Location Matters
Clarifying the Concept of Nuclear and Cytosolic CaMKII Subtypes

Shikha Mishra, Charles B.B. Gray, Shigeki Miyamoto, Donald M. Bers, Joan Heller Brown

Rationale: Differential effects of \( \delta_h \) and \( \delta_c \) 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 versus cytosolic localizations. CaMKII\( \delta \) subtype localization and its relationship to enzyme activation and target phosphorylation have not, however, been systematically evaluated.

Objective: To determine whether CaMKII\( \delta \) subtypes are restricted to a particular subcellular location and assess the relationship of localization to enzyme activation and function.

Methods and Results: CaMKII\( \delta \) is highly expressed in mouse heart and cardiomyocytes and concentrated in sarcoplasmic reticulum (SR)/membrane and nuclear fractions. CaMKII\( \delta_h \) and \( \delta_c \) 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 CaMKII\( \delta_h \) or \( \delta_c \), even in a CaMKII\( \delta \) null background. Fluorescently tagged CaMKII\( \delta_h \) expressed in cardiomyocytes concentrates in nuclei whereas \( \delta_c \) concentrates in cytosol, but neither localization is exclusive. Mouse hearts exposed to phenylephrine show selective CaMKII\( \delta \) activation in the nuclear (versus SR) compartment, whereas caffeine selectively activates CaMKII\( \delta \) in SR (versus nuclei), independent of subtype. Compartmentalized activation extends to functional differences in target phosphorylation at CaMKII sites: phenylephrine increases histone deacetylase 5 phosphorylation (Ser498) but not phospholamban (Thr17), whereas the converse holds for caffeine.

Conclusions: These studies demonstrate that CaMKII\( \delta_h \) and \( \delta_c \) are not exclusively restricted to the nucleus and cytosol and that spatial and functional specificity in CaMKII\( \delta \) activation is elicited by mobilization of different Ca\(^{2+}\) stores rather than by compartmentalized subtype localization. (Circ Res. 2011;109:1354-1362.)

Key Words: Ca\(^{2+}/\)calmodulin-dependent protein kinase ■ nuclear localization ■ heart ■ splice variants ■ sarcoplasmic reticulum

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). The expression of CaMKII is elevated in animal HF models and human HF patients. Transgenic overexpression of the predominant cardiac isoform CaMKII\( \delta \) elicits hypertrophy and HF, whereas genetic deletion or inhibition of CaMKII\( \delta \) prevents HF development. Two splice variants, CaMKII\( \delta_h \) and \( \delta_c \), are known to be present in cardiac myocytes. The CaMKII\( \delta_h \) and \( \delta_c \) subtypes have been implicated in distinct cardiomyocyte functions, 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. Oxidation can also lead to CaMKII activation. Downstream targets phosphorylated by CaMKII\( \delta \) 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\(^+\)/Na\(^+\) channel subunit. CaMKII\( \delta \) may also regulate gene transcription, for example, by phosphorylation of type II histone deacetylas (HDACs), which derepress myocyte enhancer factor-2 (MEF2), through AP-1 or GATA4.

CaMKII\( \delta_h \) and \( \delta_c \) subtypes differ only by the presence of an 11–amino acid nuclear localization sequence (NLS) in CaMKII\( \delta_h \). The Schulman laboratory established and we subsequently confirmed that heterologously expressed CaMKII\( \delta_h \) primarily localizes to the nucleus, whereas \( \delta_c \) is found primarily in the cytosol. Accordingly, we postulated different functions of the 2 subtypes, with nuclear \( \delta_h \) involved in hypertrophic gene regulation and cytosolic \( \delta_c \) in the regulation of Ca\(^{2+}\) handling and ion channels. This was supported by early findings using isolated neonatal rat ventricular myocytes and the differential phenotypes that we observed in the CaMKII \( \delta_h \) and
nuclear (Nuc) fractions. The purity of these fractions was analyzed by differential centrifugation using minor modifications of a published procedure. To compare expression levels in different subcellular compartments, equal portions (eg, 50%, 100%) of each fraction were loaded onto SDS gels and analyzed by immunoblotting. Distribution of green fluorescent protein (GFP)-tagged CaMKIIb and c in adult mouse ventricular myocytes (AMVM) was visualized by confocal microscopy after adenoviral overexpression. Isolated hearts were perfused in the Langendorff mode and treated with vehicle, phenylephrine (PE) or caffeine. AMVMs were isolated from wild type and transgenic (TG) mice models. More specifically, CaMKIIb TG mice primarily develop cardiac hypertrophy whereas hypertrophy in the δc TG mice rapidly transitions to HF characterized by severely disrupted cytosolic Ca2+ handling. Subsequent work directly comparing the 2 lines showed that δb and δc 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. In the studies presented in the present report, we more extensively investigate the localization of CaMKIIδb and δc 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 Ca2+. The findings reported here demonstrate that CaMKII δb is indeed concentrated in the nucleus and CaMKII δc 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 Ca2+ 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
An expanded Methods section is available in the Data Supplement at http://circres.ahajournals.org.

Subcellular fractionation of mouse ventricle was performed by differential centrifugation using minor modifications of a published procedure. To compare expression levels in different subcellular compartments, equal portions (eg, 50%, 100%) of each fraction were loaded onto SDS gels and analyzed by immunoblotting. Distribution of green fluorescent protein (GFP)-tagged CaMKIIb and c in adult mouse ventricular myocytes (AMVM) was visualized by confocal microscopy after adenoviral overexpression. Isolated hearts were perfused in the Langendorff mode and treated with vehicle, phenylephrine (PE) or caffeine. AMVMs were isolated from wild type and transgenic (TG) mice models. More specifically, CaMKIIb TG mice primarily develop cardiac hypertrophy whereas hypertrophy in the δc TG mice rapidly transitions to HF characterized by severely disrupted cytosolic Ca2+ handling. Subsequent work directly comparing the 2 lines showed that δb and δc 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. In the studies presented in the present report, we more extensively investigate the localization of CaMKIIδb and δc 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 Ca2+. The findings reported here demonstrate that CaMKII δb is indeed concentrated in the nucleus and CaMKII δc 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 Ca2+ 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.

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 CaMKIIδb and δc. Two bands were clearly evident in the WT and absent in the CaMKIIδ KO mouse heart (Figure 1A). The difference in mobility of these bands is consistent with the inclusion of an 11–amino acid (2 kDa) NLS in CaMKIIδc. Quantification of the individual bands indicates that CaMKIIδb is the more predominant splice variant, with approximately 60% of the total endogenous CaMKII migrating as the δb subtype, and just under 40% as δc (Figure 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 Ca2+ ATPase (SERCA2a) and Lamin A/C, respectively (Figure 2A). To compare CaMKII protein expression among these fractions, the entire volume of each fraction was loaded onto SDS gels. Immunoblotting for CaMKII 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), whereas less than 20% was in the cytosolic fraction and a smaller percentage was associated with the mitochondrial fraction (Figure 2B). The subtype composition of CaMKIIδ in each subcellular fraction was then analyzed by separately quantifying the individual δb and δc bands, as seen in Figure 3A.

Non-standard Abbreviations and Acronyms

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<tr>
<th>Abbreviation</th>
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<tr>
<td>AMVM</td>
<td>adult mouse ventricular myocyte</td>
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<tr>
<td>CaMKII</td>
<td>Ca2+/Calmodulin dependent protein kinase II</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<td>HDAC</td>
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<td>phospholamban</td>
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<tr>
<td>Rho-GDI</td>
<td>Rho GDP-dissociation inhibitor</td>
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<tr>
<td>RyR2</td>
<td>ryanodine receptor</td>
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<tr>
<td>SERCA</td>
<td>sarcoplasmic reticulum Ca2+ ATPase</td>
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<td>transgenic</td>
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<td>VDAC</td>
<td>voltage-dependent anion channel</td>
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Figure 1. Expression of endogenous CaMKII δ in mouse ventricle and cardiomyocytes. Ventricular tissue and adult mouse ventricular myocytes (AMVM) were isolated from WT and CaMKII δ knockout (δKO) mice, lysed, and subjected to Western blotting. A, Representative blots of CaMKII δ in WT and AMVM δ knockout (δKO) mice, lysed, and subjected to Western blotting. A, Representative blots of CaMKII δ expression in ventricular lysate and AMVM lysate demonstrating 2 bands absent in the δKO. B, Quantitative analysis of the relative abundance of CaMKII δb and δc in WT ventricular tissue. n=8. *P<0.01.
We assumed that the phenotypic differences observed in these TG lines correlated with differential increases in expression of $\delta_C$ and $\delta_C$ in the nuclear versus SR compartments, respectively. To reevaluate this assumption in light of our findings on the distribution of endogenous CaMKII subtypes, we isolated and fractionated ventricular tissue from CaMKII$\delta_B$ and CaMKII$\delta_C$ TG mice. As in the experiments above, the entire volume of each fraction was loaded onto SDS gels to compare CaMKII protein expression among these fractions (Online Figure I). The percentage of the total CaMKII$\alpha$ transgene in each subcellular compartment was quantified and the data from a series of experiments averaged and shown in Figure 4A. Remarkably, whereas CaMKII$\delta_B$ TG mice show a high concentration of CaMKII$\delta_B$ in cardiomyocyte nuclei, based on immunofluorescence staining, subcellular fractionation indicates that significant amounts of CaMKII$\delta_C$ are also present outside of the nucleus in the cytosolic and SR/membrane fractions (Figure 4A). In the CaMKII$\delta_C$ TG mice, immunostaining revealed relative exclusion of CaMKII$\delta_C$ from the nucleus, but, whereas most CaMKII$\delta_C$ is in the SR/membrane fraction, the CaMKII$\delta_C$ subtype is clearly detectable in the nuclear fraction as well (Figure 4A). Thus distribution of the transgenes, like that of endogenous CaMKII$\delta$ subtypes, is not exclusive.

CaMKII$\delta$ is believed to exist as a multimer of 12 subunits. Nuclear versus cytosolic localization can be significantly affected by changing the expression ratio of $\delta_B$ and $\delta_C$ splice variants, consistent with heteromultimerization of these subtypes. Multimerization of transgenically expressed CaMKII$\delta_C$ with endogenous CaMKII$\delta_B$ could promote its localization to the nucleus, whereas multimerization of CaMKII$\delta_B$ with endogenous CaMKII$\delta_C$ could lead to its exclusion from the nuclear compartment. To test the hypothesis that the broad and relatively nonselective subcellular distribution of the CaMKII$\delta_B$ and $\delta_C$ subtypes results from their heteromultimerization, we crossed the CaMKII$\delta_B$ and $\delta_C$ TG mice with the CaMKII$\delta$ KO mice previously developed in our laboratory. Progeny from these crosses were shown to express only a single CaMKII$\delta$ subtype ($\delta_B$ or $\delta_C$) in the CaMKII$\delta$ null background. Interestingly, the subcellular distribution of the CaMKII$\delta_B$ and $\delta_C$ transgenes expressed in the CaMKII$\delta$ null background (Figure 4B) was not appreciably different from that of the $\delta_B$ and $\delta_C$ transgenes expressed in the WT background (Figure 4A). To examine the distribution of the $\delta_B$ and $\delta_C$ subtypes in a manner that does not require cell disruption and fractionation we infected AMVMs from $\delta_B$KO mice with GFP-tagged CaMKII$\delta_B$ and $\delta_C$ adeno virus. Myocytes infected with CaMKII$\delta_B$ and visualized by confocal microscopy clearly showed accumulation of the overexpressed protein in the nuclear compartment, but a significant amount of CaMKII$\delta_B$ was also seen outside the nucleus, distributed in a striated pattern corresponding to T-tubule organization (Figure 5A). Line scan quantification of fluorescent intensity of a 1-μm-thick plane from several different cells showed the fluorescence intensity of CaMKII$\delta_B$ in the nucleus to be 2.69 (±0.08) times higher than that of CaMKII$\delta_B$ in the cytosol; expressed another way, CaMKII$\delta_C$ fluorescence intensity outside of the nuclear compartment is approximately one-third that in the nucleus. Experiments were also carried out using GFP-tagged CaMKII$\delta_C$, and showed prominent localization coincident with the striated patterns of the cardiomyocyte

This analysis revealed that both subtypes were present in every compartment examined (Figure 3B). Importantly, CaMKII$\delta_B$ was detected not only in the nuclear compartment, but also in the SR/membrane compartment; conversely CaMKII$\delta_C$, though abundant in the SR/membrane, was also clearly present in the nuclear compartment (Figure 3B). We have generated cardiac-specific CaMKII$\delta_B$ and $\delta_C$ TG mice and characterized these lines in numerous studies. We assumed that the phenotypic differences observed in these TG lines correlated with differential increases in expression of $\delta_B$ and $\delta_C$ in the nuclear versus SR compartments, respectively. To reevaluate this assumption in light of our findings on the distribution of endogenous CaMKII subtypes, we isolated and fractionated ventricular tissue from CaMKII$\delta_B$ and CaMKII$\delta_C$ TG mice. As in the experiments above, the entire volume of each fraction was loaded onto SDS gels to compare CaMKII protein expression among these fractions (Online Figure I). The percentage of the total CaMKII$\alpha$ transgene in each subcellular compartment was quantified and the data from a series of experiments averaged and shown in Figure 4A. Remarkably, whereas CaMKII$\delta_B$ TG mice show a high concentration of CaMKII$\delta_B$ in cardiomyocyte nuclei, based on immunofluorescence staining, subcellular fractionation indicates that significant amounts of CaMKII$\delta_C$ are also present outside of the nucleus in the cytosolic and SR/membrane fractions (Figure 4A). In the CaMKII$\delta_C$ TG mice, immunostaining revealed relative exclusion of CaMKII$\delta_C$ from the nucleus, but, whereas most CaMKII$\delta_C$ is in the SR/membrane fraction, the CaMKII$\delta_C$ subtype is clearly detectable in the nuclear fraction as well (Figure 4A). Thus distribution of the transgenes, like that of endogenous CaMKII$\delta$ subtypes, is not exclusive.

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Significant perinuclear CaMKII\(\delta_c\) staining was also observed. Line scan quantification of CaMKII\(\delta_c\) fluorescence intensity showed the nuclear \(\delta_c\) signal to be 0.44 (±0.09) of that in the extranuclear compartment; expressed another way the fluorescence intensity of CaMKII\(\delta_c\) 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 CaMKII\(\delta_c\), result from phosphorylation of SR targets involved in \(Ca^{2+}\) handling. Because the SR/membrane fraction obtained from the protocol used in the Figures 2–4 is heterogeneous (eg, it includes sarcolemmal membranes), we optimized a sucrose density gradient separation

**Figure 4. Comparative subcellular distribution of CaMKII\(\delta_b\) and \(\delta_c\) subtypes in transgenic versus transgenic in CaMKII \(\delta\) null background.** Ventricular tissue isolated from \(\delta_b\) TG, \(\delta_b\)/\(\delta\)-KO, and \(\delta_c\)-KO mice was harvested and fractionated into cytosolic, mitochondrial, SR/membrane and nuclear fractions and subjected to Western blotting. The distribution of transgenically expressed CaMKII \(\delta_b\) and \(\delta_c\) is examined in the WT background (A) or in the CaMKII \(\delta\) null background (B). n=4.

**Figure 5. GFP-tagged CaMKII\(\delta_b\) and \(\delta_c\) expressed in AMVMs isolated from \(\delta\)-KO mice.** AMVMs isolated from \(\delta\)-KO mice were infected with adenovirus expressing GFP-tagged CaMKII \(\delta_b\) (A) or GFP-tagged CaMKII \(\delta_c\) (B). 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 \(\mu\)m. Background measurements taken from noninfected AMVMs were averaged and subtracted from the fluorescence intensity measurements of the GFP-tagged enzyme.
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 (Figure 6A) and nuclear (Figure 6B) fractions to contain some mitochondria (VDAC staining) but otherwise show little cross contamination. Immunoblots from the purified SR and nuclear fractions show 2 CaMKII bands (Figure 6C and 6D). Whereas the lower band, CaMKII-B, is the more abundant in the SR (Figure 6C), and CaMKII-C is predominant in the nuclear fraction (Figure 6D), the 2 subtypes are clearly not exclusively segregated to a specific compartment.

The finding that CaMKII-B and CaMKII-C subtypes colocalize 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 10 mmol/L caffeine (to release SR Ca
2+/H11001) or perfused them for 15 minutes with 100 nmol/L PE (to increase nuclear Ca
2+/H11001) levels.17,38 Hearts were then fractionated to obtain purified SR and nuclei (as described in Figure 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 (Figure 7A). In contrast, PE treatment increased P-CaMKII levels in the nuclear fraction but not in the SR. Thus caffeine selectively activates whichever CaMKII 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 CaMKII-B or CaMKII-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 2 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 (Figure 8) or CaMKII-B TG (Online Figure II), treatment with caffeine lead to significant increases in phosphorylation of PLN at Thr17 while treatment with PE did not lead to PLN phosphorylation (Figure 8A). Conversely, treatment with PE increased phosphorylation of HDAC5 at Ser498, whereas caffeine did not increase phosphorylation (Figure 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 Figure III). Additionally, we observed no increase in phosphorylation of these substrates at their putative CaMKII phosphorylation sites in CaMKII δ- KO mice treated with caffeine or PE (Figure 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

CaMKII\( \delta \) and \( \delta_c \) subtypes, which differ only by the inclusion of a nuclear localization sequence, are present in the mouse heart ventricle at similar protein levels (Figure 1). Seminal papers from the Schulman laboratory describing these 2 splice variants,8,21 along with our early studies in which we expressed CaMKII\( \delta_B \) and \( \delta_C \) in neonatal rat ventricular myocytes (NRVMs),22 supported the notion that CaMKII\( \delta_B \) would be localized to and signal in the nucleus, whereas \( \delta_C \) would localize to and signal outside of the nucleus. These conclusions were based on studies in which CaMKII was heterologously expressed in COS cells or NRVMs.22,39,40

Subsequently, we generated CaMKII\( \delta_B \) or \( \delta_C \) 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 CaMKII\( \delta_B \) and \( \delta_C \) in neonatal rat ventricular myocytes (NRVMs),22 supported the notion that CaMKII\( \delta_B \) would be localized to and signal in the nucleus, whereas \( \delta_C \) would localize to and signal outside of the nucleus. These conclusions were based on studies in which CaMKII was heterologously expressed in COS cells or NRVMs.22,39,40

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Discussion

CaMKII\( \delta_B \) and \( \delta_C \) subtypes, which differ only by the inclusion of a nuclear localization sequence, are present in the mouse heart ventricle at similar protein levels (Figure 1). Seminal papers from the Schulman laboratory describing these 2 splice variants,8,21 along with our early studies in which we expressed CaMKII\( \delta_B \) and \( \delta_C \) in neonatal rat ventricular myocytes (NRVMs),22 supported the notion that CaMKII\( \delta_B \) would be localized to and signal in the nucleus, whereas \( \delta_C \) would localize to and signal outside of the nucleus. These conclusions were based on studies in which CaMKII was heterologously expressed in COS cells or NRVMs.22,39,40

Subsequently, we generated CaMKII\( \delta_B \) or \( \delta_C \) 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 CaMKII\( \delta_B \) and \( \delta_C \) in the nucleus and \( \delta_C \) 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 CaMKII\( \delta_B \) and \( \delta_C \) 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 2 subtypes: nuclear effects on gene expression leading to hypertrophy in the CaMKII\( \delta_B \) TG mice, and effects on SR protein phosphorylation and \( \text{Ca}^{2+} \) handling leading to HF development in the CaMKII\( \delta_C \) TGs.

The more extensive analysis presented in the current manuscript was motivated by our observation that endogenous CaMKII\( \delta \) is found in both the SR/membrane and nuclear compartments isolated from mouse ventricle and that there are 2 CaMKII\( \delta \) immunoreactive bands in both of these compartments (Figures 3A and 6). That these bands are absent in CaMKII\( \delta \) knockout mouse hearts (Figure 1A) indicates that they are both CaMKII\( \delta \) gene products, whereas the fact that they differ in mobility by approximately 2 kDa suggests that they represent \( \delta_C \) and the 11 amino acid larger NLS containing \( \delta_B \).21 The ability of various CaMKII isoforms and subtypes to form heteromultimers9,36 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 CaMKII\( \delta_B \) in the absence of CaMKII\( \delta_C \) did not restrict its localization to the nucleus nor was \( \delta_C \) confined to the cytosolic/SR compartment when expressed in the absence of \( \delta_B \) (Figure 4B). Thus heteromultimers of CaMKII\( \delta_B \) and \( \delta_C \) appear unlikely to account for the indiscriminate distribution of these subtypes. We cannot rule out the possibility that other minor cardiac CaMKII isoforms, including CaMKII\( \gamma \) and \( \beta \), heteromultimerize with CaMKII\( \delta \) 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 CaMKII\( \delta_B \) is not restricted to the nuclear compartment, and \( \delta_C \) is not excluded from the nuclear compartment.

Because 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 CaMKIIδC or δC. Data obtained by confocal imaging shows extensive accumulation of GFP-tagged CaMKIIδC in the nucleus, consistent with what we reported previously.32 Notably however, quantitative analysis confirmed that δC is not wholly restricted to the nucleus; indeed δC 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 CaMKIIδC appeared largely consistent with the earlier studies from our laboratory suggesting that δC 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 CaMKIIδC nuclear fluorescence intensity does not include what appears to be a prominent pool of GFP-tagged perinuclear CaMKIIδC; whether this represents mitochondria, SR, or other cellular organelles in confluence with the nucleus.

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 100 nmol/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. Quantified data are from n=5. *P<0.01.

this compartment of CaMKIIδC probably would be included in our nuclear fractionation and thus contribute to higher estimates for the proportion of nuclear CaMKIIδC 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 CaMKIIδC, 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 judgment as to which approach give the most valid estimate of CaMKIIδC and δC in each compartment, all of the preparations and approaches utilized here lead us to the same conclusions: the CaMKIIδC and CaMKIIδC 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 Ca2+ 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 CaMKIIδC or δC in the nuclear compartment with little change in activation of either subtype at the SR; conversely, caffeine activates both CaMKIIδC and δC in the SR, with little change in activation of either subtype in the nuclear compartment (Figure 7). Several published studies have highlighted the importance of localized Ca2+ stores and subsequent compartmentalized signaling within the cardiomyocyte,41–45 In cardiomyocytes, the majority of inositol triphosphate receptors (IP3R2) are located on the nuclear envelope and our previous work demonstrated that endothelin-1 and PE increase Ca2+ release from nuclear IP3 insensitive stores.17,38 Thus we believe that the selectivity of PE for inducing nuclear CaMKII activation reflects Ca2+ mobilization through IP3 insensitive 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 [Ca2+]i increase in the cleft region as a result of SR Ca2+ 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 Ca2+ 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 Figures III and IV) and shown to be selectively increased after addition of caffeine (Figure 8). Phosphorylation of the nuclear transcriptional regulator HDACS at a site indicated by previous studies and in Online Figures III and IV to be a CaMKII phosphorylation site17,46,47 was selectively increased after PE treatment (Figure 8). Agonist selectivity in substrate phosphorylation was demonstrated in studies using both WT (Figure 8) and CaMKIIδC TG (Online Figure II) mice. The high basal level of PLN and HDAC phosphorylation seen in the CaMKIIδC TG heart (Online Figure II) precluded detection of further agonist
induced increases, although it does indicate that both of these substrates are in vivo targets for CaMKIIδC. Notably our previous analysis of the CaMKII δC TG mouse heart demonstrated increased PLN and RyR2 phosphorylation associated with dysfunctional Ca2+ handling and heart failure phenotype.3,32 The reason that we did not see RyR2 and PLN phosphorylation and Ca2+ handling changes in the CaMKIIδC TG mice32 may be that the level of CaMKII transgene expression is lower in the SR of the CaMKII δC TG mice than in the SR of CaMKII δC TG mice (Online Figure V). We do find, however, that both PLN and RyR2 are highly phosphorylated in neonatal rat cardiomyocytes after adenosine expression of equal levels of either CaMKII δC or CaMKII δC (data not shown), supporting the notion that either subtype can phosphorylate these SR targets.

In conclusion, we demonstrate for the first time that CaMKIIδB and δC 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 CaMKIIδB subtypes is also dependent on the nature of the stimulus. The evidence for nonselective CaMKIIδB subtype localization is particularly interesting and challenging with regard to understanding mechanisms by which CaMKIIδB could subserve a protective role, and δC a more deleterious role in cardiomyocyte survival and heart disease.12,48,49

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Disclosures
None.

References
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 (CaMKII\(\delta\)) is the predominant cardiac isoform and is present as 2 major splice variants (subtypes): CaMKII\(\delta_B\) or CaMKII\(\delta_C\), which contains a nuclear localization sequence, and CaMKII\(\delta_B\), which does not contain a nuclear localization sequence.

- Based on cellular or transgenic overexpression, the 2 subtypes are differentially localized and accordingly serve different functions: CaMKII\(\delta_B\) regulates gene expression and cell survival whereas CaMKII\(\delta_C\) regulates Ca\(^{2+}\) handling and cell death.

What New Information Does This Article Contribute?

- The 2 CaMKII\(\delta\) subtypes are not as exclusively localized as previously believed: the SR compartment contains considerable amounts of CaMKII\(\delta_B\) and the nuclear compartment contains significant amounts of CaMKII\(\delta_C\).

- Two Ca\(^{2+}\) mobilizing agonists, caffeine and phenylephrine, differentially activate CaMKII\(\delta\) in accordance with enzyme localization (caffeine in SR, phenylephrine in nucleus) and increase substrate phosphorylation (phospholamban and HDAC-5), independent of CaMKII\(\delta\) subtype.


- Specificity in CaMKII\(\delta\) signaling results from compartmentalized, rather than subtype specific, activation.

This study was designed to test the concept that CaMKII\(\delta_B\) and CaMKII\(\delta_C\), the 2 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 2 subtypes. Using hearts from mice in which only one of the 2 subtypes was expressed (\(\delta_B\) or \(\delta_C\) transgenic mice in a CaMKII\(\delta\) 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 CaMKII\(\delta\) and \(\delta_C\). Strikingly, regardless of subtype, caffeine activated CaMKII in the SR compartment and increased phosphorylation of the CaMKII substrate, phospholamban, whereas phenylephrine only activated CaMKII\(\delta_C\) in the nucleus and increased phosphorylation of the nuclear CaMKII target, HDAC-5. These findings question the accepted notion of strict “nuclear” versus “cytoplasmic” isoforms of CaMKII\(\delta\), while demonstrating that there is compartmentalized activation of CaMKII\(\delta\) and its functional targets in cardiomyocytes.
Location Matters: Clarifying the Concept of Nuclear and Cytosolic CaMKII Subtypes
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SUPPLEMENTAL MATERIAL
for
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METHODS

Transgenic Mice—Transgenic mice expressing either the cytoplasmic CaMKIIδC or the nuclear CaMKIIδB in the heart were generated as described previously.1,2 KO mice lacking CaMKIIδ were generated as described previously.3 CaMKIIδB and δC mice were crossed with CaMKIIδ KO mice (F1), and heterozygous offspring carrying the transgene were inbred with the ones without the transgene (F2). Genotypes used for experiments were WT, δKO, CaMKII δB and δC TG and CaMKII δB and δC TG mice in the δ null background. All mice used in these studies were black swiss background, and WT littermates were used as controls. All mice used in the present study were of mixed gender and 5-6 weeks of age, unless otherwise noted. 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.

Preparation of heart tissue extract and cell lysate for western blot analysis—Each mouse was cervically dislocated and placed in supine position. An incision was made parallel to the diaphragm and the rib cage was separated by cutting parallel to the sternum to visualize the heart. The heart was excised and immediately rinsed in ice cold PBS to remove blood. Atria were removed and the ventricles were snap frozen in liquid nitrogen. Frozen hearts were pulverized and powder was homogenized using a tissue homogenizer (Tissuemiser, Fisher Scientific) in ice cold RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris (pH7.4), 1% NP-40, 1% of sodium deoxycholate, 0.1% of SDS, 0.2 mmol/L EDTA) supplemented with protease and phosphatase inhibitors (100 μmol/L Na3VO4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mmol/L p-nitrophenyl phosphate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L NaF). Cardiomyocytes were washed 3 times in ice cold PBS and then harvested in the RIPA buffer formulation described above. Tissue and cell lysates incubated 4°C for 20 min, and lysates were clarified by centrifugation at 14,000 RPM at 4°C. Protein concentration was measured using Bradford analysis.

Western Blotting—Western blot analysis was carried out as described previously.3,27 Lysates were mixed with LDS sample buffer and reducing agent, heated at 80°C for 10 minutes. Equal amounts of protein (10-40 μg), or equal volumes of fractions were loaded onto SDS-PAGE (Invitrogen NuPage system), run, transferred to PVDF membranes (Millipore), blocked in 5% milk in TBS/Tween for 1 hour and the resulting blot probed using antibodies at a 1:1000 dilution in 3% BSA/TBS-tween. The antibodies used for immunoblotting were as following: rabbit CaMKIIδ antibody (a gift from Don Bers laboratory, University of California Davis, Davis, CA), mouse anti-phospholamban (Upstate Biotechnology), rabbit anti-phospho-phospholamban (Thr17) (Cyclacel, Dundee, UK), rabbit Rho-GDI (Cell signaling technology), rabbit VDAC/porin (Calbiochem), mouse SERCA2a (Thermo/Affinity Bioreagents),
mouse Lamin A/C (Cell Signaling Technology), mouse anti-phospho-CaMKII (Thermo/Affinity Bioreagents), and rabbit anti-phospho-HDAC5 Ser498 (Signalway Antibody). Following primary antibody incubation, blots were washed with TBS-tween (10 min x 3 times) and incubated with secondary antibodies (1:5000) in 5% milk/TBS-Tween for 30-60 minutes. Anti-rabbit or anti-mouse secondary antibody (Sigma) was used at 1:10,000 dilution.

**Tissue Fractionation** - Tissue was fractionated using differential centrifugation as described previously. Briefly, flash frozen hearts were pulverized and homogenized using a Dounce glass tissue grinder. The homogenate was centrifuged for 10 min at 600 × g, the pellet washed three times and then re-suspended in nuclear extract buffer (HEPES 20 mmol/L, 25% Glycerol, NaCl 420 mmol/L, MgCl₂ 1.5 mmol/L, EDTA 0.2 mmol/L). The resulting supernatant is the Nuclear Fraction. The supernatant from the first centrifugation was centrifuged at 5000 × g (to remove mitochondria) and the resulting supernatant then centrifuged at 100,000 × g. The supernatant is the Cytosolic Fraction and the pellet, after being washed 3 times and re-suspended in RIPA buffer (NaCl 150 mmol/L, Tris 20 mmol/L, 1% Triton X 100, 0.1% SDS), is the SR/membrane fraction.

**SR purification** – Freshly isolated myocytes from 4 mouse hearts were suspend in homogenization buffer and using a dounce homogenizer, cells were homogenized using 15-20 strokes on setting B. Cell lysates were spun at 10,000 RPM to clarify lysates and pellets resuspended in homogenization buffer and homogenize for 10 strokes at setting B. Following a spin @10,000 rpm for 8min, supernatant was collected and loaded onto a fresh sucrose gradient consisting of 1ml of 20%, 2ml of 27%, 2 ml 30%, 2ml of 34%, 3ml of sup with 72% sucrose (1.5 ml +1.5ml 72%). Gradients were spun at 22000rpm for 24hrs @4C. SR fraction was harvested from the interface of the 27% and 30% gradient.

**AMVM isolation** – Adult mouse ventricular myocytes were isolated from WT and CaMKIIδ KO mice (4 weeks old, weighing 23–25 g). Hearts were removed, cannulated and perfused via the aorta at 37° C at a rate of 3ml/min. Hearts were first perfused with Ca²⁺ free buffer for 4 minutes, followed by perfusion with the buffer containing 0.25 mg/ml collagenase (Blendzyme 1, Roche) for 8-12 minutes. Atria were removed, and ventricle was dissociated by pipetting with increasingly smaller diameter transfer pipette tips. Following tissue digestion, collagenase was inactivated by addition of 10% bovine calf serum to tissue suspension. Calcium was gradually added back to a final concentration of 1mmol/L. Cells were plated on laminin-coated dishes or chamber slides in minimal essential medium/Hanks’ balanced salt solution containing 5% serum. After 1 hour, cells were washed and serum-free medium was added back. Prior to adenoviral infection, cells were serum starved for 4 hours.

**Adenoviral infection and Immunofluorescent measurements of Adult Mouse Ventricular Myocytes** – Isolated AMVMs were infected with AdCMV, GFP-tagged CaMKIIδB or CaMKIIδC at 3000-5000 viral particles/cell and cultured in serum free media for 24 hours. The GFP- tagged CaMKIIδB and δC adenovirus were a gift from Don Bers laboratory, University of California Davis, CA. Cells were fixed using 4% paraformaldehyde (20 min at room temperature), rinsed (three times) in phosphate-buffered saline (PBS) and then mounted with Vectashield with DAPI.
Langendorff Perfusion of Heart—Mice (4 weeks old, weighing 23–25 g) were heparinized (500 units/kg, intraperitoneally) and cervically dislocated. Hearts were rapidly excised, washed in ice-cold Krebs-Henseleit solution (118 mmol/L NaCl, 24 mmol/L NaHCO3, 4 mmol/L KCl, 1 mmol/L NaH2PO4, 2 mmol/L CaCl2, 1.2 mmol/L MgCl2, 12 mmol/L glucose and 10 mmol/L Hepes, pH 7.4), and cannulated via the aorta on a 20-gauge stainless steel blunt needle. Hearts were perfused at 80 mm Hg on a Langendorff apparatus using Krebs-Henseleit solution at 37 °C. Hearts were perfused with oxygenated buffer for 12 min to allow for equilibration, followed by 15 minutes perfusion with 100 nmol/L phenylephrine or vehicle, or a bolus injection of 10 mmol/L caffeine or vehicle directly into the heart. KN-93 (20 μmol/L) was added to the Krebs-Henseleit buffer used for the 12 minute equilibration perfusion as a pre-treatment. Following this pre-treatment, KN-93 was administered simultaneously with PE or caffeine. Heart was removed following perfusion/treatment and immediately rinsed in ice cold buffer, atria removed, and then flash frozen in liquid nitrogen.

Statistical Analysis - All data are reported as means±SEM. Statistical significance of differences between groups was determined using 1-way ANOVA with Tukey post hoc test. A probability value of <0.05 was considered statistically significant

REFERENCES


Online Figure I. Comparative subcellular distribution of CaMKII $\delta_B$ and $\delta_C$ isoforms in $\delta_B$ TG and $\delta_C$ TG mice. Ventricular tissue was isolated from $\delta_B$ or $\delta_C$ TG mice and fractionated into cytosol, mitochondria, SR/membrane and nuclear fractions. Equal portions were loaded onto gels for comparative purposes. Representative western blots show the relative distribution of HA-tagged CaMKII in all the fractions. Blots were probed with HA antibody to identify overexpressed CaMKII.
Online Figure II. Caffeine and PE induced phosphorylation of PLN and HDAC5 in CaMKII$\delta_B$ and CaMKII$\delta_C$ TG mice. Hearts were isolated from $\delta_B$ and $\delta_C$ TG mice, perfused with 10mmol/L caffeine or 100nmol/L phenylephrine. Ventricular homogenates were subjected to western blotting for (A) PLN phosphorylation at the CaMKII phosphorylation site, threonine-17 and (B) HDAC5 phosphorylation using an antibody to the CaMKII phosphorylation site. Quantitated data are from n=3 * p<0.01
**Online Figure III. Substrate phosphorylation is blocked by the CaMKII inhibitor KN-93.** Hearts were isolated from mice and perfused with 20μmol/L KN-93 following by a bolus injection of 10mmol/L caffeine + KN-93 or followed by perfusion of 1 μmol/L phenylephrine + KN-93. Ventricular homogenates were subjected to Western blotting for (A) PLN phosphorylation at, T-17 or (B) HDAC5 phosphorylation at S-498. n=3-4 # p<0.05 and * p<0.01
Online Figure IV. Substrate phosphorylation in response to PE and caffeine requires CaMKII. Hearts were isolated from CaMKIIδ KO mice and perfused with either 10mmol/L caffeine or 1μmol/L phenylephrine. Ventricular homogenates were subjected to Western blotting for (A) PLN phosphorylation at T-17 or (B) HDAC5 phosphorylation at S-498. n=3-4
Online Figure V. Relative expression levels of CaMKIIδ in whole heart homogenate and isolated SR fractions from CaMKIIδ TG mice. (A) Ventricular tissue isolated from WT, δKO, δn TG and δc TG mice and subjected to western blotting for T-CaMKIIδ expression. (B) Purified SR fraction from ventricle isolated from WT, δn and δc TG mice and probed for HA-tagged T-CaMKIIδ