that seen with equivalent levels of PKA (~1.5 times greater), although quantitative stoichiometric analysis was not performed. Interestingly CaMKII-phosphorylation of sorcin completely reversed the inhibitory effects of unphosphorylated sorcin on RyR₂. The authors speculated that this may serve as an additional indirect route for CaMKII-dependent modulation of channel activity. It was suggested that under conditions where increased SR Ca²⁺ release was observed, such as during chronic β -adrenergic stimulation, increased CaMKII activity may not only directly phosphorylate and activate the channel but additional mechanisms may be in place to exacerbate this effect such as CaMKII-mediated phosphorylation of the RyR₂ inhibitory protein sorcin.

4.2. Protein phosphatases

Phosphatase 1 and 2A (PP1 and PP2A) have been shown to exist as part of the RyR₂ complex (5). Both phosphatases bind to RyR₂ via adaptor proteins PR130 (LIZ1) and Spinophilin (LIZ2) respectively (6) and act in concert with protein kinases to maintain the phosphorylation status of the RyR₂ complex. Both enzymes are also important in directly modulating channel phosphorylation levels (30). Phosphatase 2B (calcineurin) has also been suggested to modulate RyR₂ activity via a Ca²⁺-dependent interaction with FKBP12/12.6 in both skeletal and cardiac muscle (81,82) although whether there is direct interaction of calcineurin with the channel remains controversial (83). There is evidence to suggest that alterations in levels of PP1 and PP2A in cell-based studies leads to altered Ca²⁺ spark frequency and SR store content (84). In addition, it appears that PP1 can reverse effects of PKA (6) and CaMKII-mediated (31) phosphorylation of RyR₂ *in vitro*. In heart failure, there is evidence that both phosphatases become dissociated from RyR₂ (5) and further evidence that where there is more CaMKII associated with the channel, there is a switch in balance

between phosphorylation/dephosphorylation with increased phosphorylation being evident (31). This is important and in examining possible mechanisms for how CaMKII may regulate RyR₂, it is intriguing to examine whether kinase interaction with either PP1 or PP2A may provide an indirect link to regulation (particularly abnormal regulation) of channel activity. Could kinase interaction/phosphorylation of either or both phosphatases lead to PP1 and/or PP2A dissociation from the channel complex and ultimately increased phosphorylation of RyR₂? Although there is no direct evidence to suggest that CaMKII may modulate RyR₂ via action on phosphatases, there is compelling separate evidence that a link may exist. In mouse heart evidence has been presented that CaMKII δ physically associates with PP2A (85). Endogenous CaMKII co-immunoprecipitated with endogenous PP2A. There was also evidence to suggest that increased CaMKII activity as observed in transgenic mice overexpressing CaMKII δ , was associated with *increased* PP2A protein levels. This was not just evident at the nucleus but was also evident in the SR. It was suggested by the authors that alterations in CaMKII activity could lead to compensatory changes in PP2A expression and activity that may contribute to changes in SR Ca²⁺ handling. Whether CaMKII can directly phosphorylate and mediate PP2A activity is not known. However, the possibility exists that this potentially compensatory mechanism for maintaining a balance in SR phosphorylation levels, could be adversely affected under conditions where CaMKII is chronically elevated. Since both proteins are part of the greater RyR₂ complex and are capable of a direct interaction, this may serve as another indirect route for CaMKII-mediated modulation of channel phosphorylation and activity.

4.3. Triadin

Phosphorylation of the triadin cytoplasmic domain by CaMKII has been shown in skeletal muscle (86,87). Triadin is a junctional SR transmembrane protein that interacts with RyR (86). No other kinase has been identified that can phosphorylate triadin and it was postulated that CaMKII-mediated phosphorylation of triadin may modulate RyR activity. Experiments examining the effects of phospho and dephospho-triadin on RyR activity have yet to be performed and this interaction has yet to be established in cardiac muscle. It is known however, that in cardiac muscle, triadin is the scaffold linking calsequestrin (an SR luminal Ca²⁺ binding protein) to RyR₂ (88). Experiments using synthetic peptides corresponding to the calsequestrin binding domain of triadin, have suggested that calsequestrin stabilizes RyR₂ through interaction with triadin (13). If triadin can be phosphorylated by CaMKII and if this leads to altered interaction with RyR₂, causing disruption of the calsequestrin-stabilising effect, this would be another potential indirect route for CaMKII modulation of channel function.

It is highly likely that there remain other components of the RyR₂ complex that have yet to be identified and therefore the scope for potential CaMKII interactions is likely to grow. Although adding to the complexity of channel regulation, this may also add to the

feasibility of targeted and selective CaMKII-dependent modulation of channel activity. Targeting CaMKII effects on the channel itself may result in an all or nothing effect whereas targeting phosphorylation of other components of the complex could give scope for a more graded approach of modulation. This may lead to a better understanding of what CaMKII-mediated phosphorylation of the larger channel complex actually means functionally. Potential routes for phosphorylation of the complex are outlined in Figure 2.

5. FUNCTIONAL CONSEQUENCES OF CaMKII-MEDIATED PHOSPHORYLATION OF THE RyR₂ COMPLEX

It has already been alluded to in this review and elsewhere (89) that, as well as Ser 2809 and Ser 2815, RyR₂ contains multiple consensus phosphorylation sites for CaMKII. That, coupled to the fact that other RyR₂-interacting proteins may also be phosphorylated and, as a result, could impose a functional effect on the channel itself, makes the functional implications of RyR₂ complex phosphorylation potentially multifaceted. A number of studies have investigated effects of CaMKII modulation using a variety of different techniques and these are outlined in Table 1. It is especially interesting to note the surge in number of studies published in the past year alone (from the time this review article was written in June 2008), reflecting the heightened interest in this area. The effects observed on RyR₂ outlined in the table, are not all consistent (varying between enhanced or reduced activity, no effect or complex effects that are difficult to interpret) and the key question remains – what, if any, is the functional purpose of CaMKII-mediated phosphorylation of RyR₂?

This may well depend on the context in which the effect is measured. Highly artificial systems may lead to results that may not accurately reflect the *in vivo* scenario. An issue surrounding the studies to date relates to the artificial nature of the various experimental procedures that have been used. Given the potential incredible complexity involved in CaMKII-mediated phosphorylation of the channel complex, current strategies that use highly artificial modes of measurement such as bilayer preparations and immunoprecipitated RyR₂ protein are limited. Interpretation of these studies has to be cautious unless they can be coupled with other less functionally invasive techniques. Recent evidence has highlighted sensitivity problems with some of the phosphorylation site-specific antibodies currently in use (90). This alone could explain some of the discrepancies observed when assessment of phosphorylated protein is made using these antibodies in isolation. Issues surrounding experimental limitations in RyR₂ phosphorylation research have recently been highlighted in a separate review (91) and it was suggested that the development of novel strategies that allow monitoring of the interaction under native conditions would be a huge step forward. Non-invasive imaging tools have been used recently to measure other interactions in the native environment and these could be applied in this area (92-94).

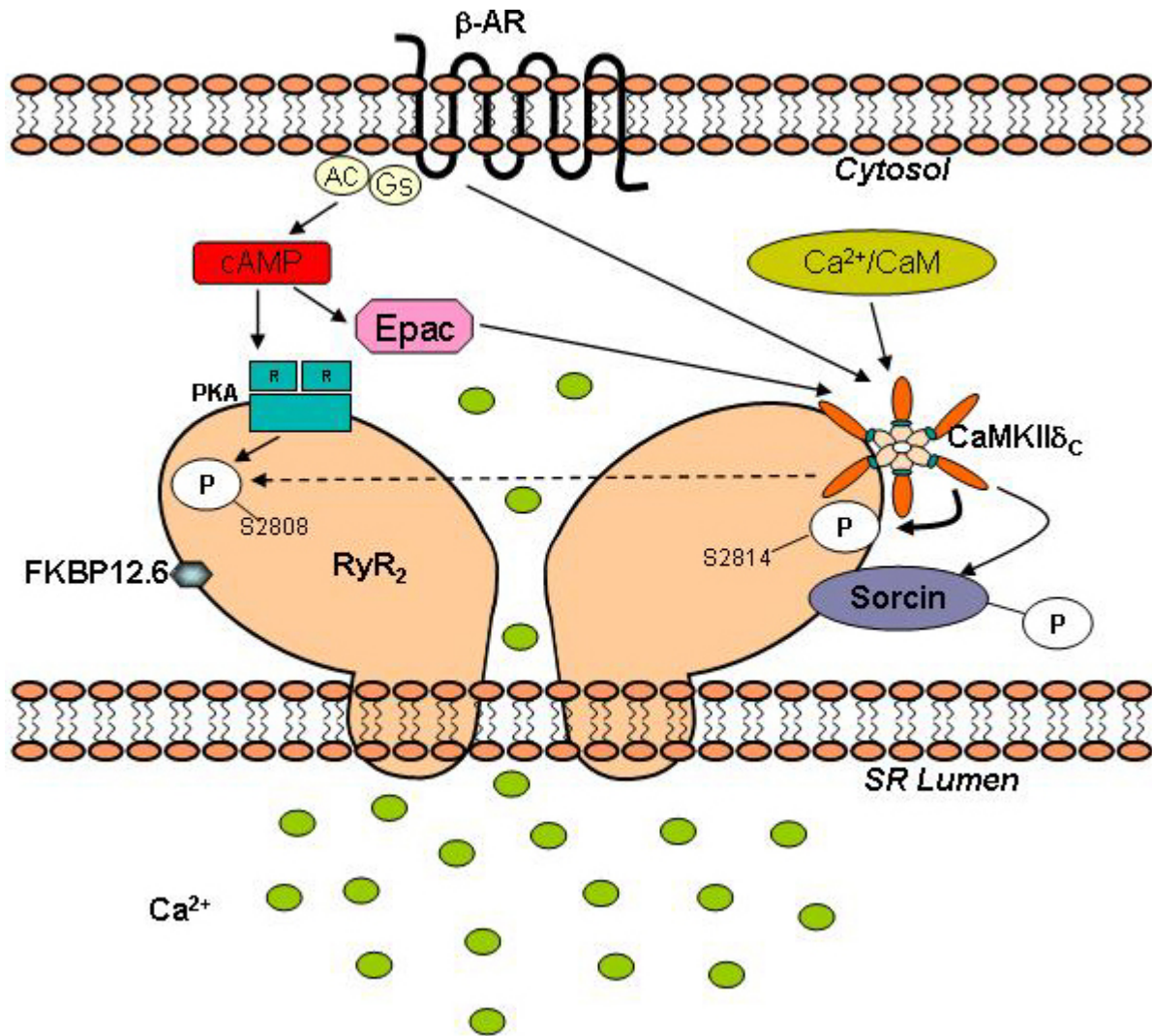


Figure 2. Model of CaMKII phosphorylation sites on the RyR₂ complex. Upon β -adrenergic receptor stimulation by catecholamines both CaMKII and PKA are activated. CaMKII can be activated via the classical route of Ca^{2+} /calmodulin and/or via the more novel cAMP/Epac pathway independent of PKA activation. Upon activation, CaMKII preferentially phosphorylates Ser 2814/2815 on RyR₂ (bold arrow) and to a lesser extent Ser 2808/2809 (dotted arrow). CaMKII also phosphorylates the inhibitory protein sorcin that exists as part of the channel complex. Phosphorylation at any of these sites promotes channel opening and an increase in Ca^{2+} release from the SR lumen into the cytosol.

The issue of whether CaMKII activates or inhibits RyR₂ has been highlighted recently (95). This has come with the publication of recent work suggesting CaMKII may act to inhibit channel function (67). This goes against the grain of most other published research suggesting that CaMKII phosphorylation (mainly detected at Ser 2815) leads to increased RyR₂ activity (7,51,52). The work suggesting inhibitory effects involved using adenoviral gene transfer to express wild type (WT), constitutively active (CA) and dominant negative (DN) CaMKII δ_C in cultured rat cardiac myocytes. The authors examined parameters including RyR₂ phosphorylation status, modulation of Ca^{2+} transients, sparks and waves. The frequency of the latter two parameters were suppressed in CA preparations. Interestingly only Ser 2809 phosphorylation was monitored

in this study and differences in basal levels of phosphorylation were noted. In reconciling these results with the rest of the literature the authors referred to previous work that had used dialysis of CA CaMKII which lead to suppression of SR Ca^{2+} release (96). Dialysis of the enzyme is a very different approach to using adenovirus-mediated gene transfer and implications for differences in the levels of activity as well as the concentration/expression of CaMKII are likely to exist. This again reflects the problem of comparisons being made between studies where very different experimental approaches, and often different species of animal, are used. In further assessing their results in the adenovirus study, the authors allude to the complexity of the *in vivo* scenario. The dynamic nature of phosphorylation *in vivo* makes

Ryr2 phosphorylation by camkii

Table 1. CaMKII-mediated effects on RyR₂ activity

Species used	Effect on RyR ₂ activity ¹	Heart Failure ²	Key findings (Direct/Indirect effects of CaMKII)	Reference
Dog	N.D.	N/A	Dual phosphorylation by CaMKII and PKA	39
Dog	↓	N/A	Level of CaM-dependent phosphorylation was four times greater than that of PKA, PKG or PKC	97
Dog	↑	N/A	Phosphorylation by CaMKII at Ser 2809. Both endogenous and exogenous CaMKII were studied	47
Cow	N.D.	N/A	CaMKII phosphorylates RyR ₂ to a much greater extent than either PKA or PKG. Strong co-association of CaMKII with RyR ₂ during purification suggesting physical interaction	44
Pig	↑	N/A	CaMKII-mediated RyR ₂ phosphorylation was additive with PKA-mediated phosphorylation. This kinase modulation was much more marked in cardiac muscle than skeletal muscle	136
Dog	↑	N/A	RyR ₂ block by physiological (Mg ²⁺) is overcome by CaMKII or PKA. It was suggested that there were multiple sites of phosphorylation on RyR ₂ with different functional consequences	49
Pig/Rabbit	↓	N/A	CaMKII inhibited RyR ₂ activity and this was reversed by treatment with acid phosphatase	50
Dog	N.D.	N/A	CaMKII and PKA phosphorylate Ser 2809 to full stoichiometry. CaMKII causes more incorporation of phosphate into RyR ₂ implying phosphorylation at additional sites (at least four in addition to Ser 2809)	51
Mouse	↑	+	CaMKIIδ _C overexpression lead to increased fractional SR Ca ²⁺ release and spark frequency in transgenic mice. Acute CaMKII inhibition normalised spark frequency suggesting direct CaMKII phosphorylation and activation of RyR ₂ in transgenics	53
Rabbit	↑	N/A	Endogenous CaMKIIδ physically associated with RyR ₂ and was largely responsible for basal phosphorylation levels. Evidence for increased number of CaMKII sites on RyR ₂ compared with PKA. Inhibition of CaMKII decreased Ca ²⁺ sparks	7
Rabbit	↑	+	Ser 2815 identified as CaMKII-selective phosphorylation site on RyR ₂ . CaMKII identified as part of RyR ₂ multi-protein complex. Defective CaMKII-mediated RyR ₂ phosphorylation in heart failure	54
Rabbit	↑	+	Increased RyR ₂ -associated CaMKII in heart failure and increased phosphorylation at Ser 2815 and Ser 2809. Inhibition of CaMKII (but not PKA) inhibited SR Ca ²⁺ leak in failing cardiomyocytes	31
Mouse/Human	N.D.	—	Phosphorylation of Ser 2814 by CaMKII was not altered in mice after myocardial infarction	18
Rabbit	↑	N/A	Overexpression of CaMKIIδ _C resulted in increased RyR ₂ phosphorylation at Ser 2815 and Ser 2809. Increased SR Ca ²⁺ leak and reduced SR content	56
Mouse	↑	N/A	Both endogenous and exogenous CaMKII increased resting SR Ca ²⁺ release. Endogenous CaMKII phosphorylated RyR ₂ to the same extent as pre-activated exogenous CaMKII	32
Rat	N.D.	N/A	CaMKII phosphorylation at Ser 2808/Ser 2814 is not responsible for development of frequency dependent acceleration of relaxation. Inconsistencies between RyR ₂ phosphorylation site-specific antibodies were highlighted	90
Rabbit	↑	N/A	β-adrenergic stimulation leads to CaMKII-dependent phosphorylation of RyR ₂ which increases diastolic Ca ²⁺ leak. This is not PKA dependent and may reflect the situation in heart failure	33
Mouse	↑	N/A	Transgenic mice with AIP targeted to the SR displayed decreased diastolic Ca ²⁺ leak and markedly reduced RyR ₂ phosphorylation at Ser 2815. Frequency dependent acceleration of relaxation was inhibited.	130
Rat	↓	N/A	Adenoviral expression of CaMKIIδ in rat myocytes caused depression of Ca ²⁺ sparks and waves	67
Rat	↑	N/A	Epac activates RyR ₂ and increases Ser 2815 phosphorylation via CaMKII	65
Mouse	↑	N/A	RyR ₂ activity may be modulated indirectly via CaMKII-mediated phosphorylation of sorcin	11
Rat	↑	N/A	Ser-2815 phosphorylation correlates with increased RyR ₂ activity. CaMKII predominates over PKA in modulation of SR Ca ²⁺ release following β-adr stimulation	57

¹RyR₂ activity measurements vary between studies and include (³H) ryanodine binding, single channel measurements from planar bilayer reconstitution studies and Ca²⁺ spark measurements where a combination of spark frequency, duration, width and peak have been monitored. N.D. refers to studies where RyR₂ activity was not measured. ² The majority of studies quoted in the table have not examined the role of CaMKII in modulating RyR₂ in heart failure. Where indicated, the study has shown that CaMKII-mediated phosphorylation and activation of RyR₂ is increased in heart failure (+) or that there is no change in phosphorylation or activation status (-).

extrapolation from this type of *in vitro* work very limited. The possibility of different isolated preparations being subject to different intrinsic bias will almost certainly affect interpretation of the result. Earlier work where an inhibitory effect of CaMKII on RyR₂ had been noted included the use of single channel studies and ryanodine binding studies using SR microsomes (50, 97). Both types

of preparations are highly artificial and again intrinsic differences in levels of basal phosphorylation and/or activation were not studied in detail. Even at the time these studies were performed (1991 and 1995), there was already other evidence to suggest that CaMKII-mediated phosphorylation activated RyR₂ (47). The authors of the 1995 study suggested that differences in species, coupled

with different experimental solutions could have lead to the seemingly opposing effects. It seems unlikely that these differences in observations could be due to differences between species. Regions of diversity in RyR₂ are located between residues 1310-1423, 1815-1903 and 4208-4489 and the phosphorylation sites in question are outwith these regions and appear to be well conserved between species (91). It would appear more likely that the differences observed are a result of the different experimental conditions and approaches taken.

When deciding what the functional effects of CaMKII-mediated phosphorylation of RyR₂ are, it is critical to remember that this Ca²⁺ release channel and the function it performs do not exist in isolation; i.e. CaMKII phosphorylates and modulates other Ca²⁺ handling proteins (e.g. L-type Ca²⁺ channel and phospholamban (PLB)) that will ultimately influence SR Ca²⁺ handling. In addition to this, the issue of whether preparations are 'stimulated' or not will affect interpretation. Due to these complicating issues, interpretation of effects is difficult (highlighting the rationale behind the more artificial techniques that are currently in use). The development of sophisticated means of measuring SR Ca²⁺ release or leak are important but again not without their limitations. Measurements that take into account SR Ca²⁺ content and that use tools such as tetracaine to block SR Ca²⁺ leak can be used to show selective effects on SR release through RyR₂ (98). These allow a more direct quantification of release than relying on measurement of Ca²⁺ sparks during diastole. However, tetracaine experiments are usually performed against a background of negligible or clamped plasma membrane ionic fluxes so experimental limitations have to be considered. The importance of SR Ca²⁺ content in influencing altered leak has also been considered. Recent work has suggested that increased diastolic Ca²⁺ leak is dependent on increased SR Ca²⁺ content (99). Specifically, this work showed that in isolated rat cardiac myocytes, caffeine-induced increases in RyR₂ P_o did not cause diastolic Ca²⁺ release under control conditions. However, when cells were stimulated with isoproterenol, Ca²⁺ waves were observed in the steady state. The authors concluded that increasing P_o of RyR₂ alone is not sufficient to cause diastolic Ca²⁺ release and that maintenance of SR Ca²⁺ content was necessary. Again however, the limitations of experimental approach have to be considered. There is evidence to the contrary that in heart failure increased SR Ca²⁺ leak is observed at normal-low SR Ca²⁺ load reflecting perhaps the increased abnormality of the situation. In the normal situation during β -adrenergic stimulation, increased SERCA activity will be an important factor leading to increased SR content and NCX will be important in preserving cytoplasmic Ca²⁺ levels. The role of CaMKII in regulating these phenomena by phosphorylating not only the RyR₂ complex but also the SERCA/PLB complex (100) has been demonstrated but the relationship between SR Ca²⁺ content and release/leak will change depending on whether measurements are taken during the resting steady state or during acute or chronic β -adrenergic stimulation. Previous work had suggested that CaMKII activation in response to β -adrenergic stimulation was slow and was therefore responsible for mediating the long-term effects of

sustained stimulation (101). However, more recent work has suggested CaMKII is activated early after stimulation and can therefore lead to rapidly increased phosphorylation of Ca²⁺ handling proteins (33). It seems likely that the CaMKII effect will be complex and will persist during both acute and chronic stimulation. Currently there is strong evidence to suggest that CaMKII plays a key role in RyR₂ phosphorylation and activation during β -adrenergic stimulation. Evidence for PKA-mediated phosphorylation of RyR₂ at Ser-2808 following stimulation is highly controversial (27) (see above section 3.2).

The predominant effect of CaMKII over PKA on SR Ca²⁺ release was elegantly demonstrated in a recent study where cardiac myocytes from PLB knock-out mice were used (33). By removing this major additional substrate for both kinases from the SR, the authors were able to examine effects on RyR₂-mediated Ca²⁺ release without potentially confounding effects on SR Ca²⁺ load mediated via PLB phosphorylation. Interestingly, CaMKII was still able to cause increased Ca²⁺ spark activity in cells from PLB knock-out mice, whereas PKA could not. The authors concluded that this reflected a direct effect of CaMKII on RyR₂ activity whereas the increase in Ca²⁺ release seen with PKA was indirect and dependent on phosphorylation of at least one other SR substrate (PLB) that influences SR Ca²⁺ load. This study actually strengthened previous data suggesting that PKA did not affect Ca²⁺ sparks (19) and highlighted the potential for quite different effects of both kinases on RyR₂ activity. Whether this is due to each kinase phosphorylating different residues on the protein (PKA has been suggested to phosphorylate Ser 2030 (30) whereas CaMKII phosphorylates Ser 2815 (54)) with differential effects on RyR₂ is currently not known.

The functional consequences of CaMKII-mediated RyR₂ phosphorylation cannot be explained in a 'one size fits all' type statement. This is because of the multiple components involved in cardiac Ca²⁺ handling and because of the difficulty in making interpretations from assessment of one component of that process in isolation. From the evidence we have to date it would appear that CaMKII phosphorylation of RyR₂ does lead to increased SR Ca²⁺ release, and that this is more marked when β -adrenergic stimulation is applied. Interpretation of previous studies has to be context- and technique-dependent. Unfortunately this does not always happen, contributing to the complicated nature this field is in today. Spontaneous Ca²⁺ release observed after β -adrenergic stimulation can be reversed by CaMKII inhibitors such as KN-93 and autocamtide-2-related inhibitory peptide II (AIP) (33). These inhibitors can actually reduce Ca²⁺ release to below control levels (7,33) suggesting endogenous CaMKII does contribute to basal phosphorylation and activity of RyR₂. However, by using a method that globally inhibits CaMKII, it has to be acknowledged that the observed alterations in RyR₂ activity may be attributed to effects on other Ca²⁺ handling proteins in addition to RyR₂. It is important to reiterate that until we have techniques that allow CaMKII-dependent phosphorylation to be monitored in real time *in vivo* under both basal and stimulated conditions, we have to

justify any previous conclusions within the context of the study in question.

6. ROLE FOR CaMKII-MEDIATED RyR₂ COMPLEX PHOSPHORYLATION IN HEART FAILURE – OPENING THE FLOOD -GATES TO ARRHYTHMOGENESIS

Defective RyR₂ function is evident in heart failure (1,102). In addition, mutations in RyR₂ that cause channelopathies are associated with stress-induced malignant tachycardia which can lead to sudden cardiac death (103-105). In heart failure there is evidence that physical dissociation of stabilizing proteins from the RyR₂ complex (5) results in 'leaky' channels that are more active at a lower (Ca^{2+}) (5, 106). The role of increased kinase activity leading to increased phosphorylation and alteration of RyR₂ function in heart failure is evident but remains controversial. As mentioned previously, both PKA and CaMKII have been suggested to phosphorylate RyR₂. Initial work performed in 2000 had suggested PKA-mediated phosphorylation of RyR₂ could lead to altered channel function in heart failure (5). However, more recent work has suggested that CaMKII plays a key role in phosphorylation of RyR₂ in cardiac disease (31) and current evidence suggests that CaMKII predominates over PKA to increase RyR₂ P_o in both normal and diseased heart (107).

It has been well documented that CaMKII expression and activity is elevated in cardiac hypertrophy and heart failure and this appears to be a common feature across species, including humans (37, 52, 106-111). As well as showing that heart failure leads to an increase in CaMKII, the inverse has been demonstrated i.e. increased CaMKII expression and activity leads to heart failure. Overexpression of CaMKII δ_C in mouse and rabbit cardiac myocytes leads to dilated cardiomyopathy with pronounced changes in cardiac myocyte Ca^{2+} handling (53) and arrhythmogenesis (111). CaMKII-dependent arrhythmias are further enhanced following β -adrenergic stimulation and this increase in incidence of arrhythmias ties in with the exacerbation of CaMKII effects on RyR₂ activity in the presence of β -stimulation. It could be that PKA-mediated phosphorylation of RyR₂ following stimulation causes structural alterations that makes the channel a more accessible substrate for CaMKII. This has not been tested but it could explain why experimental values of potential CaMKII sites on RyR₂ varies dramatically.

Overexpression studies have also shown that although increased levels of CaMKII lead to increased RyR₂ Ca^{2+} sensitivity during diastole and systole, this does not involve dissociation of FKBP12.6 (54). Interestingly, the key site for phosphorylation associated with increased CaMKII activity is confirmed as Ser 2815. Increased SR Ca^{2+} leak and fractional release were shown to be associated with increased RyR₂ phosphorylation at Ser 2809 and Ser 2815 but with increases of 71% and 171% respectively (56). In heart failure this difference is even more pronounced with RyR₂ phosphorylation increased by 30% at Ser 2809 and 105% at Ser 2815 and increased co-association of CaMKII with RyR₂ as shown by immunoprecipitation (31). This phosphorylation data,

combined with difference in effects on FKBP12.6, again highlights the probability that CaMKII and PKA-mediated phosphorylation of the RyR₂ complex act at different sites and have different overall mechanistic effects on the channel. This is something that has been highlighted previously but has not been examined in detail in any studies to date. That CaMKII is largely responsible for increased SR Ca^{2+} leak in heart failure is demonstrated by the effects of inhibition of the enzyme. Treatment of isolated myocytes from CaMKII-overexpressing hearts with KN-93 was shown to normalize Ca^{2+} spark frequency (53). Of particular interest, KN-93 (and not H-89) was shown to block SR Ca^{2+} leak and significantly enhance SR content in myocytes from failing hearts (31). In an attempt to correlate sites of phosphorylation with functional effects, it would be interesting to correlate normalization of SR leak with changes (decreases) in phosphorylation at specific sites on RyR₂ (in this case Ser 2815).

As well as increased kinase activity in heart failure, other components of the channel complex may also be altered; most notably, RyR₂ itself. Studies from human tissue and animal models of heart failure have suggested that RyR₂ expression may be reduced (112,113) or unchanged (114) however studies showing reduction have often examined expression in selective regions of the heart (transmural sections) rather than performing global measurements. Sub-endocardial expression of RyR₂ is reduced in a rabbit model of left ventricular dysfunction (112) as well as in dogs with left ventricular hypertrophy (113). Fractional release was only decreased in sub-endocardium and not in the sub-epicardium (112). These studies highlight the heterogeneity that exists within the heart and again reflect the importance of the technique used to assess expression. In studies where global RyR₂ measurements were made, more controversial results were obtained with some studies suggesting down-regulation (115, 116) and others suggesting no change (117). Expression levels of a protein do not necessarily always coincide with similar changes in activity of that protein and this should be borne in mind when attempting to relate biochemical changes to functional consequences. These studies examining local changes in RyR₂ also raise important issues about global versus local expression and activity of proteins. In heart failure, it is likely that differential effects on RyR₂ are observed throughout the heart which, if measured globally, may be masked (117). In future work where therapeutic intervention is considered, selective targeting to specific regions of the heart is likely to be an important issue. Understanding how localized regions of the heart differ from each other at the molecular level will be important in designing appropriate and effective intervention strategies. This is certainly applicable to the RyR₂ complex which is likely to exhibit differences in expression, interaction with CaMKII δ (as well as other components of the complex) and Ca^{2+} sensitivity, depending on which region of the heart is studied. Previous work has highlighted this regional variability in Ca^{2+} handling protein expression in heart failure (118) where myocardial infarction causes very distinct levels of insult to the heart with consequent differential changes in Ca^{2+} handling. Regional alterations in CaMKII in heart failure

have not been explored but it seems likely that the interaction, phosphorylation and consequent altered channel function will not be uniform throughout the heart. Future studies designed at correlating regional CaMKII-RyR₂ interactions transmurally and also with degree of cardiac insult will be important in establishing a detailed picture of this relationship in normal and failing hearts.

An interesting point regarding alterations in protein expression in heart failure is that where RyR₂ levels have been shown to be reduced, there appears to be an increase (~2-fold) in inositol trisphosphate receptor (IP₃R) levels (31, 119). The functional importance of this effect has not been studied, however it is known that CaMKII can also associate with and modulate IP₃R function in the heart (64). IP₃Rs are predominantly expressed at the nuclear envelope in cardiac myocytes however whether this expression pattern changes in heart failure is not known. Expression of IP₃Rs in these cells is tiny compared with RyR₂s however it will be of interest to determine whether there is a potential relationship between these two proteins and if there is some significance to this reciprocal expression in heart failure. Future studies should also be designed to address the relationship between CaMKII modulation of the two Ca²⁺ release channels, and whether different variants of CaMKII may interact with and modulate these proteins in cardiac disease. Understanding the dynamics of a potential relationship will be important in defining the potential impact this may have in heart failure.

Experimental variation in heart failure studies is an important consideration when interpreting results. Sources of variation between heart failure studies include the animal models that have been used and also the specifics of whether regional effects have been measured (as indicated above). Another important point to consider in heart failure studies examining the phenomenon of RyR₂ phosphorylation, is that most have been performed at a single pre-determined time point from cells or tissue isolated from experimental animal models or from failing human hearts. This gives no perspective on the dynamics of the relationship between CaMKII and RyR₂ or the continually changing nature of the relationship in the earlier stages of disease onset progressing from normality to hypertrophy to heart failure. Recent work from a rabbit coronary artery ligation model where several time points post-ligation have been studied, suggests that CaMKII is elevated acutely (1 week following ligation) as well as chronically (32 weeks following ligation) (120). Both expression and activity of CaMKII δ were measured in this study. Interestingly, at 1 week post-ligation, expression of CaMKII δ is more significantly increased. At 32 weeks post ligation, activity of CaMKII δ is more significantly increased. The progressive nature of heart disease is something that has largely been ignored in studies to date, certainly those investigating alterations in Ca²⁺ handling. These results highlight the fact that investigations performed at a single time point are only reflecting a 'snapshot' of what is actually a dynamic and progressive condition. Although CaMKII remains elevated throughout progression of the condition, the nature of kinase elevation changes and this will certainly have an impact on the Ca²⁺

handling protein substrates that are affected. Correlation of the phosphorylation of RyR₂ at Ser 2815 with relative CaMKII levels associated with the channel complex and ultimately alterations in RyR₂ function will be important. Examination of this relationship at different time points as heart failure progresses will provide a much better handle on whether 2815 phosphorylation (either transient or persistent) correlates directly with changes in diastolic SR Ca²⁺ leak and is an important component of the failing heart acutely and chronically. Identifying key changes in RyR₂ (such as Ser 2815 phosphorylation) that correlate with RyR₂ dysfunction may provide important clues for future therapeutic targeting.

7. THERAPEUTIC STRATEGIES FOR NORMALISING SR Ca²⁺ LEAK IN HEART FAILURE – IS MODULATION OF LOCAL CaMKII ACTIVITY A SILVER BULLET FOR TREATING HEART DISEASE?

It has been questioned whether heart failure is a disease of CaMKII over-activity (121). From evidence presented to date it appears that increased CaMKII activity is certainly a central feature of pathological β -adrenergic signaling in heart failure. It should be evident from this review and others that elevated CaMKII activity will impose a range of effects on Ca²⁺ handling in the cardiac myocyte, however what has been established and what is of relevance to this review, is that CaMKII directly (and indirectly) regulates SR Ca²⁺ leak and RyR₂ phosphorylation in heart failure. An arrhythmogenic rabbit model of nonischaemic heart failure has been used to demonstrate this effect (31). The hallmark characteristics of this model are arrhythmias, contractile dysfunction and sudden death (122). The enhanced diastolic Ca²⁺ leak characteristic of this model is dangerous for a number of reasons. It can lead to reduced SR content and systolic function. It may promote increased Ca²⁺-dependent pathological gene transcription. Finally, it may lead to increased inward Na⁺-Ca²⁺ exchanger current resulting in delayed afterdepolarisations and arrhythmias. It seems then that selectively inhibiting a central causative factor of increased SR Ca²⁺ leak, namely CaMKII δ , would be an important way forward in developing more selective therapeutic intervention for heart disease. A more selective approach to modulation of CaMKII, particularly CaMKII targeted to the RyR₂ complex, could provide a means of selectively controlling the dangerously high SR Ca²⁺ leak observed in heart failure.

Early work using CaMKII inhibitory peptides including AC3-I suggested that CaMKII inhibition prevented development of the arrhythmogenic transient inward current observed in isolated rabbit ventricular cardiac myocytes (123). Other studies using the organic CaMKII inhibitor KN-93, showed inhibition of the excessive Ca²⁺ leak observed in heart failure, as well as enhanced SR content and improved cardiac inotropy (31). An issue with using KN-93 however, is the relative specificity of this inhibitor for CaMKII at higher concentrations where other non-specific effects may be apparent. In particular, it has been shown to exert

antagonistic effects on the L-type Ca^{2+} channel (124). Different, more selective approaches for CaMKII inhibition are therefore needed. A genetic mouse model of cardiac CaMKII inhibition was recently developed where a conserved region of the CaMKII regulatory domain was targeted with cDNA encoding an inhibitory peptide (AC3-I) that had been used in earlier studies and was highly selective for CaMKII (125). These mice provided the chance to study the effects of chronic CaMKII inhibition in a setting where Ca^{2+} handling protein expression remained unchanged. AC3-I and wild-type mice were subjected to myocardial infarction or isoproterenol cardiomyopathy. The AC3-I mice showed a substantial reduction in pathological remodeling in both circumstances. There was also a compensatory upregulation of L-type Ca^{2+} current (PKA-mediated) that was balanced by action-potential shortening to preserve basal and β -adrenergic stimulated cell contraction. The authors also referred to compensatory PKA-mediated phosphorylation of Ca^{2+} channels in this model, suggesting that when CaMKII is unavailable there is scope for the PKA pathway to act as a reserve for maintenance of normal RyR₂ activity. This highlights the interesting and largely uninvestigated concept of overlap between PKA and CaMKII in regulation of cardiac Ca^{2+} handling in heart disease. Concrete evidence that PKA could 'step in' for CaMKII to maintain SR function under conditions where the latter kinase is inhibited is lacking. Complete inhibition of CaMKII was not observed in AC3-I mice (there was ~40% inhibition) and this is an important point. Total inhibition of CaMKII would not only eliminate the pathological effects of the increased kinase activity in heart failure, it would also destroy the positive inotropic effects of CaMKII required for normal cardiac function. These include RyR₂ activation and increased fractional SR Ca^{2+} release as well as PLB phosphorylation and regulation of SERCA 2a activity (126) in addition to L-type Ca^{2+} channel (127) and Na^{+} channel regulation (128). It seems highly unlikely that even if PKA can offer some compensatory effects when CaMKII is inhibited, that total compensation for a complete lack of CaMKII activity would be a possibility.

Targeted approaches for inhibiting CaMKII are currently being investigated. Targeting can involve different degrees of focus and selectivity; either targeting to the SR or homing in further to target a particular SR protein such as RyR₂. The difficulty with this sort of focus is in ensuring that the strategy chosen is selective enough to allow inhibition only to occur at the chosen microdomain of the cell. The potential advantages of this approach are that 'fine-tuning' of CaMKII effects would be allowed. The ability to selectively manipulate CaMKII effects on SR leak without gross effects on other elements of cardiac Ca^{2+} handling could provide more information on the benefits of more selective therapeutics in treatment of heart disease. Different approaches have been taken to inhibit CaMKII only at the SR. Transgenic mice were produced using a synthetic gene expression unit which included sequences encoding a tetramer of AIP (a CaMKII inhibitory peptide) and an SR localization signal (129). The localization signal was a truncated PLB transmembrane domain with mutations to ablate any PLB inhibition of SERCA.

Interestingly these mice showed a ~30% decrease in RyR₂ phosphorylation at Ser 2809 and β -adrenergic stimulation was able to increase phosphorylation at this residue. Ser 2815 phosphorylation was not examined in this study. This study focused predominantly on examination of SR uptake linking this with inhibition of PLB phosphorylation at Thr 17. The effects observed on RyR₂ however, did suggest that targeting was not selective to longitudinal SR as was initially expected. A separate study using the same mice examined the possibility that SR-targeted CaMKII inhibition was able to (i) inhibit SR Ca^{2+} leak (and RyR₂ phosphorylation at Ser 2815), (ii) inhibit frequency dependent acceleration of relaxation and (iii) leave L-type Ca^{2+} current unaltered (130). This study verified that CaMKII inhibition was targeted to the SR. It strongly suggested however that, as previously observed, effects of CaMKII inhibition using this gene expression unit, were not restricted to the longitudinal SR since marked effects on RyR₂ were observed. Phosphorylation at Ser 2815 was decreased by ~62% in SR-AIP versus wild type mice when normalized to RyR₂ expression level. This suggested that effects of the PLB-targeted AIP were also evident in junctional SR. Interestingly CaMKII-mediated effects on the L-type Ca^{2+} channel were abolished in SR-AIP mice. This was unexpected and the authors suggested could be a result of the very close proximity of junctional SR (RyRs) to the L-type Ca^{2+} channel (131) or that there was some predisposition of SR-localized CaMKII to interact with the Ca^{2+} channel in the sarcolemma. The other possibility is of course that the targeting of CaMKII inhibition is not as achievable may have been originally hoped.

In order to build upon the approach used previously (132) and to attempt to selectively inhibit RyR₂-associated CaMKII, it would be essential to construct a synthetic expression unit that incorporated a component of the RyR₂ complex (and not PLB) as well as the peptide inhibitor (AIP) of CaMKII. However the problem with this sort of approach is that we do not know enough about the specificity of expression of all SR regulatory proteins so we cannot develop highly specific approaches of delivery if they are based upon this sort of strategy. Delivery would have to be directed at RyR₂ itself, and only to the SR. Selective inhibition would also probably need to focus on the CaMKII-selective 2815 epitope since other identified targets on the RyR₂ complex are also subject to modulation by PKA. Inhibition of CaMKII phosphorylation at Ser 2808/2809 or of sorcin, could well be compensated for by the PKA pathway. Similarly, targeting Epac as an activator of CaMKII-mediated 2815 phosphorylation may not be selective since this will have additional roles within the cell that could also be affected. It is likely that we have much more to learn about CaMKII localization and activation mechanisms within cardiac myocytes, particularly given the variety of proteins that are substrates for the enzyme and the dynamic nature of this enzyme's function.

Selective inhibition of the open probability of RyR₂ as a method of reducing diastolic Ca^{2+} release has recently been investigated using another approach (132). Application of selective concentrations of tetracaine to ventricular myocytes that had been previously exposed to

isoproterenol decreased diastolic Ca^{2+} release and, as a result, increased the systolic Ca^{2+} transient. The authors of this study excluded any effects of tetracaine on NCX, SERCA or the L-type Ca^{2+} channel however it is likely that effects are dose-dependent and therefore careful consideration of the concentration used would be important. Although tetracaine could not be used therapeutically, this study is important to consider in the context of this review since it highlights the possibility of *selective* modulation of RyR₂ being a potential target for development of novel anti-arrhythmic therapies. Building upon the strategies outlined above for selective targeting of CaMKII inhibition could form the basis for such an anti-arrhythmic agent.

Aside from the possibility of selective CaMKII inhibition, it is likely that some current non-selective therapies incorporate CaMKII inhibition as part of their activity profile. It seems likely that effects on CaMKII activity may underlie at least some of the positive effects of current β -receptor blocking therapy although this has not been examined in detail. Additionally, recent interest has focused on K201 (formerly called JTV-519), a derivative of 1,4-benzothiazepine (133). This has cardioprotective and anti-arrhythmic properties but its mode of action is yet to be fully established. It has been suggested that K201 may decrease the excessive SR Ca^{2+} leak that contributes to arrhythmias by stabilizing RyR₂. This was suggested to be mediated by prevention of FKBP12.6 dissociation from the channel (106). However, more recent work has suggested that while K201 suppresses RyR₂ activity, it does so without affecting the RyR₂ – FKBP12.6 interaction (134). The effects of K201 do not appear to be restricted to RyR₂ since other work has suggested that it may also exert inhibitory effects on SERCA activity (135). That the anti-arrhythmic properties of K201 are due to effects on a number of Ca^{2+} handling proteins is likely. Whether there may be a common underlying mechanism to explain these effects has not been considered. Inhibition of CaMKII δ could explain the reported effects of K201 on both RyR₂ and SERCA and may be an unrecognized mode of action for this drug.

Is modulation/inhibition of RyR₂-targeted CaMKII δ a silver bullet for heart disease? Based on current evidence, it is safe to say that it certainly forms part of the formula for a silver bullet. How big a part is not yet clear. Enhancement of diastolic RyR₂ Ca^{2+} leak by increased CaMKII δ associated with the channel in the failing heart certainly increases the probability of arrhythmias occurring, but it cannot be solely responsible for the complex functional changes that underlie systolic dysfunction. Herein lies the challenge of trying to define the importance of one component (CaMKII-RyR₂) in isolation, in what is a dynamic and multi-factorial process. Decreasing CaMKII activity targeted to the SR normalizes RyR₂ activity and improves cardiac myocyte function. What we do not know at present is how effective decreasing CaMKII activity would be if it were only applied to RyR₂-targeted CaMKII. Although current SR-targeted inhibition strategies have demonstrated effects on RyR₂, other SR (and sarcolemmal) proteins are affected by this inhibition, complicating

interpretation of what processes are fundamentally important. A crucial therapeutic difficulty exists in attempting to only alter the pathological profile of CaMKII-mediated RyR₂ phosphorylation while leaving the ‘house-keeping’ CaMKII activity largely unaltered. Therapeutic advances *in vivo* will only come from a better understanding of each of the key components of Ca^{2+} handling dysregulation in heart failure defined at the molecular, cellular and organ level. More specifically for the scenario outlined here, a better understanding of CaMKII substrates within the greater RyR₂ complex (and how they modulate channel activity) will be important. Specific targeting of these downstream effectors (as well as, or instead of, CaMKII) may give scope to remove at least some of the pathological effects of ‘hyper’ CaMKII activity on RyR₂ while leaving the underlying activity relatively untouched. This will rely heavily on the development of new experimental approaches such as those alluded to in this review as well as recognizing the limitations and variations that certain approaches may have. The hurdles involved in moving this area forward are many but can be largely overcome by appreciation of the experimental and conceptual limitations that have gone before. By developing more selective intervention strategies that can also be applied *in vivo* to dampen the pathological effects of increased CaMKII activity, it should be possible to determine which CaMKII sensitive components of the RyR₂ complex are effective for modulating both systolic and diastolic function in heart failure.

8. SUMMARY AND PERSPECTIVE

Phosphorylation of RyR₂ by CaMKII plays a key role in mediating the physiological and pathological function of SR Ca^{2+} release. Sites of phosphorylation include those sites on RyR₂ itself, as well as those on accessory proteins that form part of the larger channel complex. Recent identification of Ser 2815 as a key CaMKII phosphorylation site on RyR₂, has allowed a better understanding of how changes in phosphorylation status at this site relate to changes in channel activity, and the importance that this may have in normal inotropic function as well as in defective function in heart disease. Increasing evidence suggests that increased CaMKII-mediated phosphorylation of RyR₂ is a key mechanism underlying channel over-activity in heart failure. As such, the possibility of selectively modulating this activity with a view to therapeutic management of this effect, has recently received considerable attention. Evaluation of the results of reducing CaMKII phosphorylation of RyR₂ has revealed a beneficial effect in reduction of diastolic Ca^{2+} leak and the propensity for arrhythmias. Future challenges in this area include (i) a better understanding of the interaction and overlap between the PKA and CaMKII pathways in RyR₂ phosphorylation, (ii) identification of other CaMKII phosphorylation sites on RyR₂ and their functional relevance and (iii) identification of other CaMKII substrates (both luminal and cytosolic) that form part of the larger RyR₂ channel complex and may contribute to the functional effects that have been observed but are not fully understood. Novel, selective and controlled inhibition of

CaMKII-targeted to RyR₂ will be an important step in defining the contribution this interaction really has in mediating abnormal Ca²⁺ release and SR dysfunction in heart failure.

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Abbreviations: RyR₂: ryanodine receptor type 2; CaMKII: Ca^{2+} /calmodulin dependent protein kinase type II; SERCA: sarco/endoplasmic reticulum Ca^{2+} ATPase; PLB: phospholaman; PKA: cAMP-dependent protein kinase; SR: sarcoplasmic reticulum; ECC: excitation-contraction coupling; CICR: Ca^{2+} -induced Ca^{2+} release; NCX: Na^{+} - Ca^{2+} exchanger; FKBP: FK506-binding protein; PPI: phosphatase I; PP2A: phosphatase 2A.

Key Words: Ryanodine Receptor, Calcium, Sarcoplasmic Reticulum, Cam Kinase, Phosphorylation, Cardiac, Review

Send correspondence to: Susan Currie, SIPBS, University of Strathclyde, 27 Taylor Street, Glasgow G4 0NR, Scotland, UK, Tel: 00-44-141 548 2405, Fax: 00-44-141-552 2562, E-mail: susan.currie@strath.ac.uk

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