International Journal of Cardiology 181 (2015) 288–296

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Cardiac CaMKIIδ splice variants exhibit target signaling specificity and confer sex-selective arrhythmogenic actions in the ischemic-reperfused heart☆

James R. Bell [a], Antonia J.A. Raaijmakers [a], Claire L. Curl [a], Melissa E. Reichelt [a], Tristan W. Harding [a], Aier Bei [a], Dominic C.H. Ng [b], Jeffrey R. Erickson [c], Martin Vila Petroff [d], Stephen B. Harrap [a], Lea M.D. Delbridge [a,*]

[a] Department of Physiology, University of Melbourne, Victoria, Australia
[b] Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Victoria, Australia
[c] Department of Physiology, University of Otago, Dunedin, New Zealand
[d] Centro de Investigaciones Cardiovasculares, Conicet La Plata, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, La Plata 1900, Argentina

ARTICLE INFO

Article history:
Received 8 August 2014
Received in revised form 24 October 2014
Accepted 23 November 2014
Available online 26 November 2014

Keywords:
Cardiac myocytes
Ischemia/reperfusion
Ca^{2+} regulation
Ventricular arrhythmia
Sex-specific

ABSTRACT

Background: Ischemia-related arrhythmic incidence is generally lower in females (vs males), though risk is selectively increased in women with underlying cardiopathology. Ca^{2+}/calmodulin dependent kinase II (CaMKII) has been implicated in ischemia/reperfusion arrhythmias, yet the role of CaMKII in the ischemic female heart has not been determined. The aim of this study was to define the role and molecular mechanism of CaMKII activation in reperfusion arrhythmias in male/female hearts.

Methods and results: Male and female rat hearts and cardiomyocytes were subjected to multiple arrhythmogenic challenges. An increased capacity to upregulate autophosphorylated CaMKII (P-CaMKII) in Ca^{2+}-challenged female hearts was associated with an enhanced ability to maintain diastolic function. In ischemia/reperfusion, female hearts (vs male) exhibited less arrhythmias (59 ± 18 vs 548 ± 9, s, $p < 0.05$), yet had augmented P-CaMKII (2.69 ± 0.30 vs 1.50 ± 0.14, rel. units, $p < 0.05$) and downstream phosphorylation of phospholamban (1.71 ± 0.42 vs 0.90 ± 0.10, $p < 0.05$). In contrast, hypertrophic female hearts had more reperfusion arrhythmias and lower phospholamban phosphorylation. Isolated myocyte experiments (fura-2) confirmed Ca^{2+}-handling arrhythmogenic involvement. Molecular analysis showed target specificity of CaMKII was determined by post-translational modification, with $CaMKII\delta_B$ and $CaMKII\delta_C$ splice variants selectively co-localized with autophosphorylation and oxidative modifications of CaMKII respectively.

Conclusions: This study provides new mechanistic evidence that CaMKIIδ splice variants are selectively susceptible to autophosphorylation/oxidation, and that augmented generation of P-$CaMKII\delta_B$(Thr287) is associated with arrhythmia suppression in the female heart. Collectively these findings indicate that therapeutic approaches based on selective CaMKII splice form targeting may have potential benefit, and that sex-selective CaMKII intervention strategies may be valid.

1. Introduction

Ischemic heart disease is the leading cause of death in women and men, albeit with nuanced etiology and outcomes. Ischemia-related arrhythmic incidence is generally lower in females (vs males) [1–3], although mortality is increased after an ischemic event [4]. The risk of sudden cardiac death in women (not men) substantially increases when there is a myocardial oxidative stress state, such as occurs in hypertrophic pathologies [5–7].

Experimentally, we and others have shown that non-diseased female hearts exhibit a relative resistance to acute ischemia/reperfusion pathologies [8–10], including reduction in the severity of reperfusion arrhythmias, though the mechanisms responsible have not been elucidated. This female resilience is absent when hearts have an underlying hypertrophic pathology [9] and may be related to a down-regulation of the phosphoinositide 3-kinase/Akt (PI3-K/Akt) pathway [9]. Furthermore, we and others have reported fundamental sex steroid dependent differences between males/females in excitation–contraction coupling and cardiomyocyte Ca^{2+} handling processes [11,12], with Ca^{2+} challenged female cardiomyocytes exhibiting lower operational Ca^{2+} levels [13–15].

☆ Statement of authorship: All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

* Corresponding author at: Cardiac Phenomics Laboratory, Department of Physiology, University of Melbourne, VIC 3010, Australia.
E-mail address: lmd@unimelb.edu.au (L.M.D. Delbridge).

http://dx.doi.org/10.1016/j.ijcard.2014.11.159
0167-5273/

Given that Ca^{2+} overload is a key instigator of ischemia/reperfusion arrhythmias, interest has focused on the actions of Ca^{2+}/calmodulin dependent kinase II (CaMKII) and the therapeutic potential of its inhibition in a number of myocardial disease contexts. CaMKII is a ubiquitously expressed key signaling intermediate activated in response to alterations in cellular Ca^{2+} levels [16]. In the myocardium, CaMKII is expressed primarily as two splice variants (δ_B and δ_C) [17], and Ca^{2+}-dependent activation can be maintained through post-translational modification, including autophosphorylation and oxidation (P-CaMKII(Thr287) & ox-CaMKII(Met281/2) respectively) [18]. When active, CaMKII phosphorylates and functionally modulates many of the ion channels and transporters centrally involved in cardiac excitation-contraction coupling [16]. CaMKII activation is known to both augment loading and promote leakage of the cardiomyocyte internal sarcoplasmic reticulum (SR) Ca^{2+} store in a context specific manner [12,19–23], though selective transporter targeting processes are not yet delineated.

It is notable that studies of the role of CaMKII in arrhythmogenesis have exclusively been performed in male animal models despite the documented significant sex/gender differences in cardiomyocyte Ca^{2+} handling and ischemia/reperfusion responses. There is a surprising knowledge deficit regarding the role of CaMKII in the ischemic female heart. The lower Ca^{2+} operational levels observed in female myocytes could suggest a differential pattern of CaMKII activation associated with altered susceptibility to Ca^{2+}-dependent arrhythmogenesis.

New strategies are required to achieve sex-selective therapeutic efficacy, and CaMKII represents a high-potential interventional target. The aim of this study was to define how different CaMKII post-translational modifications mediate arrhythmias and inotropic status in response to high Ca^{2+} and ischemic challenges in male and female hearts. We demonstrate that CaMKIIδ splice variants are selectively modified by autophosphorylation and oxidation. We report that upregulation of the δ_B-associated P-CaMKII(Thr287) occurs concomitantly with a suppression of reperfusion arrhythmias in the female heart. In settings of both Ca^{2+} and ischemia provocations, we provide novel evidence that selective CaMKII post-translational modifications are associated with differential downstream signaling outcomes — and that CaMKII activation is hence not invariably associated with arrhythmogenesis, as has been previously reported to be the case (in studies which have involved male hearts only) [24]. These findings significantly refine the current view of CaMKII signaling processes. The results presented indicate that therapeutic approaches based on selective CaMKII splice form targeting may have potential, and that sex-selective CaMKII intervention strategies may be valid.

2. Methods

2.1. Animals

Male and female Sprague–Dawley (SpD) rats were obtained from the Animal Resources Centre (WA, Aus). The colony of Normal Heart Rats (NHR) and Hypertrophic Heart Rats (HHR) was derived as previously reported (see Supplementary material) [9,25,26]. All rats were age-matched (12–16 weeks) and maintained under identical conditions at the Biological Research Facility at the University of Melbourne, Australia. Experiments were conducted and animals handled in the manner specified by the NHMRC/CSIRO/ACC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997) and the EU Directive 2010/63/EU for animal experiments, with approval and oversight of the project by the University of Melbourne Animal Ethics Committee. Vaginal cytology was evaluated for estrous state and confirmed the non-cycling estrous status of female rodents (as previously described for segregated male/female housing conditions [27]). Rats were anesthetized with sodium pentobarbitone (60 mg/kg) and injected with sodium heparin (200 IU) via the femoral artery, prior to heart excision.

2.2. Cardiomyocyte Ca^{2+} transient measurements

Cardiomyocytes were isolated from hearts (SpD, NHR, HHR) by enzymatic digestion as described previously (see Supplement material) [28]. Cardiomyocytes were loaded with the Ca^{2+} fluorescent dye, Fura-2 AM (2.5 μmol/L), and superfused with a HEPES–Krebs buffer on an inverted light microscope. Cells were field stimulated and microfluorimetric measurements of cardiomyocyte Ca^{2+} were performed (IonOptix, Milton, MA, USA). Ca^{2+} transient amplitude was determined by the difference between peak systolic and diastolic Ca^{2+} ($F_{360/380}$). The rate of cytosolic Ca^{2+} removal in diastole was measured by determining the rate constant of Ca^{2+} signal decay (Tau, ms; exponential fit from 50% decay to 1/e). Male and female cardiomyocytes were subjected to one of three protocols; (i) superfused with serially increasing concentrations of $CaCl_2$ (1, 2, 3, 4, 5 mmol/L) for 3 min each (25 °C, 0.5 Hz; n = 15–18 cells, N = 6 hearts), (ii) superfused with simulated ischemia solution (136 mmol/L NaCl, 8 mmol/L KCl, 0.35 mmol/L $NaH_2PO_4 \cdot H_2O$, 1.05 mmol/L $MgSO_4 \cdot 7H_2O$, 2.0 mmol/L $CaCl_2$, 10 mmol/L HEPES, 0 mmol/L glucose, 10 mmol/L lactate, pH 6.8, N_2 gas saturation; n = 9–12 cells, N = 5–7 hearts, [29,30]) in the absence/presence of CaMKII inhibitor (KN93 hydrochloride, 2.5 μmol/L; Sigma-Aldrich, NSW, Australia), and (iii) superfused with isoproterenol (10 nmol/L) for 5 min (37 °C, 4 Hz) prior to 30 s non-paced to determine vulnerability to spontaneous contraction (n = 21–25 cells, N = 4–5 hearts). All data were analyzed off-line using IonWizard (IonOptix, Milton, MA, USA). Control experiments for the KN93 compound showed Ca^{2+} transients were not modified by the analogue agent KN92 (Fig. S1).

2.3. Isolated heart preparation

Isolated hearts were perfused aerobically and heart function was monitored and analyzed (ADInstruments, Bella Vista, NSW, Australia) as previously described (see Supplement material) [31,32] throughout one of four pathological perfusion protocols; (i) high Ca^{2+} (4 mmol/L) perfusate for 2 min, (ii) 20 min global ischemia (37.0 °C) and 2 min reperfusion, (iii) 20 min global ischemia and 10 min reperfusion in the absence/presence of the CaMKII inhibitor, KN93 hydrochloride (0.5 μmol/L; Sigma-Aldrich, NSW, Australia) for 10 min immediately prior to ischemia and throughout reperfusion (n = 8 hearts), (iv) hydrogen peroxide (200 μmol/L) for 2 min. Left ventricular pressure measurements were performed using a fluid-filled balloon connected to a pressure transducer (MLT844) and recorded on a MacLab data acquisition system (ADInstruments, Bella Vista, NSW, Australia). The balloon was inflated to produce an end-diastolic pressure of 4 mm Hg and the volume kept constant throughout the perfusion protocol. Arrhythmia quantification relied on mechanical analysis, in order to directly evaluate ventricular functional outcomes of electrical instability (rather than infer function indirectly from electrical record, [32]).

2.4. Immunoblotting

Left ventricular tissue was homogenized and fractionated (Fig. S2) as previously described [33]. Homogenate was reconstituted into 2× SDS sample buffer and equal volumes were loaded onto polyacrylamide gels for SDS-PAGE and subsequent immunoblot analysis. Antibody selection, previous validation details, and immuno-imaging methods are provided in the Supplementary material.

2.5. Statistical analysis

Results are presented as mean ± SEM. Comparisons between two groups with normally distributed data was performed with a Student's unpaired t-test. Data from experiments with two groups assessed at multiple time-points were evaluated by a one-way analysis of variance (ANOVA) with repeated measures. Experiments incorporating two groups with two characteristics were assessed by two-way ANOVA with Fisher's least significant difference (LSD) post-hoc analysis. The statistical significance of the difference between correlation coefficients was evaluated by comparing z scores to calculate the observed value of z_{obs}. Differences were considered significant at $P < 0.05$. All statistical calculations were performed using SPSS v.21.0 (SPSS, Chicago, IL).

2.6. On-line Supplementary material

Supplementary materials (Figs. S1–S8) may be located in the on-line supplement.

3. Results

3.1. Accentuated CaMKII response associated with preserved diastolic function in Ca^{2+}-challenged female myocytes and hearts

Functional evidence shows that female cardiomyocytes operate at a lower operational Ca^{2+} level than males [12,22], but the contribution of CaMKII signaling to these differences is unknown. Though the absolute concentration of cardiomyocyte CaMKII in males and females is not known, the relative basal expression of CaMKII (δ_B and δ_C analyzed either individually or combined) was comparable in male and female adult rat hearts under normoxic conditions (Figs. 1 & S3). Similarly, expression of the sarcoplasmic reticulum Ca^{2+} release channel (RyR2), sarcoplasmic reticulum Ca^{2+} ATPase 2a (SERCA2a), phospholamban (PLB), and the SERCA2a:PLB ratio were not different in male and female hearts (Fig. 1), as were levels of ox-CaMKII(Met281/2) and

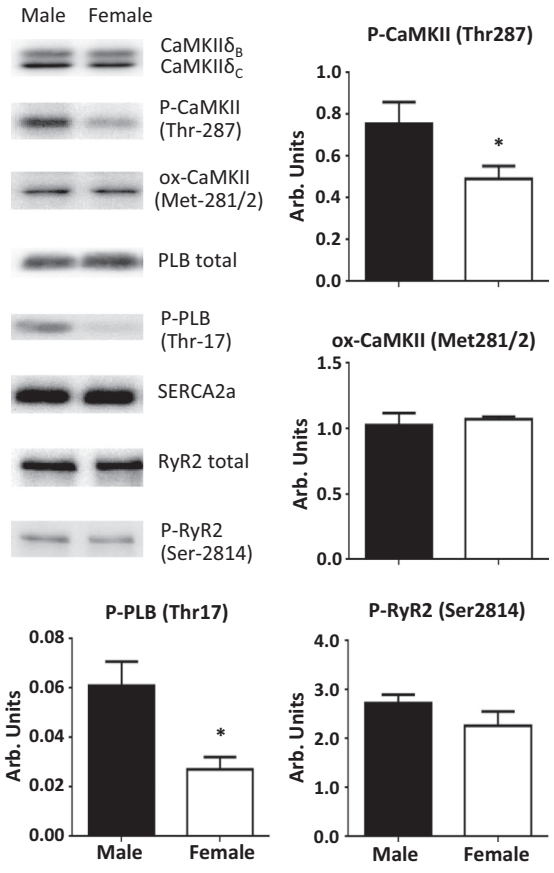

Fig. 1. Basal CaMKII activation is suppressed in the female heart. Western blot analysis of total homogenate from aerobically perfused hearts showed levels of P-CaMKII(Thr287) and the downstream substrate, P-PLB(Thr17), were significantly lower in female vs male hearts. No difference was observed in ox-CaMKII(Met281/2) or P-RyR2(Ser2814), nor in the expression of total CaMKII, PLB, SERCA2a or RyR2 between male and females (*$p < 0.05$, Student's t-test, mean ± SEM, n = 8/group).

phosphorylated RyR2 at the serine 2814 residue (P-RyR2(Ser2814)). In contrast, P-CaMKII(Thr287) and phosphorylation of PLB at the threonine 17 residue (P-PLB(Thr17)) were lower in female hearts, suggesting less basal activity of CaMKII in these hearts consistent with a lower operational Ca^{2+} level.

The sex-specific functional effects of extracellular Ca^{2+} (Ca^{2+}_o) challenge on diastolic stability, systolic response and CaMKII activation were evaluated in isolated myocytes and Langendorff hearts. The rise in diastolic Ca^{2+} in cardiomyocytes superfused with high concentrations (4 & 5 mmol/L) of Ca^{2+}_o was lower in females than males (Fig. 2A), indicating an enhanced capacity in the females to clear cytosolic Ca^{2+} in diastole. Indeed, in hearts perfused with 4 mmol/L Ca^{2+} (2 min), females maintained diastolic function, while male left ventricular end diastolic pressure (LVEDP) deteriorated significantly (Fig. 2B). Within 90 s of the switch to high Ca^{2+} (4 mmol/L), male hearts exhibited an increase in LVEDP that was absent in female hearts. Moreover, female hearts displayed an increase in relaxation rate (dP/dt min) relative to basal levels. There was also a trend (non-significant) towards decreased ectopic incidence in Ca^{2+}-challenged female hearts (vs males), though heart rate was not different throughout the high Ca^{2+} perfusion (female vs male, heart rate, bpm; 281 ± 12 vs 276 ± 17, p = ns). High Ca^{2+} challenge elicited an augmented CaMKII activation in females compared to males, with an accentuated increase in P-CaMKII(Thr287) and P-PLB(Thr17), but not P-RyR2(Ser2814) (Fig. 2C). This enhanced female CaMKII recruitment in response to a Ca^{2+} challenge may account for the greater capacity to maintain diastolic function and cytosolic Ca^{2+} levels. These findings suggest the possibility that CaMKII activation may confer benefit in certain settings.

3.2. Augmented CaMKII activation despite fewer reperfusion arrhythmias in female hearts

Cytosolic Ca^{2+} loading is an underlying cellular etiology of ischemia/reperfusion pathologies, including reperfusion arrhythmias, and CaMKII activation is implicated. Thus, we sought to determine whether CaMKII recruitment and/or signaling actions were attenuated in female hearts subjected to ex vivo ischemia/reperfusion. At an early time-point of reperfusion (2 min) previously reproducibly shown to be associated with maximal CaMKII recruitment [30], females exhibited augmented P-CaMKII(Thr287) generation and substrate phosphorylation of P-PLB(Thr17) and P-RyR2(Ser2814) (Fig. 3A). Extrapolating from previous reports of data from male animals, these phosphorylation events would predict an increase in arrhythmias in reperfused female hearts. However, arrhythmic incidence and severity were significantly decreased in female hearts during the first 10 min of reperfusion (Fig. 3B & C). CaMKII inhibition (KN93) reduced the total duration and incidence of ventricular tachycardia and/or fibrillation (VT and/or VF) in males (as we have reported previously) [34], but had no effect in female hearts. These findings suggest pleiotropic effects of CaMKII on arrhythmia in reperfusion. CaMKII activation may not be invariably pro-arrhythmic and in stress-responsive female hearts may be associated with beneficial arrhythmia suppression.

Female and male isolated cardiomyocytes exhibited distinctly different responses to simulated 'ischemia/reperfusion'. Marked elevation in diastolic Ca^{2+} and suppression of Ca^{2+} transient amplitude was observed in control male myocytes (Fig. 4A & B). Simulated 'reperfusion' was associated with some recovery of Ca^{2+} transient kinetics for both control male and female myocytes (shorter 'tau'). Treatment with KN93 revealed that in female myocytes, a more robust systolic function was sustained (Ca^{2+} transient amplitude, Fig. S4) through greater dependence on CaMKII activation, as evidenced by marked transient prolongation in KN93-treated female cardiomyocytes (longer tau; Fig. 4C). Ca^{2+} transients were not modified by the KN93 analogue agent KN92 (Fig. S1). Thus, data derived from both intact hearts (Figs. 2 & 3) and isolated myocytes (Fig. 4) exposed to Ca^{2+} load or ischemic conditions provided evidence of an important role for P-CaMKII(Thr287) signaling in stimulating SR Ca^{2+} uptake in stress-response settings, which may contribute to the relative suppression of arrhythmias in female hearts (Fig. 3C).

3.3. Underlying hypertrophic pathology undermines arrhythmia suppression in females

As women are more susceptible to sudden cardiac death when an underlying left ventricular hypertrophy is present [5,7], we assessed cardiac CaMKII activation and contractile arrhythmogenicity in the female Hypertrophic Heart Rat (HHR) — a polygenic model of primary hypertrophy [9,25]. Compared to control NHR, HHR female hearts were more arrhythmic immediately post-ischemia, exhibiting more extensive VT and/or VF in the first 10 min of reperfusion (Fig. 5A). In isoproterenol-treated adult cardiomyocytes, following suspension of electrical pacing, spontaneous beats occurred earlier and more frequently in HHR females vs NHR (Fig. 5B). Immediately post-ischemia, a suppressed P-PLB(Thr17) response was seen in the hypertrophied female hearts (Fig. 5C). Interestingly no difference was observed in P-CaMKII(Thr287), or in ox-CaMKII(Met281/2) between HHR and NHR hearts, and equivalent levels of P-RyR2(Ser2814) were also observed. Expression levels of CaMKII, PLB, SERCA2a and RyR2 were not different (Fig. S5). These data show that females can be vulnerable to arrhythmias in the presence of an underlying cardiac hypertrophy, which may be related to a downregulation of SR Ca^{2+} reuptake mediated via the CaMKII-specific phosphorylation site of PLB.

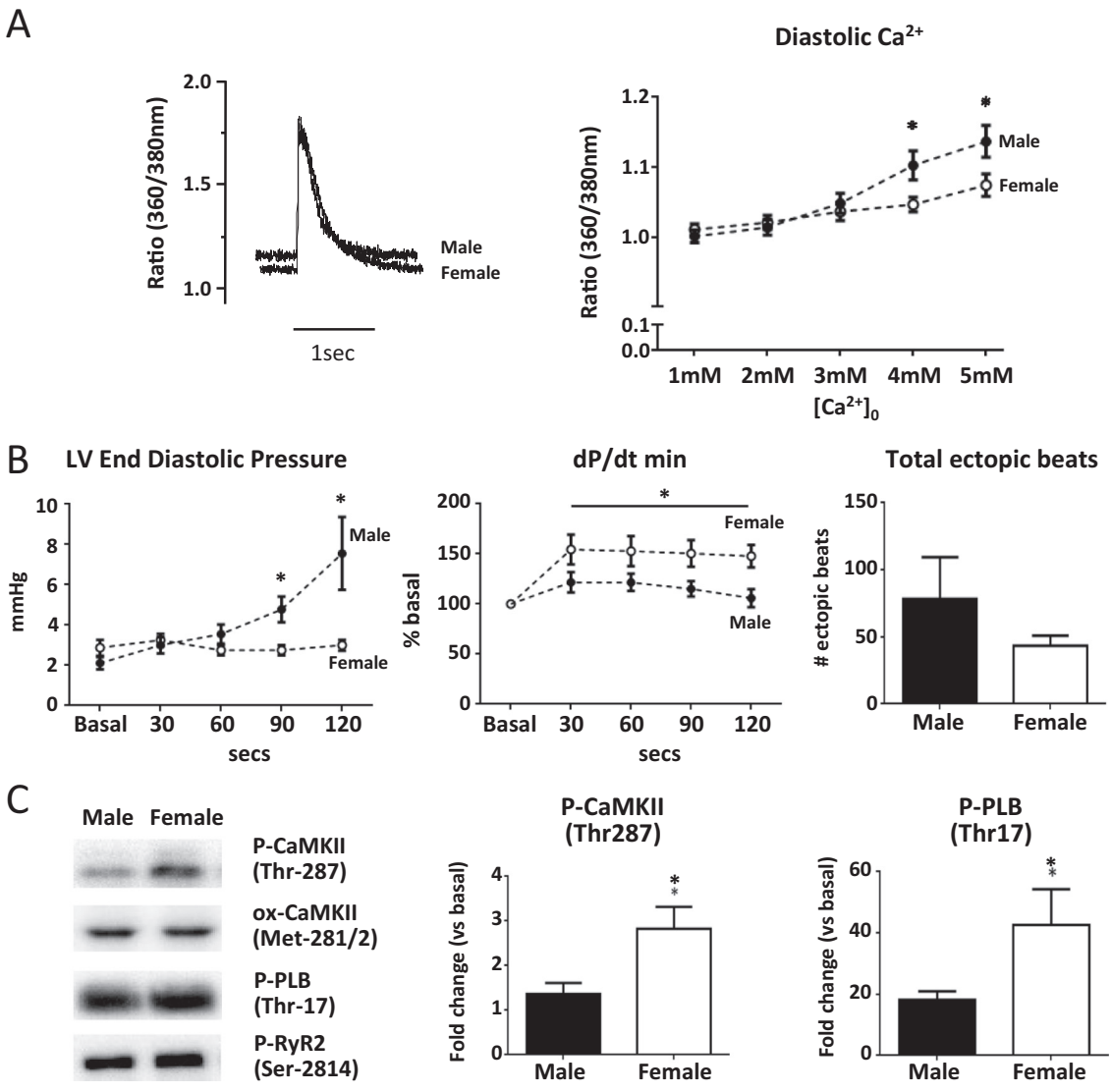

Fig. 2. Accentuated CaMKII response to Ca^{2+} challenge associated with preserved diastolic function in female myocytes and hearts. A, Representative trace of Fura2 Ca^{2+} transients from male and female cardiomyocytes shows that diastolic Ca^{2+} is greater in males vs females when challenged with high concentration (5 mM) of extracellular Ca^{2+} (*$p < 0.05$, individual concentration analysis, mean ± SEM, n = 17 myocytes from N = 6 hearts/group). B, In isolated hearts perfused with 4 mM Ca^{2+} Krebs, male hearts exhibited increased LVEDP which was absent in female hearts. This was associated with a greater increase in the dP/dt_{min} (vs basal) in the female hearts and a trend towards less ectopic incidence (*$p < 0.05$, individual time-point analysis, mean ± SEM, n = 8–9 hearts/group). C, At the end of the 2 min high Ca^{2+} challenge, the relative increase in P-CaMKII(Thr287) and P-PLB(Thr17) (fold change vs 1.4 mM Ca^{2+} control) was greater in females vs males (*$p < 0.05$, Student's t-test, mean ± SEM, n = 7 hearts/group).

3.4. CaMKIIδ post-translational modification has a role in determining downstream target signaling specificity

In contrast to previous reports, our findings suggest that CaMKII may not always be pro-arrhythmic. Specifically, a reduced arrhythmogenic propensity was evident when P-CaMKII(Thr287) recruitment was augmented coincident with an increased phosphorylation of the downstream target, phospholamban (P-PLB(Thr17)) (Fig. 3). Thus evidence of a relationship between CaMKII activation type and specific downstream signaling outcome was sought.

To assess the specificity of P-/ox-CaMKII for P-PLB(Thr17) and P-RyR(Ser2814), the relative phosphorylation/oxidation of CaMKII was directly compared to the extent of PLB or RyR phosphorylation on an individual heart basis (Fig. 6). For each protein analyzed, values for individual hearts were normalized, assigning the largest a value of 1 and the lowest a value 0, with intermediate values spread proportionally. As the fundamental properties of CaMKII target specificity were being assessed, all values for male and female hearts were incorporated in this analysis.

In hearts perfused with 200 μM H_2O_2 to increase oxidation of CaMKII, a striking inverse relationship was observed between the extent of ox-CaMKII(Met281/2) and P-PLB(Thr17). That is, high P-PLB(Thr17) values were associated with low ox-CaMKII(Met281/2) and vice versa (Fig. 6A). This contrasts with the proportional relationship evident between P-CaMKII(Thr287) and P-PLB(Thr17) (Fig. 6B) in hearts perfused with high Ca^{2+} to optimally increase CaMKII autophosphorylation. These differences in P-CaMKII(Thr287) and ox-CaMKII(Met281/2) ability to phosphorylate PLB were confirmed by regression analysis (Fig. S6). For P-RyR(Ser2814), the extent of phosphorylation conferred by both ox- and P-CaMKII types showed similar patterns (Fig. 6C & D respectively, and Fig. S6B). These relationships did not differ between male and female hearts (Fig. S7). Taken together, these data indicate that the type of CaMKII post-translational modification confers target protein phosphorylation selectivity, thereby determining the arrhythmogenic properties of CaMKII. Hence, in arrhythmia-resistant reperfused female hearts, the elevated P-CaMKII(Thr287) is linked with augmented P-PLB(Thr17), increased SR Ca^{2+} uptake and lower cytosolic Ca^{2+} levels.

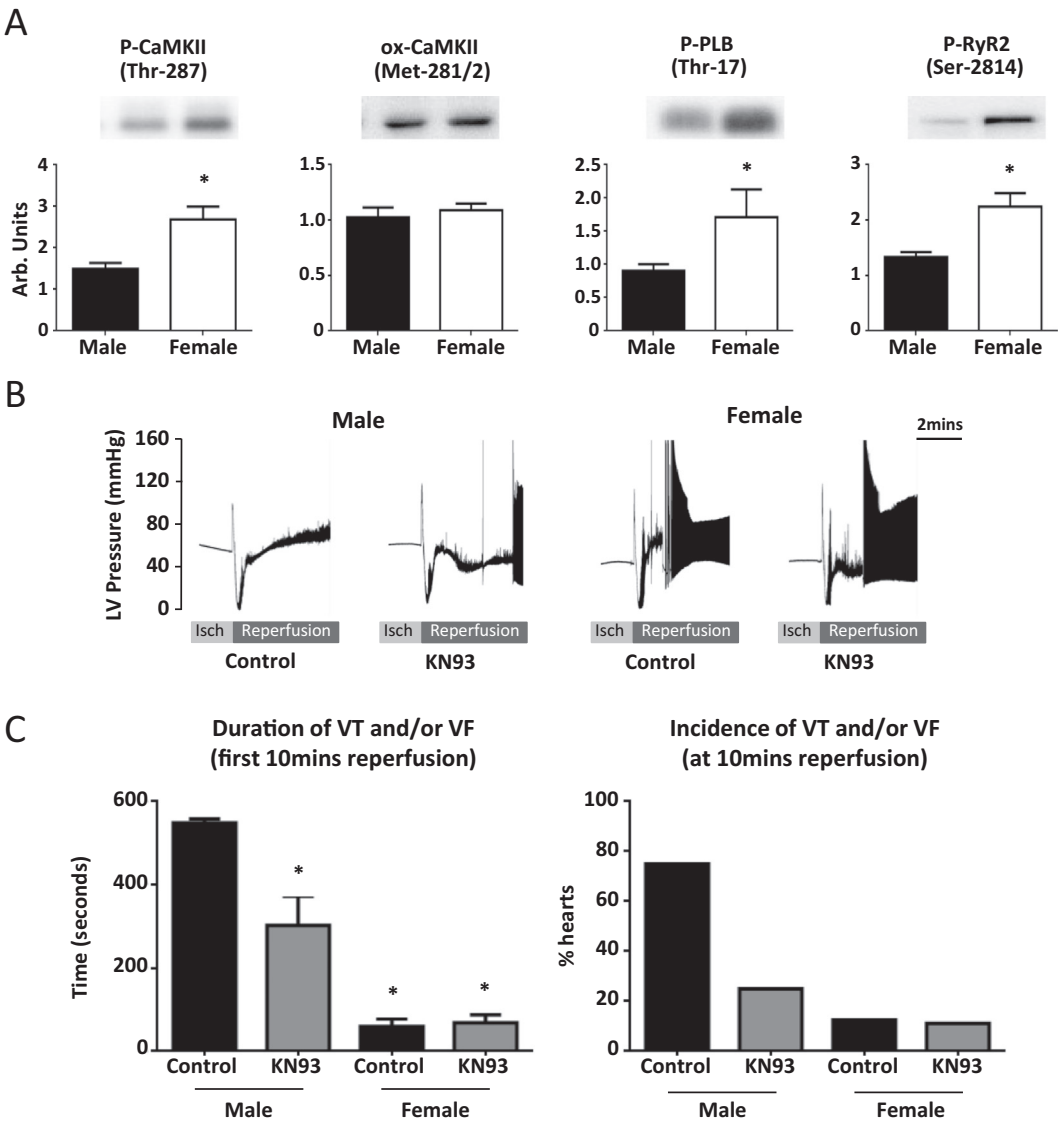

Fig. 3. Augmented CaMKII activation in reperfusion, despite less arrhythmias in female hearts. A, In isolated hearts at 2 min reperfusion, augmented P-CaMKII(Thr287) generation and substrate phosphorylation of P-PLB(Thr17) and P-RyR2(Ser2814) was observed in females (*$p < 0.05$, Student's t-test, mean ± SEM, n = 7 hearts/group). B, Time-compressed representative traces show the left ventricular (LV) pressure in early reperfusion. C, In non-treated hearts, both the total duration of ventricular tachycardia and/or fibrillation during 10 min reperfusion and the incidence at 10 min reperfusion was significantly lower in females vs males. Perfusing hearts with KN93 significantly decreased the duration/incidence of these arrhythmias in male hearts, but not female hearts (*$p < 0.05$ vs male control, two-way ANOVA with post-hoc analysis, mean ± SEM, n = 8–9 hearts/group).

3.5. Differential susceptibility of CaMKIIδ splice variants to autophosphorylation and oxidation

Conventionally (i.e. in males), CaMKIIδ_C is understood to mediate arrhythmogenic activity while CaMKIIδ_B is largely (but not exclusively) restricted to nuclear transcriptional regulation. Limited evidence indicates that CaMKIIδ splice variants may exert opposing actions in pathological settings associated with ischemia/reperfusion [35–37]. We are not aware of any studies investigating a link between post-translational modification type and splice variant activity. Male and female normoxic heart samples were fractionated according to density and triton solubility — fractions nominally termed 'cytosolic' (i.e. supernatant), 'membrane' (triton-soluble) and 'nuclear' (including myofilament, triton-insoluble). Using all values from male and female hearts, Fig. 7 shows that CaMKIIδ_B was predominantly localized in the nuclear/myofilament fraction (80%), contrasting with CaMKIIδ_C which was somewhat enriched in the membrane fraction (66%). Most compelling were the observations that, P-CaMKII(Thr287) and ox-CaMKII(Met281/2) closely co-localized with CaMKIIδ_B and CaMKIIδ_C respectively, suggesting a differential susceptibility of splice variants to autophosphorylation and oxidative modifications. These findings suggest that the reciprocal relationship between P-CaMKII(Thr287) and the suppression of arrhythmias may at least partly be conferred by the selective recruitment of the δ_B splice variant, exerting beneficial actions through currently unknown mechanisms [36]. We did not detect sex differences in the localization of CaMKII splice variants or post-translational modifications, indicating that the signaling modes of CaMKII are conserved between sexes. Together these results indicate that the observed differences in CaMKII signaling activation in male and female hearts are attributable to subcellular environmental conditions that selectively promote the modification and recruitment of different CaMKII splice variants.

4. Discussion

This study provides new mechanistic evidence that CaMKIIδ splice variants are selectively susceptible to autophosphorylation/oxidation, and that when upregulation of the δ_B-associated P-CaMKII(Thr287) is

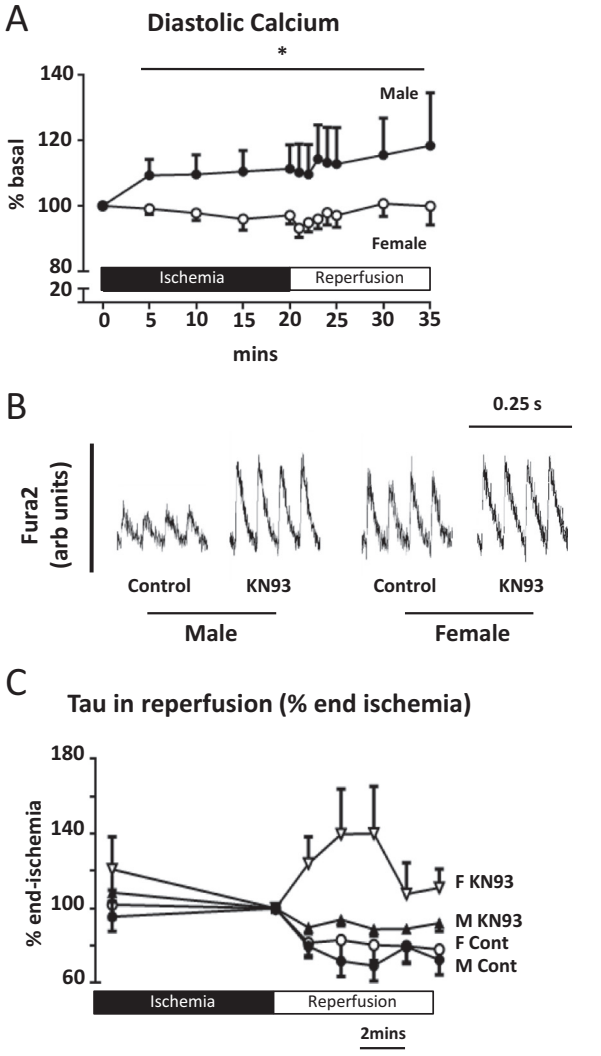

Fig. 4. CaMKII inhibition in 'simulated' ischemia/reperfusion selectively improves male myocyte function. A, Male cardiomyocytes exhibited a greater rise in diastolic Ca^{2+} vs females (*$p < 0.05$, ANOVA with repeated measures, mean ± SEM, n = 6–8 myocytes, N = 5 hearts/group). B, Representative traces of Ca^{2+} transients from male and female cardiomyocytes in early reperfusion show that transient amplitude was suppressed in control male myocytes not treated with KN93 (see Fig. S4). C, In the initial 5 min of simulated reperfusion, tau (time constant of transient decline) decreased rapidly in both male and female control cardiomyocytes. This reduction was attenuated in male cardiomyocytes treated with KN93. In female cardiomyocytes, tau actually increased in early reperfusion (*$p < 0.05$, two-way ANOVA with repeated measures, mean ± SEM, n = 5–8 myocytes, N = 3–5 hearts/group). Control experiments for the KN93 compound showed Ca^{2+} transients were not modified by the analogue agent KN92 (Fig. S1).

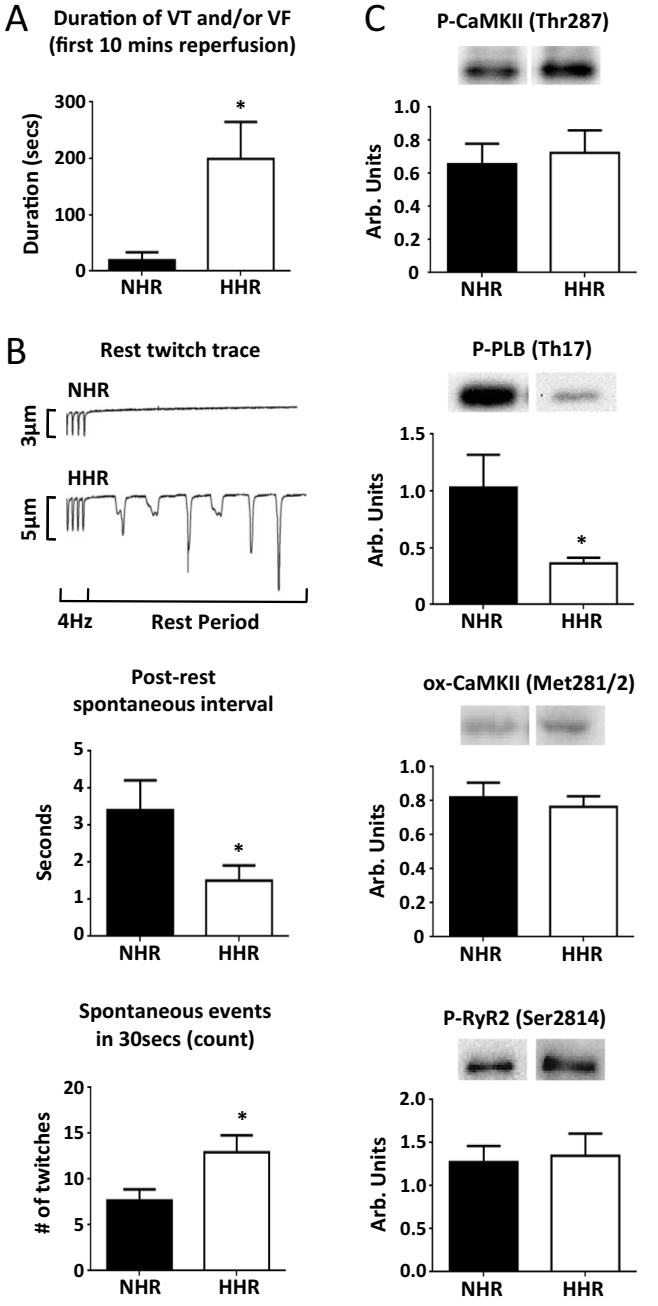

Fig. 5. Underlying cardiac hypertrophic pathology in female increases arrhythmia vulnerability, yet attenuates CaMKII-mediated SERCA function. A, Female HHR hearts subjected to ischemia/reperfusion were more susceptible to ventricular tachycardia and/or fibrillation in early reperfusion vs NHR (*$p < 0.05$, Student's t-test, mean ± SEM, n = 8–10 hearts/group). B, Non-paced female HHR cardiomyocytes exhibited spontaneous activity earlier and to a greater extent in the presence of isoproterenol (10 mmol/L) vs NHR controls (*$p < 0.05$, Student's t-test, mean ± SEM, n = 21–25 myocytes, N = 4–5 hearts/group). C, In female HHR hearts subjected to ischemia and 2 min reperfusion, no change was observed in CaMKII post-translational modification or P-RyR2(Ser2814). However, there was a significant attenuation of P-PLB(Thr17) in female HHR, indicative of reduced SERCA2a activity (*$p < 0.05$, Student's t-test, mean ± SEM, n = 8 hearts/group). Data showing the total expression of these proteins is shown in Fig. S5.

accentuated, concomitant reduction in arrhythmogenicity is observed. Through comparison of male and female hearts, our findings demonstrate in a physiologically relevant setting, that CaMKII activation is not invariably associated with arrhythmogenesis, as has been previously understood (predicated on studies involving male hearts) [24]. In both intact heart and isolated cardiomyocytes, using two different arrhythmogenic challenges (ischemia/reperfusion, high $Ca^{2+}{}_o$) we demonstrate a differential relationship between CaMKII post-translational modification type and arrhythmic propensity in male and female myocardium. Splice variant localization analysis demonstrates that the P-CaMKII(Thr287) activation (linked with beneficial arrhythmia suppression action) is predominantly mediated by the δ_B splice-form, while ox-CaMKII activation (Met281/2) linked with deleterious pro-arrhythmic action occurs substantially via the δ_C splice-form. The splice variant forms show contrasting subcellular localization patterns. The P- and ox-CaMKII types exhibit differential selectivity in activating downstream signaling targets. Collectively these findings indicate therapeutic approaches based on selective CaMKII splice form targeting may have potential, and that sex-selective CaMKII intervention strategies may be valid. Given the ubiquitous tissue occurrence of CaMKII, our findings offer insight into mechanisms underlying context-specific CaMKII isoform signaling processes — and how signaling processes in general may be nuanced in a sex-dependent manner.

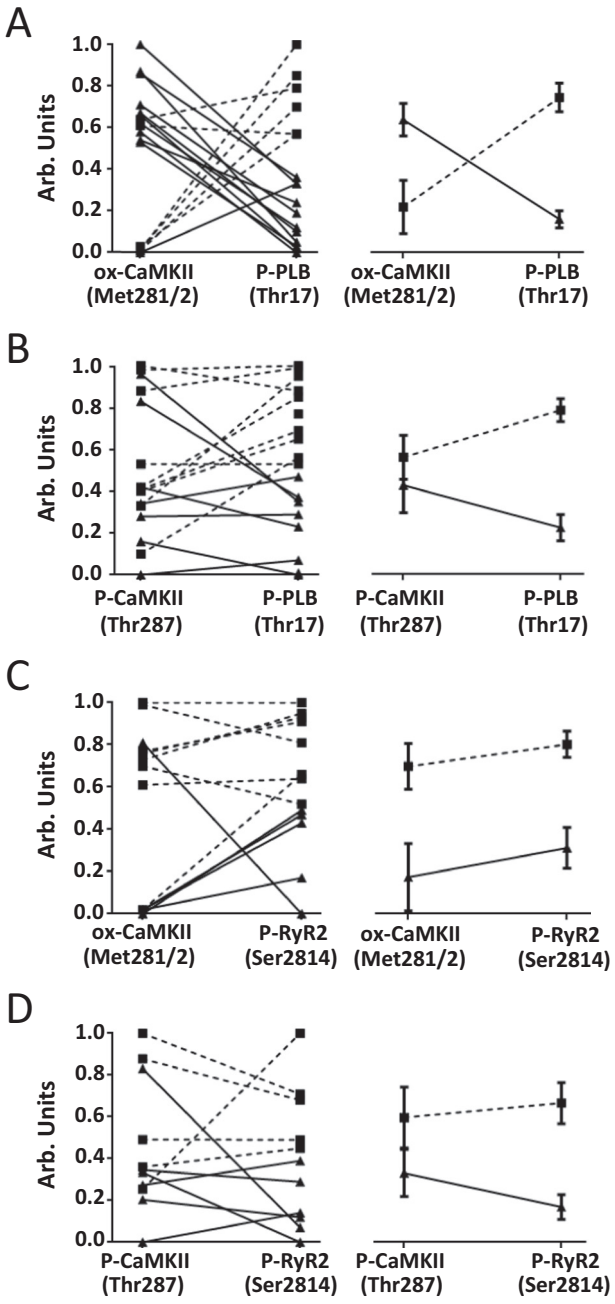

Fig. 6. CaMKII post-translational modification influences substrate specificity. Analysis of specificity of P-/ox-CaMKII for P-PLB(Thr17) and P-RyR(Ser2814). For each protein analyzed, values for individual hearts (all values from male and female hearts) have been normalized, assigning the largest a value of 1 and the lowest a value 0, with intermediate values spread proportionally. Individual and mean data are shown (top and bottom graphs respectively for each panel), with hearts exhibiting highest or lowest levels of P-PLB(Thr17) or P-RyR(Ser2814) linked to their corresponding CaMKII value by a dotted or intact line respectively. A, When oxidation of CaMKII was promoted (H_2O_2), an inverse relationship between ox-CaMKII(Met281/2) and P-PLB(Thr17) was observed. Low ox-CaMKII(Met281/2) levels were associated with high P-PLB(Thr17). B, In hearts perfused with high Ca^{2+}, to optimally increase autophosphorylation, P-CaMKII(Thr287) and P-PLB(Thr17) levels were modulated in parallel. C & D, In contrast, for P-RyR2(Ser2814), similar relationships were observed for both ox-CaMKII(Met281/2) and P-CaMKII(Thr287). Differences in P-CaMKII(Thr287) and ox-CaMKII(Met281/2) ability to phosphorylate PLB were confirmed by regression analysis (see Fig. S6). These data indicate that the type of CaMKII post-translational modification confers target protein phosphorylation selectivity.

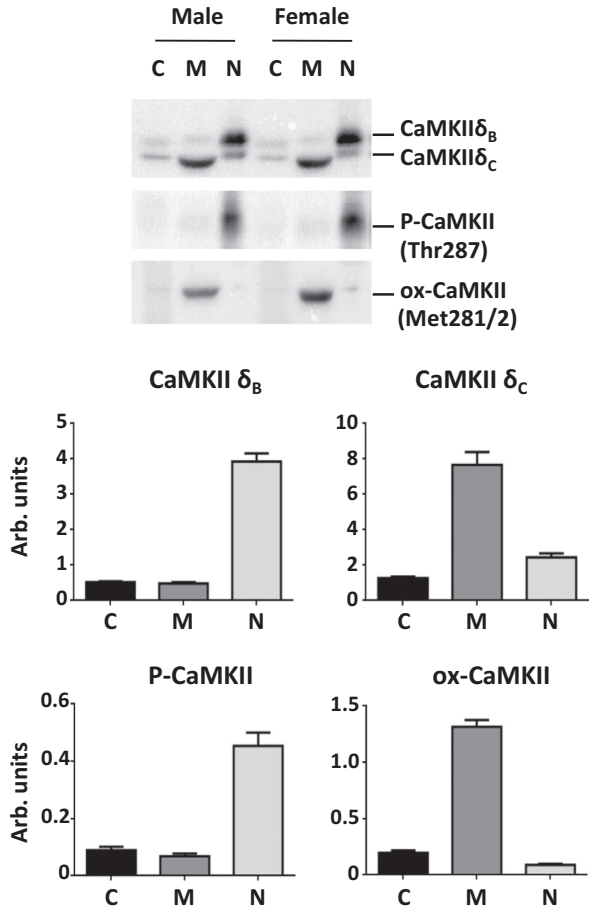

Fig. 7. CaMKII post-translational modification is selectively associated with different CaMKIIδ splice variants. CaMKIIδ$_B$ was predominantly localized in the nuclear/myofilament fraction, contrasting with CaMKIIδ$_C$ which was enriched in the membrane fraction. P-CaMKII(Thr287) and ox-CaMKII(Met281/2) closely co-localized with CaMKIIδ$_B$ and CaMKIIδ$_C$ respectively. C, cytosolic (soluble fraction); M, membrane (Triton-soluble); N, nuclear/myofilament (Triton-insoluble) fractions (Fig. S2). Localisation profiles for these splice variants and post-translational modifications in stress response settings are shown in Fig. S8.

4.1. Augmented CaMKII recruitment is not pro-arrhythmic in female hearts

Female hearts exhibited lower basal P-CaMKII(Thr287) and P-PLB(Thr17) levels. This is consistent with both the lower operational Ca^{2+} level reported in female cardiomyocytes [12,38] and a lower cardiac CaMKII enzymic activity described in female hearts [39]. This may benefit the female myocyte in dealing with Ca^{2+} load stresses, conferring a greater capacity to increase SERCA2a activity (through augmented phospholamban phosphorylation) and enhance cytosolic Ca^{2+} uptake into SR. Indeed, compared with males, female cardiomyocytes were better able to maintain diastolic Ca^{2+} and isolated hearts were better able to maintain end diastolic pressure.

Augmented activation of CaMKII was associated with more robust reperfusion recovery in female hearts, and was associated with arrhythmia suppression. This was initially a surprising observation as we and others have shown (in males) that inhibiting CaMKII activation (by multiple different interventions) in ischemia/reperfusion substantially reduces the incidence of arrhythmias in early reperfusion [34,40]. While activation levels of ox-CaMKII(Met281/2) in male and female hearts were similar, recruitment of P-CaMKII(Thr287) was markedly higher in the arrhythmia 'protected' female heart (Fig. 3A, C).

Our isolated myocyte experiments identify the cellular Ca^{2+} handling mechanisms involved, showing that CaMKII-mediated actions on

PLB and SERCA2a (promoting SR Ca^{2+} uptake and maintaining diastolic cytosolic Ca^{2+} levels) were particularly prominent in females. CaMKII inhibition in female myocytes revealed more marked reliance on CaMKII activation to manage SR Ca^{2+} uptake. In the isolated heart, the minimal 'net' impact of CaMKII inhibition on arrhythmic activity in females (i.e. Fig. 3C) reflects the coincident suppression of both ox- and P-CaMKII signaling pathways. In contrast, in the male hearts, where P-CaMKII(Thr287) activation is more modest, the overall effect of CaMKIII inhibition is arrhythmia suppression. We also show in a disease setting, that when the intrinsic female capacity to mount an enhanced P-CaMKII(Thr287) response is abrogated, arrhythmic vulnerability is apparent. Indeed, in female hearts with an underlying hypertrophic phenotype (HHR), the augmented P-PLB(Thr17) was absent and the arrhythmogenic responses to reperfusion heightened (Fig. 5). It is not clear why a concurrent reduction in P-CaMKII(Thr287) was not evident (at this time point), though this may reflect a diminished Akt expression in female HHR hearts [9]. Akt has previously been linked with phosphorylation of the canonical CaMKII-specific Thr17 residue of PLB [41].

Arrhythmia suppression in reperfusion in females is observed even though P-RyR2(Ser2814) levels are higher than male levels. Increased P-RyR2(Ser2814) might be expected to confer SR leakiness and promote depolarizing arrhythmogenic current (via Na^{+}–Ca^{2+} exchange) [42,43], but this proposition is not consistent with the observed capacity of 'reperfused' female myocytes to maintain low diastolic Ca^{2+} levels (Fig. 4). CaMKII regulation of RyR2 is complex and understanding not yet resolved [24,40,44,45]. It is possible that local RyR2 redox environment modifies the outcome of CaMKII activation, and that subtly different sex-specific conditions pertain. Our data are consistent with very recent findings that CaMKII actions on PLB and RyR can have contrasting actions on ischemia/reperfusion injury, with CaMKII-mediated P-PLB(Thr17) opposing and P-RyR2(Ser2814) exacerbating ischemia/reperfusion injury [46].

4.2. CaMKII and arrhythmogenic properties — activation type, splice variants and subcellular localisation

A key finding to emerge from this study is that oxidized and phosphorylated CaMKII types exhibit different interactions in relation to phospholamban phosphorylation (P-PLB(Thr17)) (Fig. 6). Our observations suggest a role for the type of post-translational modification of CaMKII in determining its target specificity, and/or a modulated susceptibility of PLB to phosphorylation by CaMKII in pro-oxidant conditions. Both these scenarios suggest that settings where P-CaMKII(Thr287) is augmented (as in the reperfused, normotrophic female heart) may selectively stimulate P-PLB(Thr17) and SERCA activity to promote cytosolic Ca^{2+} removal, maintain low diastolic Ca^{2+} and reduce arrhythmic vulnerability. Here we show that these post-translational modifications may be selective for either the CaMKIIδ_B or CaMKIIδ_C splice variants (Fig. 7), with P-CaMKII(Thr287) closely co-localized with CaMKIIδ_B in the nuclear/myofilament fraction, and ox-CaMKII(Met281/2) enriched in the membrane fraction with CaMKIIδ_C. Splice variant involvement in specific cardiopathology states has been previously identified [36,47,48] — and here we advance this understanding by extending splice form links to post-translational modification signaling targets. Deleterious actions of CaMKII have been attributed to the δ_C splice variant, including the promotion of Ca^{2+} sparks and reduction of SR Ca^{2+} levels, in contrast to the more favorable actions conferred by CaMKIIδ_B in oxidative stress settings [36].

It is important to emphasize that no sex differences were observed in splice variant compartmentalization or co-association with P-/ox-post-translational modifications. This implies that the fundamental properties of the CaMKII signaling mechanisms are not different between the sexes. Rather, sex difference in the extent of splice variant post-translational modifications with significant impact on downstream signaling outcomes is apparent, and this may have important implications in determining male/female responses to acute ischemia/reperfusion. The augmented P-CaMKII(Thr287) in early reperfusion in female, with specific upregulation of CaMKIIδ_B in these hearts may be of benefit in suppressing reperfusion arrhythmias. In contrast, in males lower P-CaMKII(Thr287) (and presumably less recruitment of CaMKIIδ_B) may be less effective in offsetting the actions of ox-CaMKIIδ_C to promote SR Ca^{2+} leak and arrhythmogenesis.

It is noteworthy that our observations of CaMKII isoform and type signaling specificity derive from physiologic settings, where all innate signaling components are intact and operational. Studies using genetic models of CaMKII manipulation have provided important insight — albeit always with the reservation that non-physiological redundant signaling adaptations must to be considered. In particular, interpretation of findings from knockout models to evaluate isoform contributions to signaling pathways in the heart is problematic, as it has been demonstrated that even when the predominant δ_B and δ_c forms are substantially ablated, residual CaMKII enzymatic activity is significant apparently due to ectopic isoform expression [49]. Here we are able to exploit inherent sex contrast, to advance understanding of the mechanisms underlying cardiomyocyte CaMKII responses in 'wild-type' context.

4.3. Study limitations and conclusions

The findings reported here identify CaMKII signaling differentials in the intact heart at a single timepoint in the reperfusion period. Further evaluation of a range of timepoints, and an extended time-course are required to fully characterize the response. In addition, new experiments which evaluate sex-specificity of responses in different disease settings will provide important insights into the range of clinical contexts where sex-selective interventions might be anticipated to be of greatest efficacy.

In conclusion, this study shows differential activation and modification of CaMKII in hearts of male and female rodents, provides the first evidence that CaMKII exhibits splice variant specific post-translational modification, and further elucidates the relationship between CaMKII activation and arrhythmogenesis. These findings imply that, in addition to the development of global CaMKII inhibitors as a first-line defense against lethal ventricular arrhythmias, further benefit may be gained by targeting an upregulation of an autophosphorylated form of the CaMKIIδ_B splice variant as a highly selective anti-arrhythmic intervention. Importantly, the efficacy of these interventional strategies may be sex-specific and dependent on the occurrence of underlying myocardial co-pathologies.

Conflicts of interest

None declared.

Acknowledgments

Career support was provided through the National Heart Foundation of Australia (PF11M5999) and University of Melbourne R. Douglas Wright Faculty Trust (JRB), the National Health and Medical Research Council (628841; MER), Australian Research Council (FT120100193; DCHN), and Marsden Fund of the Royal Society of New Zealand (UOO1303; JRE). Research support was provided through Grant-in-Aid from the National Heart Foundation of Australia (G10M5122; LMDD, JRB & CLC).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijcard.2014.11.159.

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