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Pinning down the CaMKII targets in the L-type Ca^{2+} channel: an essential step in defining CaMKII regulation

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With each heartbeat, intracellular Ca^{2+} in a myocyte transiently increases about 10-fold, from ~ 100 nM to a peak of ~ 1 μM . In subcellular domains, such as near the mouth of an L-type Ca^{2+} channel (LTCC), the peak is even higher. These rapid oscillations in Ca^{2+} , commencing with Ca^{2+} entry through the LTCC that in turn triggers the release of intracellular Ca^{2+} stores from the sarcoplasmic reticulum via the ryanodine receptor type 2, are central to excitation–contraction coupling. In the midst of these large beat-to-beat changes in internal Ca^{2+} , comparatively subtle Ca^{2+} signals control so much more than excitation–contraction coupling. A decade of research has shown that Ca^{2+} signals also trigger arrhythmogenic afterdepolarizations, initiate gene expression that leads to hypertrophy, and induce apoptosis. So, how does Ca^{2+} control all this among the waves of the beat-to-beat Ca^{2+} changes driving excitation–contraction coupling?

Part of the answer lies with a fundamental molecule sitting at the hub of many Ca^{2+} -dependent signaling pathways, the Ca^{2+} /calmodulin-dependent kinase II (CaMKII). Upon activation, CaMKII phosphorylates numerous downstream targets including ion channels and transcriptional regulators. Focusing on events at the sarcolemma, CaMKII-dependent phosphorylation and consequent regulation of ion channels affects the integrated cardiomyocyte Ca^{2+} signaling dynamics. Phosphorylation of LTCCs or ryanodine receptors directly increases Ca^{2+} entry, contributing to many downstream signaling pathways including the positive force-frequency relationship¹. As Ca^{2+} influx through the LTCC is the initiator of the downstream Ca^{2+} signaling cascades, CaMKII phosphorylation of LTCCs also creates a form of direct feed-forward control. In addition to the LTCC and ryanodine receptor, phosphorylation of other ion channels shapes the cardiac action potential, thus affecting LTCC gating and indirectly controlling Ca^{2+} entry.

Special features equip CaMKII to orchestrate these critical Ca^{2+} signaling pathways among the noise of the large oscillatory changes driving excitation–contraction coupling. Foremost,

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Conflicts of Interest: None.

CaMKII is endowed with the capacity to modulate its activity in response to changes in the amplitude and frequency of Ca^{2+} signals. Its activity also can be modulated by oxidation² or β -adrenergic signaling³, thereby integrating CaMKII-dependent Ca^{2+} signaling with other signaling pathways. Additionally, a fraction of myocyte CaMKII is tethered to LTCCs⁴, poised to decode changes at the source of the Ca^{2+} signaling cascade. Untangling the contributions of this web of signaling pathways is a major focus of current research.

One problem restraining progress has been that many of CaMKII's downstream targets (the LTCC is an especially good example) have been recalcitrant in revealing the functionally relevant phosphorylation sites, thus preventing a complete understanding of CaMKII's complex actions. This is not a problem unique to CaMKII, however. The site(s) on the LTCC targeted by protein kinase A (PKA) for adrenergic potentiation of the Ca^{2+} current remain unidentified more than 40 years after the phenomenon was described. Serine 1928, an unambiguous PKA target and the former leading candidate for the PKA effector site on the LTCC, was finally disqualified only after a S1928A knockin mouse model demonstrated unaltered β -adrenergic potentiation of the LTCC current⁵. For CaMKII, multiple candidate phosphorylation sites have been identified in both the LTCC's pore-forming α_{1C} subunit and the auxiliary β_2 subunit, but without a clear understanding of how these sites individually or collectively contribute to functional changes in the LTCC current.

The α_{1C} subunit and the β_2 subunit were shown to be CaMKII targets and individual phosphorylation sites were identified by testing consensus phosphorylation sites with *in vitro* kinase assays^{4, 6, 7}. The contributions of the phosphorylation sites on either subunit were then investigated using "elimination" models with conflicting results as to which sites are dominant: the significance of the α_{1C} phosphorylation sites was examined with a knockin mouse model (S1512 and S1570 were mutated to the non-phosphorylatable A), which showed reduced CaMKII-dependent potentiation of the LTCC⁸; the role of the candidate phosphorylation site in β_2 (T498 in the β_2a splice variant) was tested by viral overexpression of mutated β_2 subunits in isolated rabbit cardiomyocytes, which also reduced LTCC current potentiation⁹.

The explicit conclusion from each report is that the phosphorylation site(s) on the studied subunit are the main regulators of the CaMKII-dependent effects upon the LTCC. Each of the models has clear strengths, and so supports the role of the subunit studied in the respective report; yet neither model demonstrated complete absence of CaMKII-dependent effects, leaving open the possibility for other CaMKII targets. For example, the knockin mouse model fully replaced the endogenous α_{1C} subunit with the non-phosphorylatable mutant⁸. Stoichiometry among the different LTCC subunits and among other key Ca^{2+} -regulatory molecules was unperturbed. Moreover, variables such as heart rate and contractile function could be measured *in vivo*; that they were unaffected suggests that the knockin strategy produced no aberrant changes to the overall electrophysiological properties in the heart. For these reasons, a knockin strategy should be considered the gold-standard for evaluating the role of specific phosphorylation sites. Nevertheless, mice are not the perfect physiologic models. The faster murine heart rate leaves less room for an exercise- or adrenergic-induced heart rate increase and differences with other species in properties such as the force-frequency relationship, a major readout of CaMKII-dependent effects on the LTCC current, makes studying effects of CaMKII on the LTCC more challenging.

On the other hand, expression of a phosphorylation-deficient β_2a subunit by lentivirus in adult rabbit ventricular myocytes also reduced LTCC potentiation attributable to CaMKII⁹. Faster and more economical than creating a knockin model, viral overexpression permitted examination of multiple mutants, allowing the investigators to probe not only the specific phosphorylation site but also the significance of a candidate tethering site between CaMKII

and $\beta 2a$. Additionally, the viral overexpression strategy enables investigation in various non-mouse models that may more accurately translate to human disease, a feature successfully exploited in this study to tie CaMKII to early afterdepolarizations. These advantages, however, come with their own concessions. Atop this list is that the endogenous $\beta 2a$ subunit remains and that the stoichiometry among LTCC subunits is not preserved. Indeed, the LTCC current density in myocytes overexpressing $\beta 2a$ was significantly larger than in untransfected cells. Also, after viral infection myocytes need to be cultured; this can trigger dedifferentiation and associated loss of myocyte-specific morphological characteristics.

So, is it the α_{1C} sites or the β_2 sites? Our opinion is that it is probably both—and that sites on other factors associated with the channel might also be targeted by the tethered CaMKII and contribute to the overall regulation by CaMKII. The LTCC is a macromolecular complex containing not only the channel subunits α_{1C} and β_2 and tethered CaMKII, but also several other signaling components, such as calcineurin (a phosphatase that can oppose the actions of CaMKII), the PKA anchoring protein AKAP79, and the β_2 -adrenergic receptor. With this abundance of known and potential CaMKII targets and modulators, the strengths of both models should be employed going forward. In the context of a knockin model, the potential contribution of other sites in other components of the complex could be preliminarily tested with the viral expression approach and confirmed with the additional knockin.

Going forward, identifying the functionally important CaMKII target sites is an important challenge. Inhibition of CaMKII by a peptide that eliminated all kinase activity blocked pathological remodeling after myocardial infarction¹⁰. Whether phosphorylation of α_{1C} and/or $\beta 2a$ underlies the negative effects of CaMKII in the post-myocardial infarction setting has not yet been investigated, but it is interesting to speculate that an agent specific for one target might provide additional benefits. In our opinion, a combination of the approaches outlined above offer the best way forward to begin to answer questions such as this. In a larger context, this issue is not restricted to the LTCC; other reviews in this series will focus on CaMKII actions upon other ion channels for which specific phosphorylation sites have not yet even been identified (e.g., the cardiac Na^+ channel, $Na_v1.5$).

Moreover, we should not ignore the possibility that some effects of CaMKII may be independent of its kinase activity, as suggested for the modulation of P/Q-type Ca^{2+} channels in adult rat hippocampal pyramidal neurons¹¹. A truly multifunctional molecule, CaMKII's numerous pathways of activation and many targets make elucidating its mechanisms of action a formidable challenge. Ultimately, unlocking the mystery of this fundamental molecule could be the key to understanding the pathophysiology behind cardiac hypertrophy, sudden cardiac death, and heart failure, providing new targets for novel therapeutic modalities.

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Abbreviations

CaMKII	Ca^{2+} /calmodulin-dependent kinase II
LTCC	L-type Ca^{2+} channel
PKA	protein kinase A

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