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Location matters: clarifying the concept of nuclear and cytosolic CaMKII subtypes

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Abstract

Rationale—Differential effects of δ_B and δ_C subtypes of Ca^{2+}/calmodulin dependent protein kinase (CaMKII) on cardiomyocyte Ca^{2+} handling and survival have been suggested to result from their respective nuclear vs. cytosolic localizations. CaMKIIδ subtype localization and its relationship to enzyme activation and target phosphorylation has not, however, been systematically evaluated.

Objective—To determine whether CaMKIIδ subtypes are restricted to a particular subcellular location and assess the relationship of localization to enzyme activation and function.

Methods and Results—CaMKIIδ is highly expressed in mouse heart and cardiomyocytes and concentrated in sarcoplasmic reticulum (SR)/membrane and nuclear fractions. CaMKIIδ_B and δ_C subtypes differ by a nuclear localization sequence, but both are present in nuclear and SR/membrane fractions. Nonselective subtype distribution is also seen in mice overexpressing CaMKIIδ_B or δ_C, even in a CaMKIIδ null background. Fluorescently-tagged CaMKIIδ_B expressed in cardiomyocytes concentrates in nuclei whereas δ_C concentrates in cytosol but neither localization is exclusive. Mouse hearts exposed to phenylephrine (PE) show selective CaMKIIδ activation in the nuclear (vs. SR) compartment whereas caffeine selectively activates CaMKIIδ in SR (vs. nuclei), independent of subtype. Compartmentalized activation extends to functional differences in target phosphorylation at CaMKII sites: PE increases histone deacetylase 5 phosphorylation (Ser498) but not phospholamban (Thr17), while the converse holds for caffeine.

Conclusions—These studies demonstrate that CaMKIIδ_B and δ_C are not exclusively restricted to the nucleus and cytosol, and that spatial and functional specificity in CaMKIIδ activation is elicited by mobilization of different Ca^{2+} stores rather than by compartmentalized subtype localization.

Keywords

Ca^{2+}/Calmodulin dependent protein kinase; nuclear localization; heart; splice variants; sarcoplasmic reticulum

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Disclosures
None.

Introduction

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional serine/threonine kinase critical for Ca^{2+} signaling in cardiomyocytes. Our work and that of others has implicated CaMKII in the development of cardiac hypertrophy and heart failure (HF).[1–7] The expression of CaMKII is elevated in animal HF models and human HF patients.[1, 2] Transgenic overexpression of the predominant cardiac isoform, CaMKIIδ, elicits hypertrophy and HF, while genetic deletion or inhibition of CaMKIIδ prevents HF development. [3–7] Two splice variants, CaMKIIδ_B and δ_C, are known to be present in cardiac myocytes. [8] The CaMKIIδ_B and δ_C subtypes have been implicated in distinct cardiomyocyte functions[2], but the exclusivity of their localization, potential selectivity in activation mechanisms, and relationship of localization and subtype to functional outcomes have not been well defined.

CaMKII is activated by Ca^{2+}/calmodulin binding to the enzyme. The resultant conformational change favors subsequent autophosphorylation of the enzyme to a Ca^{2+}-independent activated form. [9] Oxidation can also lead to CaMKII activation.[10] Downstream targets phosphorylated by CaMKIIδ include proteins important for the modulation of Ca^{2+} handling such as phospholamban (PLN), ryanodine receptors (RyR2), voltage sensitive L-type Ca^{2+} channels, and the $Na_v1.5$ Na^+ channel subunit [1, 2, 11–14] CaMKIIδ can also regulate gene transcription, for example by phosphorylation of type II histone deacetylases (HDACs) which derepress myocyte enhancer factor-2 (MEF2),[15–17] or through AP-1 [18, 19] or GATA4.[20]

CaMKIIδ_B and δ_C subtypes differ only by the presence of an 11 amino acid NLS in CaMKIIδ_B. [8, 21] The Schulman laboratory established and we subsequently confirmed that heterologously expressed CaMKIIδ_B primarily localizes to the nucleus, whereas δ_C is found primarily in the cytosol.[8, 21, 22] Accordingly, we postulated different functions of the two subtypes, with nuclear δ_B involved in hypertrophic gene regulation and cytosolic δ_C in the regulation of Ca^{2+} handling and ion channels. This was supported by early findings using isolated neonatal rat ventricular myocytes [23–30] and by the differential phenotypes that we observed in the CaMKIIδ_B and δ_C transgenic (TG) mice models.[3, 4, 11, 22, 31] More specifically, CaMKIIδ_B TG mice primarily develop cardiac hypertrophy whereas hypertrophy in the δ_C TG mice rapidly transitions to HF characterized by severely disrupted cytosolic Ca^{2+}handling. [3, 32] Subsequent work directly comparing the two lines showed that δ_B and δ_C both modulate MEF2 activity and gene expression, a result attributed to the ability of CaMKIIδ to phosphorylate HDAC in either the cytosol or the nucleus.[32] In the studies presented here we more extensively investigate the localization of CaMKIIδ_B and δ_C subtypes in the mouse heart ventricle and isolated cardiomyocytes. We further test the hypothesis that enzyme location within the myocyte determines its activation by stimuli that mobilize distinct subcellular pools of Ca^{2+}. The findings reported here demonstrate that CaMKII δ_B is indeed concentrated in the nucleus and CaMKII δ_C at the sarcoplasmic reticulum (SR), but that this localization is not exclusive, either for the endogenous or overexpressed enzyme. Additionally, we report that the nature of the stimulus and presumed site of localized Ca^{2+} release determines where CaMKII is activated and is indiscriminate with regard to enzyme subtype. Finally, we show that downstream target phosphorylation provides a functional readout of the consequences of activation of CaMKII at specific cellular locations.

Methods

Subcellular fractionation of mouse ventricle was performed by differential centrifugation using minor modifications of a published procedure.[33] To compare expression levels in

different subcellular compartments, equal portions (e.g. 50%, 100%) of each fraction were loaded onto SDS gels and analyzed by immunoblotting. Distribution of green fluorescent protein (GFP)-tagged CaMKII δ_B and δ_C in adult mouse ventricular myocytes (AMVM) was visualized by confocal microscopy following adenoviral overexpression. Isolated hearts were perfused in the Langendorff mode [34] and treated with vehicle, phenylephrine (PE) or caffeine. AMVMs were isolated from wild type and knockout as described.[35] CaMKII activation was assessed by Western blotting using a P-CaMKII antibody from Affinity Bioreagents.[4] All procedures were performed in accordance with NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. Results are reported as averages ± SEM. Statistical significance was determined using ANOVA followed by the Tukey post hoc test. $P<0.01$ was considered statistically significant. For additional details regarding the methods used, see the Online Supplemental Material.

Results

Ventricular tissue and AMVMs isolated from adult wild type (WT) and CaMKIIδ knockout (δKO) mice were analyzed by Western blotting using a CaMKIIδ antibody that recognizes both CaMKIIδ_B and δ_C. Two bands were clearly evident in the WT and absent in the CaMKIIδKO mouse heart (Fig 1A). The difference in mobility of these bands is consistent with the inclusion of an 11 amino acid (2 kD) NLS in CaMKIIδ_B. [8, 21] Quantification of the individual bands indicates that CaMKIIδ_B is the more predominant splice variant, with approximately 60% of the total endogenous CaMKII migrating as the δ_B subtype, and just under 40% as δ_C (Fig 1B). To determine which subcellular compartments contain endogenous CaMKIIδ isolated left ventricle was fractionated into cytosolic (Cyto), mitochondrial (Mito), SR/membrane (SR/mem) and nuclear (Nuc) fractions. The purity of these fractions was verified using the markers Rho GDP-dissociation inhibitor (Rho-GDI), voltage-dependent anion channel (VDAC), sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a)and Lamin A/C respectively (Fig 2A). To compare CaMKII protein expression amongst these fractions, the entire volume of each fraction was loaded onto SDS gels. Immunoblotting for CaMKIIδ revealed that there were nearly equivalent amounts of CaMKIIδ in the SR/membrane and nuclear compartments of the cell (together accounting for approximately 75% of the total enzyme) while less than 20% was in the cytosolic fraction and a smaller percent was associated with the mitochondrial fraction (Fig 2B). The subtype composition of CaMKIIδ in each subcellular fraction was then analyzed by separately quantifying the individual δ_B and δ_C bands as seen in Fig 3A. This analysis revealed that both subtypes were present in every compartment examined (Fig 3B). Importantly, CaMKIIδ_B was detected not only in the nuclear compartment, but also in the SR/membrane compartment; conversely CaMKIIδ_C, while abundant in the SR/membrane, was also clearly present in the nuclear compartment (Fig 3B).

We have generated cardiac specific CaMKIIδ_B and δ_C TG mice and characterized these lines in numerous studies. [3, 4, 31, 32] We assumed that the phenotypic differences observed in these TG lines correlated with differential increases in expression of δ_B and δ_C in the nuclear vs. SR compartments respectively. To re-evaluate this assumption in light of our findings on the distribution of endogenous CaMKII subtypes, we isolated and fractionated ventricular tissue from CaMKIIδ_B and δ_C TG mice. As in the experiments above, the entire volume of each fraction was loaded onto SDS gels to compare CaMKII protein expression amongst these fractions (Online Fig I). The percent of the total CaMKIIδ transgene in each subcellular compartment was quantitated and the data from a series of experiments averaged and shown in Fig 4A. Remarkably, while CaMKIIδ_B TG mice show a high concentration of CaMKIIδ_B in cardiomyocyte nuclei based on immunofluorescence staining, [32] subcellular fractionation indicates that significant amounts of CaMKIIδ_B are also present outside of the

nucleus in the cytosolic and SR/membrane fractions (Fig 4A). In the CaMKIIδ_C TG mice, immunostaining revealed relative exclusion of CaMKIIδ_C from the nucleus, [32] but whereas most CaMKIIδ_C is in the SR/membrane fraction, the CaMKIIδ_C subtype is clearly detectable in the nuclear fraction as well (Fig 4A). Thus distribution of the transgenes, like that of endogenous CaMKIIδ subtypes, is not exclusive.

CaMKIIδ is believed to exist as a multimer of 12 subunits. [9] Nuclear vs. cytosolic localization can be significantly affected by changing the expression ratio of δ_B and δ_C splice variants, consistent with heteromultimerization of these subtypes. [3622, 37] Multimerization of transgenically expressed CaMKIIδ_C with endogenous CaMKIIδ_B could promote its localization to the nucleus, whereas multimerization of CaMKIIδ_B with endogenous CaMKIIδ_C could lead to its exclusion from the nuclear compartment. To test the hypothesis that the broad and relatively nonselective subcellular distribution of the CaMKII δ_B and δ_C subtypes results from their heteromultimerization we crossed the CaMKIIδ_B and δ_C TG mice with the CaMKIIδKO mice previously developed in our lab. Progeny from these crosses were shown to express only a single CaMKIIδ subtype (δ_B or δ_C) in the CaMKIIδ null background. Interestingly, the subcellular distribution of the CaMKIIδ_C and δ_B transgenes expressed in the CaMKIIδ null background (Fig 4B) was not appreciably different from that of the δ_B and δ_C transgenes expressed in the WT background (Fig 4A).

To examine the distribution of the δ_B and δ_C subtypes in a manner that does not require cell disruption and fractionation we infected AMVMs from δKO mice with GFP-tagged CaMKII δ_B and δ_C adenovirus. Myocytes infected with CaMKIIδ_B and visualized by confocal microscopy clearly showed accumulation of the overexpressed protein in the nuclear compartment, but a significant amount of CaMKIIδ_B was also seen outside the nucleus, distributed in a striated pattern corresponding to T-tubule organization (Fig 5A). Line scan quantification of fluorescent intensity of a 1 micron thick plane from several different cells showed the fluorescence intensity of CaMKIIδ_B in the nucleus to be 2.69 (±0.08) times higher than that of CaMKIIδ_B in the cytosol; expressed another way, CaMKII δ_B fluorescence intensity outside of the nuclear compartment is approximately one third that in the nucleus. Experiments were also carried out using GFP-tagged CaMKIIδ_C, and showed prominent localization coincident with the striated patterns of the cardiomyocyte (Fig 5B). Significant perinuclear CaMKIIδ_C staining was also observed. Line scan quantification of CaMKIIδ_C fluorescence intensity showed the nuclear δ_C signal to be 0.44 (±0.09) of that in the extranuclear compartment; expressed another way the fluorescence intensity of CaMKIIδ_C inside the nuclear compartment is approximately half of that in the cytosol.

Many of the functional effects of CaMKII in the myocardium, in particular those ascribed to CaMKIIδ_C, result from phosphorylation of SR targets involved in Ca^{2+} handling. Because the SR/membrane fraction obtained from the protocol used in the Figs 2–4 is heterogeneous (e.g. it includes sarcolemmal membranes) we optimized a sucrose density gradient separation protocol to isolate a more purified SR fraction. We looked at purity of the SR and nuclear fractions by immunoblotting using markers for cytosol, mitochondria, SR and nucleus and found the SR (Fig 6A) and nuclear (Fig 6B) fractions to contain some mitochondria (VDAC staining) but otherwise show little cross contamination. Immunoblots from the purified SR and nuclear fractions show two CaMKIIδ bands (Fig 6C,D). While the lower band, CaMKIIδ_C, is the more abundant in the SR (Fig 6C), and CaMKIIδ_B is predominant in the nuclear fraction (Fig 6D), the two subtypes are clearly not exclusively segregated to a specific compartment.

The finding that CaMKII δ_B and δ_C subtypes co-localize in the same subcellular compartment suggested that they could also be activated in parallel. To determine whether

this is the case, we isolated hearts from WT mice, perfused them in the Langendorff mode and then either treated them with a bolus injection of 10mmol/L caffeine (to release SR Ca^{2+}) or perfused them for 15 minutes with 100nmol/L PE (to increase nuclear Ca^{2+} levels). [17, 38] Hearts were then fractionated to obtain purified SR and nuclei (as described in Fig 6) and analyzed by Western blot analysis. Phosphorylation of CaMKII at Thr286, the site of enzyme autophosphorylation, was used as a read-out for CaMKII activation. Perfusion with caffeine increased P-CaMKII in the SR fraction but not in the nuclear fraction (Fig 7A). In contrast, PE treatment increased P-CaMKII levels in the nuclear fraction but not in the SR fraction (Fig 7A). Since the resolution of the P-CaMKII antibody is not adequate to individually distinguish the endogenously expressed phosphorylated CaMKIIδ_B and CaMKIIδ_C subtypes we repeated these experiments using the δ_B and δ_C TG mice (in the CaMKIIδ null background). The data shown in Figure 7B and C demonstrate that caffeine significantly increases phosphorylation of both CaMKIIδ_B and CaMKIIδ_C in the SR (but not in the nuclear fraction). In contrast PE treatment elicits 2–3 fold increases in phosphorylation of both CaMKIIδ_B and CaMKIIδ_C in the nuclear fraction but not in the SR. Thus caffeine selectively activates whichever CaMKIIδ subtype is located at the SR but not that located in the nucleus, whereas PE selectively activates whichever CaMKII subtype is localized to the nucleus.

The experimental findings cited above suggest that CaMKIIδ_B or CaMKIIδ_C can be activated by the same agonists and could subserve similar functions. We examined functional consequences of compartmentalized CaMKII activation by measuring the phosphorylation of established CaMKII phosphorylation sites on two CaMKII targets, PLN localized to the SR and HDAC5, largely localized to the nucleus. Perfused hearts were treated with PE or caffeine using the same protocol used to examine CaMKII activation and homogenized for analysis of CaMKII substrate phosphorylation. In WT mice (Fig 8) or CaMKIIδ_B TG (Online Fig II), treatment with caffeine lead to significant increases in phosphorylation of PLN at Thr17 while treatment with PE did not lead to PLN phosphorylation (Fig 8A). Conversely treatment with PE increased phosphorylation of HDAC5 at Ser498 while caffeine did not (Fig 8B). Concomitant perfusion with KN-93, a CaMKII inhibitor, prevented caffeine induced phosphorylation of PLN Thr17 and PE induced phosphorylation of HDAC5 at Ser498 (Online Fig III). Additionally, we observed no increase in phosphorylation of these substrates at their putative CaMKII phosphorylation sites in CaMKIIδKO mice treated with caffeine or PE (Fig IV). These data demonstrate that PLN Thr17 and HDAC5 Ser498 are CaMKII phosphorylation sites and that there is specificity in the effects of caffeine and PE on CaMKII mediated phosphorylation of these substrates.

Discussion

CaMKIIδ_B and δ_C subtypes, which differ only by the inclusion of a nuclear localization sequence, are present in the mouse heart ventricle at similar protein levels (Fig 1). Seminal papers from the Schulman laboratory describing these two splice variants, [8, 21] along with our early studies in which we expressed CaMKIIδ_B and δ_C in neonatal rat ventricular myocytes (NRVMs), [22] supported the notion that CaMKIIδ_B would be localized to and signal in the nucleus whereas δ_C would localize to and signal outside of the nucleus. These conclusions were based on studies in which CaMKIIδ was heterologously expressed in COS cells or NRVMs. [22, 39, 40]

Subsequently we generated CaMKIIδ_B or δ_C TG mice and examined the HA-tagged protein by immunostaining of myocytes isolated from these mice. [3, 4, 32] Our findings were consistent with the predominant localization of CaMKIIδ_B in the nucleus and δ_C in the cytosol.[3, 4, 32] The concept that nuclear and cytosolic splice variants/subtypes subserved

different functions was supported by the distinct phenotypes that we observed in the CaMKIIδ$_B$ and δ$_C$ TG mice. We recognized that the pathological changes seen in these mouse models could be exaggerated by overexpression, but reasoned that this approach emphasized the compartment specific effects of the two subtypes: nuclear effects on gene expression leading to hypertrophy in the CaMKIIδ$_B$ TG mice, and effects on SR protein phosphorylation and Ca^{2+} handling leading to HF development in the CaMKIIδ$_C$ TGs.

The more extensive analysis presented in the current manuscript was motivated by our observation that endogenous CaMKIIδ is found in both the SR/membrane and nuclear compartments isolated from mouse ventricle and that there are two CaMKIIδ immunoreactive bands in both of these compartments (Fig 3A and 6). That these bands are absent in CaMKIIδ knockout mouse hearts (Fig 1A) indicates that they are both CaMKIIδ gene products, while the fact that they differ in mobility by approximately 2 kD suggests that they represent δ$_C$ and the 11 amino acid larger NLS containing δ$_B$.[21] The ability of various CaMKII isoforms and subtypes to form heteromultimers [9, 36] provides a feasible explanation for the appearance of either subtype in the nucleus (or SR) independent of whether it possesses an NLS. Remarkably, however, the expression of CaMKIIδ$_B$ in the absence of CaMKIIδ$_C$ did not restrict its localization to the nucleus nor was δ$_C$ confined to the cytosolic/SR compartment when expressed in the absence of δ$_B$ (Fig 4B). Thus heteromultimers of CaMKIIδ$_B$ and δ$_C$ appear unlikely to account for the indiscriminate distribution of these subtypes. We cannot rule out the possibility that other minor cardiac CaMKII isoforms, including CaMKIIγ and β, heteromultimerize with CaMKIIδ and contribute to its appearance in unexpected locations, although this seems quantitatively unlikely. Regardless of the molecular mechanism, the conclusion from our subcellular fractionation experiments is that CaMKIIδ$_B$ is not restricted to the nuclear compartment, and δ$_C$ is not excluded from the nuclear compartment.

Since subcellular fractionation does not yield complete separation of organelles and also can disrupt normal structure we extended our studies using confocal microscopy of intact AMVMs infected with GFP-tagged CaMKIIδ$_B$ or δ$_C$. Data obtained by confocal imaging shows extensive accumulation of GFP-tagged CaMKIIδ$_B$ in the nucleus, consistent with what we reported previously.[32] Notably however, quantitative analysis confirmed that δ$_B$ is not wholly restricted to the nucleus; indeed δ$_B$ fluorescence intensity outside of the nuclear compartment was approximately one third that in the nucleus. The intensity of the nuclear staining is indeed striking but this reflects, in part, the fact that the enzyme is concentrated in a very small compartment. The distribution of GFP-tagged CaMKIIδ$_C$ appeared largely consistent with the earlier studies from our lab suggesting that δ$_C$ is excluded from the nucleus. However, quantitative analysis showed that the fluorescence intensity inside the nuclear compartment was not zero, but was approximately half of that in the cytosol. Of additional note, our assessment of CaMKIIδ$_C$ nuclear fluorescence intensity does not include what appears to be a prominent pool of GFP–tagged perinuclear CaMKIIδ$_C$; whether this represents mitochondria, SR or other cellular organelles in confluence with the nucleus, this compartment of CaMKIIδ$_C$ would likely be included in our nuclear fractionation and thus contribute to higher estimates for the proportion of nuclear CaMKIIδ$_C$ in the fractionation experiments. Finally it should be noted that the insoluble fraction discarded in the low speed spin of the fractionation protocol would contain some of the total cellular CaMKIIδ, thus the percent of total calculated for each fraction is somewhat inflated. Regardless of the limitations inherent in the use of either the adenoviral overexpression or subcellular fractionation experiments, and independent of judgement as to which approach give the most valid estimate of CaMKIIδ$_B$ and δ$_C$ in each compartment, all of the preparations and approaches utilized here lead us to the same conclusions: the CaMKIIδ$_B$ and CaMKIIδ$_C$ isoforms are not restricted to specific subcellular locations.

The finding that both CaMKII subtypes are present throughout the cell raised the question of whether localization or subtype would determine when and how the enzyme was activated. We used interventions expected to mobilize Ca^{2+} from distinct cellular locations to examine CaMKIIδ activation in WT mouse hearts. This was supplemented with studies using hearts from the subtype-specific transgenics to facilitate analysis of the activation of individual subtypes. Our findings clearly demonstrated that PE increases phosphorylation of CaMKIIδ_B or δ_C in the nuclear compartment with little change in activation of either subtype at the SR; conversely caffeine activates both CaMKIIδ_B and δ_C in the SR, with little change in activation of either subtype in the nuclear compartment (Fig 7). Several published studies have highlighted the importance of localized Ca^{2+} stores and subsequent compartmentalized signaling within the cardiomyocyte.[41–43] In cardiomyocytes, the majority of inositol trisphosphate receptors (IP_3R2) are located on the nuclear envelope and our previous work demonstrated that endothelin-1 and PE increase Ca^{2+} release from nuclear IP_3 sensitive stores.[17, 38] Thus we believe that the selectivity of PE for inducing nuclear CaMKII activation reflects Ca^{2+} mobilization through IP_3 sensitive stores in or around the nucleus, although other similarly localized signaling pathways cannot be ruled out. Treatment with caffeine would instead be expected to cause a large $[Ca]_i$ increase in the cleft region as a result of SR Ca^{2+} mobilization, consistent with CaMKII activation at the SR. Thus the studies presented here demonstrate for the first time that there is compartmentalized activation of CaMKIIδ, with the cellular compartment determined by the stimulus and presumed site of Ca^{2+} release, and notably independent of subtype.

The functional relevance of compartmentalized CaMKIIδ activation was demonstrated by studies in which we examined substrate phosphorylation. Phosphorylation of the SR target, phospholamban at its well documented CaMKII-specific phosphorylation site, [5, 44, 45] was confirmed here (Online Figs III, IV) and shown to be selectively increased following addition of caffeine (Fig 8). Phosphorylation of the nuclear transcriptional regulator HDAC5 at a site indicated by previous studies and in Online Figs III and IV to be a CaMKII phosphorylation site [17, 46, 47] was selectively increased following PE treatment (Fig 8). Agonist selectivity in substrate phosphorylation was demonstrated in studies using both WT (Fig 8) and CaMKIIδ_B TG (Online Fig II) mice. The high basal level of PLN and HDAC phosphorylation seen in the CaMKIIδ_C TG heart (Online Fig II) precluded detection of further agonist induced increases, although it does indicate that both of these substrates are in vivo targets for CaMKIIδ_C. Notably our previous analysis of the CaMKII δ_C TG mouse heart demonstrated increased PLN and RyR2 phosphorylation associated with dysfunctional Ca^{2+} handling and heart failure phenotype. [3, 32] The reason that we did not see RyR2 and PLN phosphorylation and Ca^{2+} handling changes in the CaMKIIδ_B TG mice [32] may be that the level of CaMKII transgene expression is lower in the SR of the CaMKII δ_B TG mice than in the SR of CaMKII δ_C TG mice (Online Figure V). We do find, however, that both PLN and RyR2 are highly phosphorylated in neonatal rat cardiomyocytes following adenoviral expression of equal levels of either CaMKII δ_B or CaMKII δ_C (data not shown), supporting the notion that either subtype can phosphorylate these SR targets.

In conclusion, we demonstrate for the first time that CaMKIIδ_B and δ_C subtypes are not exclusively localized. We also present evidence that both subtypes can be activated at the same cellular locations and that the activation is stimulus and location dependent rather than subtype dependent. Phosphorylation of different CaMKIIδ substrates is also dependent upon the nature of the stimulus. The evidence for nonselective CaMKIIδ subtype localization is particularly interesting and challenging with regard to understanding mechanisms by which CaMKIIδ_B could subserve a protective role, and δ_C a more deleterious role in cardiomyocyte survival and heart disease.[12, 48, 49]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

AMVM	adult mouse ventricular myocyte
CaMKII	Ca^{2+}/Calmodulin dependent protein kinase II
ET-1	endothelin-1
GFP	green fluorescent protein
HDAC	histone deacetylase
HF	heart failure
KO	knock out
MEF2	myocyte enhancer factor 2
NLS	nuclear localization sequence
PE	phenylephrine
PLN	phospholamban
Rho-GDI	Rho GDP-dissociation inhibitor
RyR2	ryanodine receptor
SERCA	sarcoplasmic reticulum Ca2+ ATPase
SR	sarcoplasmic reticulum
TG	transgenic
VDAC	voltage-dependent anion channel
WT	wild type

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Novelty and Significance

What is known?

- Ca^{2+}/CaM kinase II regulates cardiac Ca^{2+} handling and plays a critical role in adverse cardiac remodeling in response to pressure overload, catecholamines and ischemic stress.
- CaMKII delta (CaMKIIδ) is the predominant cardiac isoform and is present as two major splice variants (subtypes): CaMKIIδ_B which contains a nuclear localization sequence and CaMKIIδ_C which does not.
- Based on cellular or transgenic overexpression, the two subtypes are differentially localized and accordingly serve different functions: CaMKIIδ_B regulates gene expression and cell survival whereas CaMKIIδ_C regulates Ca^{2+} handling and cell death.

What new information does the article provide?

- The two CaMKIIδ subtypes are not as exclusively localized as previously believed: the SR compartment contains considerable amounts of CaMKIIδ_B and the nuclear compartment contains significant amounts of CaMKIIδ_C.
- Two Ca^{2+} mobilizing agonists, caffeine and phenylephrine, differentially activate CaMKIIδ in accordance with enzyme localization (caffeine in SR, phenylephrine in nucleus) and increase substrate phosphorylation (phospholamban and HDAC-5), independent of CaMKIIδ subtype
- Specificity in CaMKIIδ signaling results from compartmentalized rather than subtype specific activation.

This study was designed to test the concept that CaMKIIδ_B and CaMKIIδ_C, the two predominant cardiac splice variants (subtypes) of CaMKII, serve different functions due to their distinct localizations. Surprisingly, subcellular fractionation studies revealed that all fractions examined, including mitochondria, SR/membrane and nucleus contained a mixture of the two subtypes. Using hearts from mice in which only one of the two subtypes was expressed (δ_B or δ_C transgenic mice in a CaMKIIδ knockout background), we show that this is not a result of heteromultimerization of the subtypes. We then asked whether the subtypes, if not distinctly localized, were differentially regulated. Two agonists that mobilized Ca^{2+}, caffeine and phenylephrine, were shown to activate both CaMKIIδ_B and δ_C. Strikingly, regardless of subtype, caffeine activated CaMKIIδ in the SR compartment and increased phosphorylation of the SR CaMKII substrate, phospholamban, whereas phenylephrine only activated CaMKIIδ in the nucleus and increased phosphorylation of the nuclear CaMKII target, HDAC-5. These findings question the accepted notion of strict “nuclear” vs. “cytoplasmic” isoforms of CaMKIIδ, while demonstrating that there is compartmentalized activation of CaMKIIδ and its functional targets in cardiomyocytes.

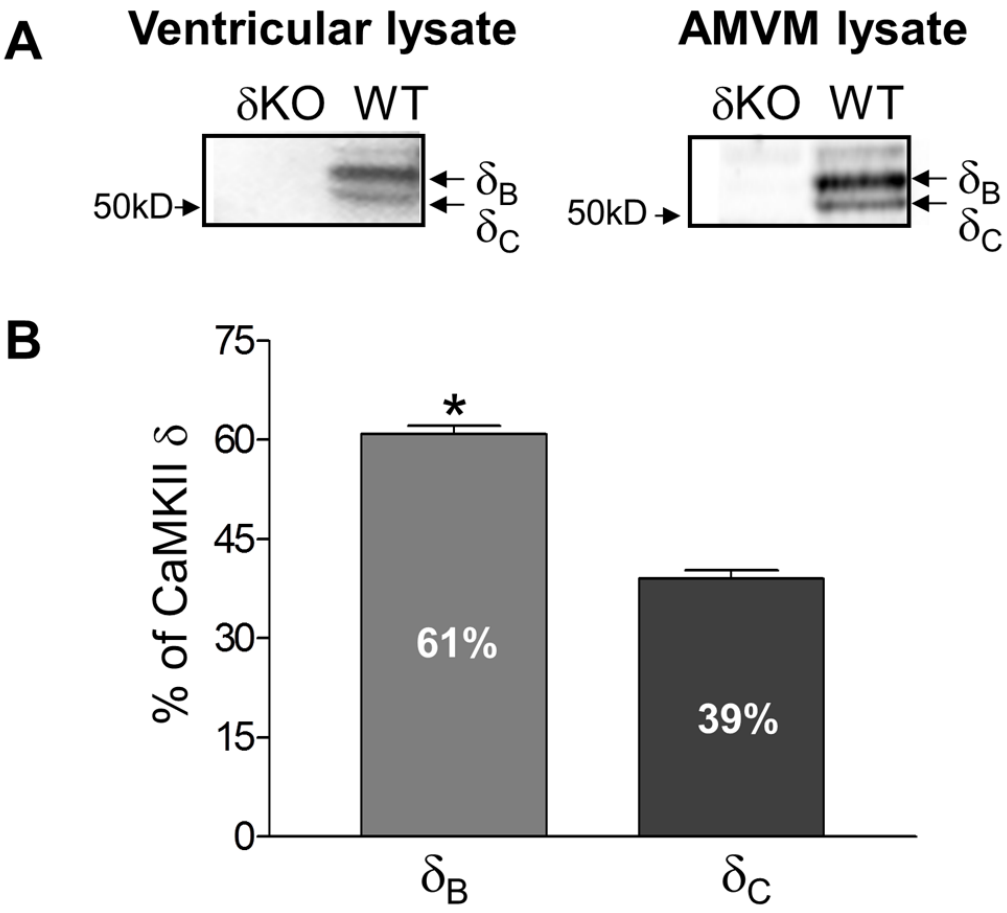

Figure 1. Expression of endogenous CaMKII δ in mouse ventricle and cardiomyocytes

Ventricular tissue and adut mouse ventricular myocytes (AMVM) isolated from wild type (WT) and CaMKII δ knockout (δKO) mice, lysed and subjected to western blotting. **(A)** Representative blots of CaMKII δ expression in ventricular lysate and AMVM lysate demonstrating two bands absent in the δKO. **(B)** Quantitative analysis of the relative abundance of CaMKII δ_B and δ_C in WT ventricular tissue (n=8). * $p<0.01$

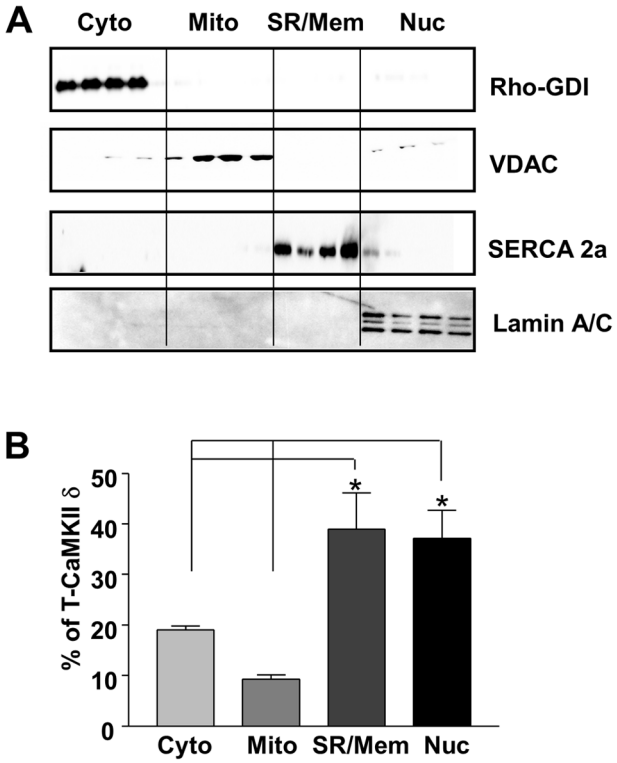

Figure 2. Subcellular distribution of endogenous CaMKII δ

(A) Western blotting of fractionated mouse ventricle for cellular markers; Rho-GDI, cytosol; VDAC, mitochondria; SERCA 2a, SR; Lamin A/C, nucleus. Each lane represents a separate heart. **(B)** Quantitative analysis of the percent of total CaMKII δ in each subcellular compartment isolated from WT mouse ventricle. For the data shown in A and B, all fractions were suspended in the same volumes and equal portions loaded for western blotting. (n=6) * $p < 0.01$

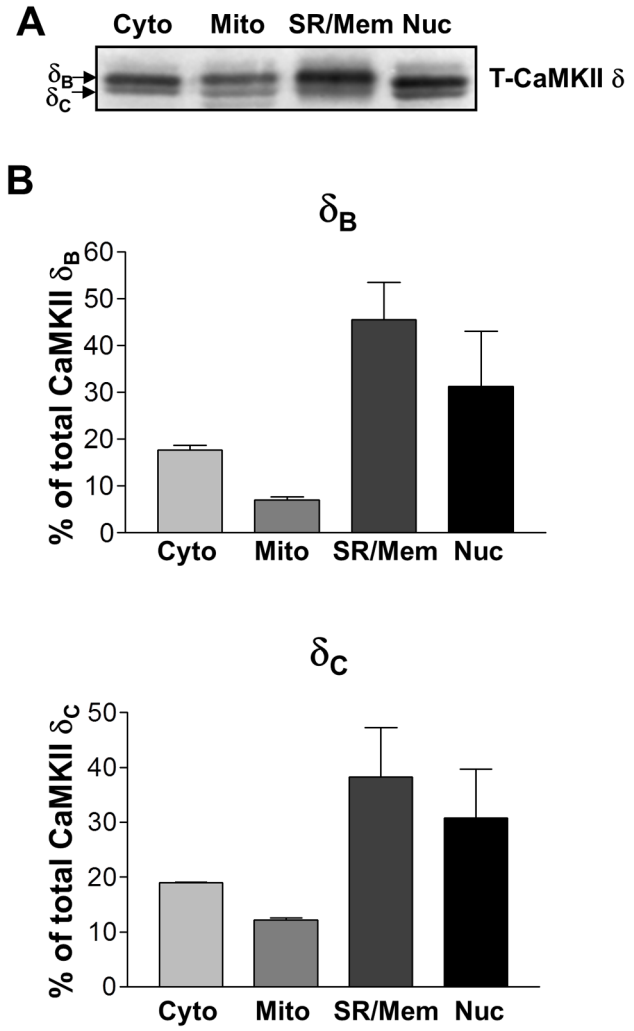

Figure 3. Subcellular distribution of the endogenous CaMKII δ_B and δ_C isoforms

Ventricular tissue was isolated from WT mice. **(A)** Representative western blot shows the relative distribution of CaMKII in the cytosolic, mitochondrial, SR/membrane and nuclear fractions. Equal portions of each fraction were loaded for western blotting. **(B)** Graphs show percent of total CaMKII δ_B or δ_C in each subcellular fraction from wild type mice. (n=6)

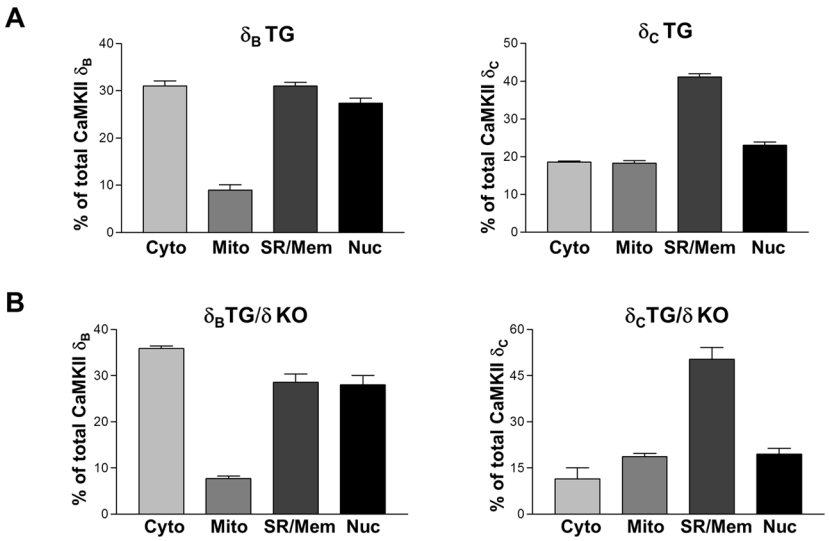

Figure 4. Comparative subcellular distribution of CaMKII δ_B and δ_C isoforms in transgenic vs. transgenic in CaMKII δ null background

Ventricular tissue isolated from δ_B TG, δ_CTG, δ_B/δKO andδ_C/δKO mice was harvested and fractionated into cytosolic, mitochondrial, SR/membrane and nuclear fractions and subjected to western blotting. The distribution of transgenically expressed CaMKII δ_B and δ_C is examined (A) in the wild type background or **(B)** in the CaMKII δ null background. (n=4)

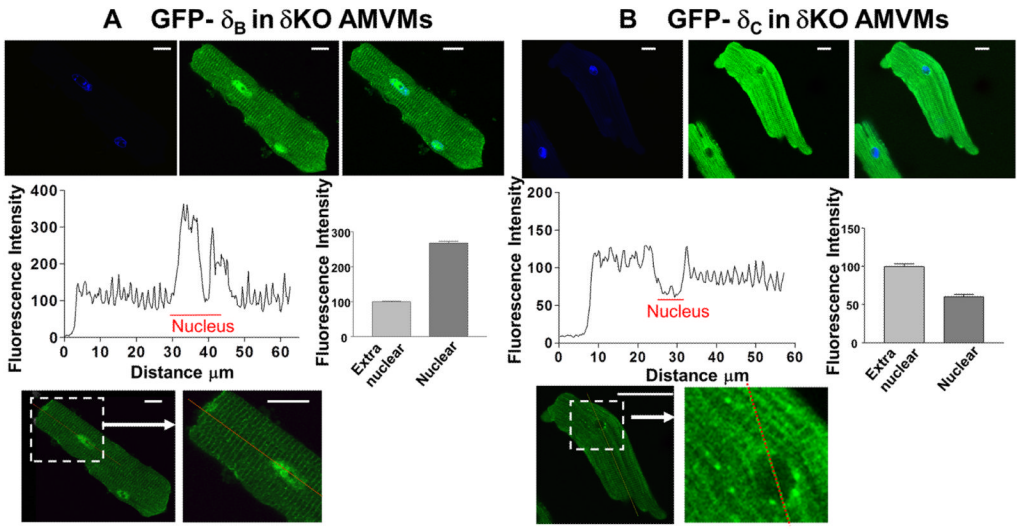

Figure 5. GFP-tagged CaMKII δ_B and δ_C expressed in AMVMs isolated from δKO mice

AMVMs isolated from δKO mice were infected with adenovirus expressing **(A)** GFP-tagged CaMKII δ_B or **(B)** GFP-tagged CaMKII δ_C. AMVMs were imaged using confocal microscopy, DAPI staining was used to identify the nuclei, and line scan quantification was used to measure fluorescence intensity and determine enzyme distribution. Scale bar is 15 microns (μm). Background measurements taken from non-infected AMVMs were averaged and subtracted from the fluorescence intensity measurements of the GFP-tagged enzyme.

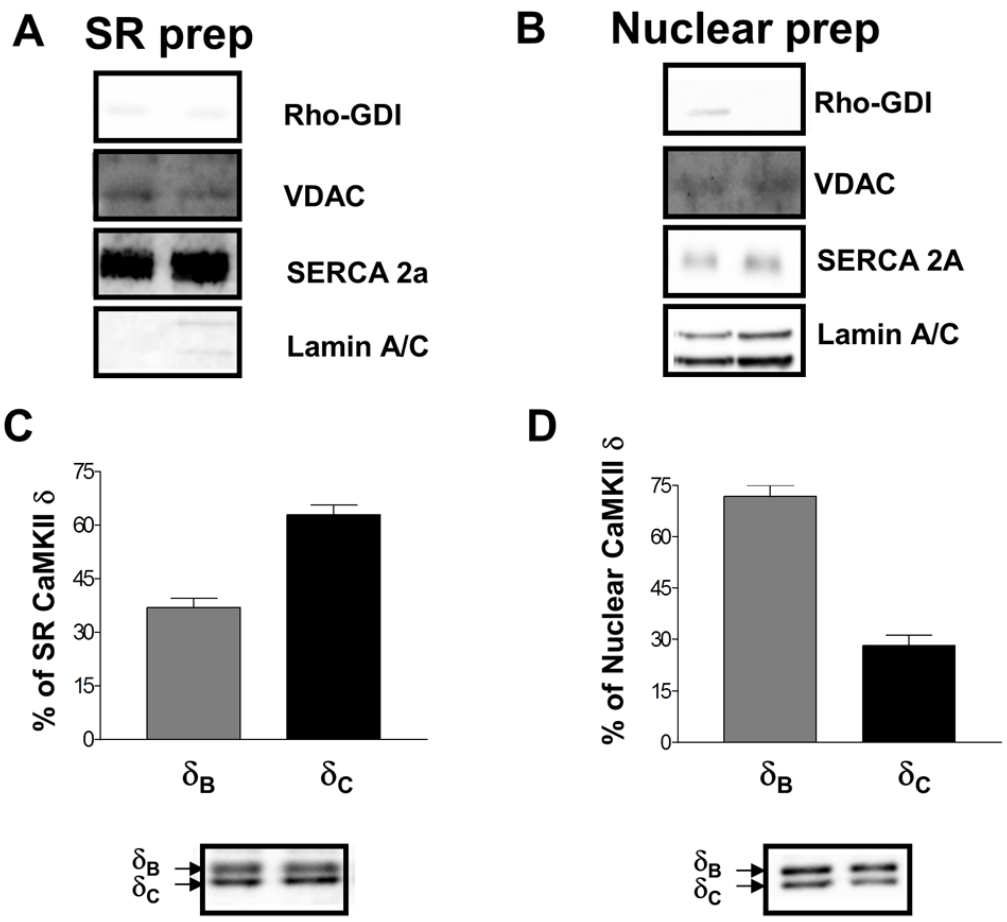

Figure 6. Distribution of CaMKII δ_B and CaMKII δ_C in purified SR and nuclei

SR and nuclear fractions were isolated from WT ventricular tissue. Representative western blots showing purity of **(A)** SR preparation or **(B)** nuclear preparation. Representative western blot and quantification of δ_B vs. δ_C in **(C)** SR or **(D)** nuclear fractions. (n=4)

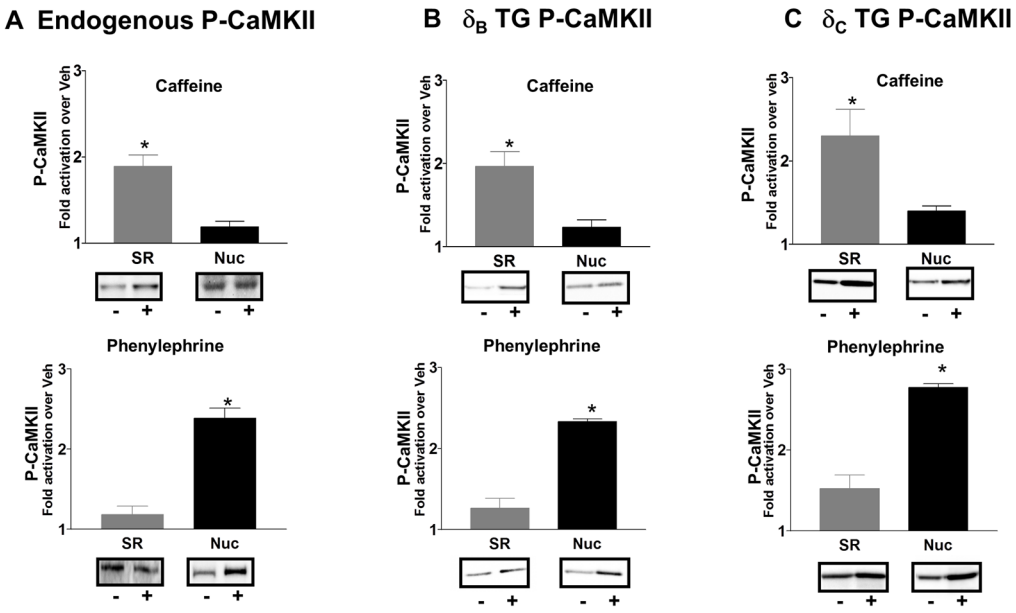

Figure 7. Caffeine activates CaMKII at the SR and phenylephrine activates CaMKII at the nucleus

SR or nuclei were isolated from ventricular tissue of WT, CaMKII δ_B or CaMKII δ_C TG mice in the CaMKII δ null background following bolus injection of 10mmol/L caffeine or 15 minutes of perfusion with 100nmol/L PE. P-CaMKII levels were measured by western blotting using an antibody directed against the CaMKII autophosphorylation site, (Thr286). Data are shown for **(A)** WT (endogenous CaMKII), **(B)** δ_B TG in the δKO background and (C) δ_C TG in the δKO background. * $p < 0.01$ for SR vs. Nuc

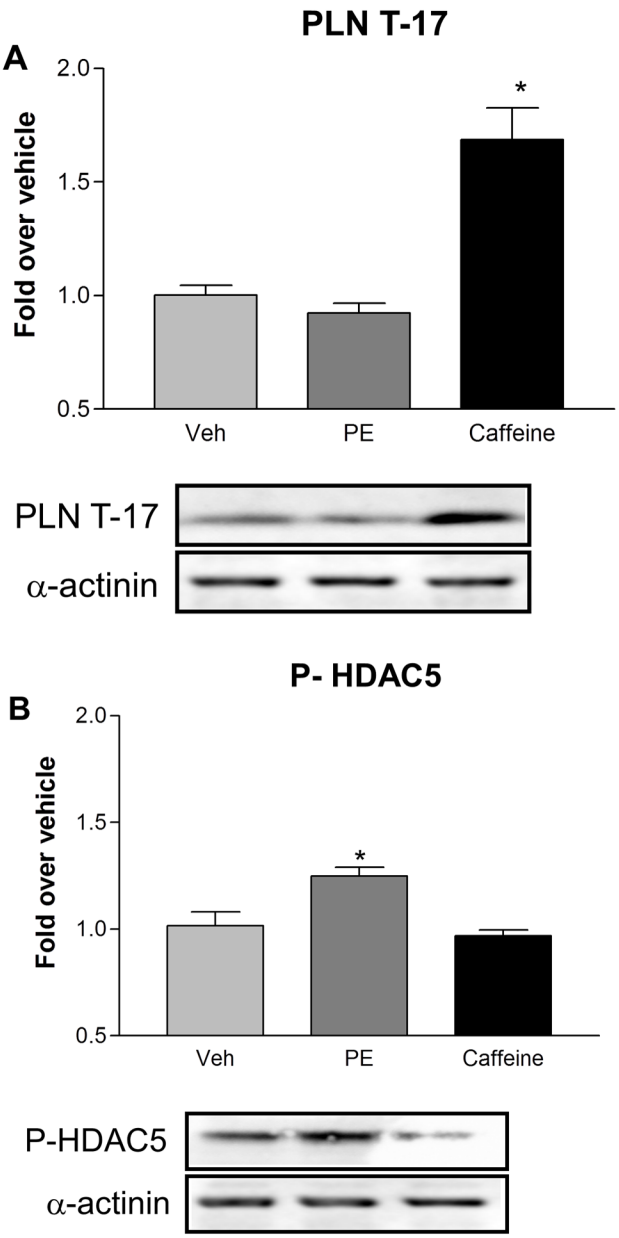

Figure 8. Caffeine preferentially increases phospholamban phosphorylation and PE preferentially increases HDAC5 phosphorylation in mouse heart

Hearts were isolated from mice perfused with 10mmol/L caffeine or 100nmol/L phenylephrine. Ventricular homogenate was subjected to western blotting for **A** PLN phosphorylation at the CaMKII phosphorylation site, threonine-17 and **B.** HDAC5 phosphorylation using an antibody for the CaMKII specific epitope. Quantitatated data are from n=5 * $p<0.01$