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Crossing Signals: relationships between β -adrenergic stimulation and CaMKII activation $\pm$

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CaMKII is a ubiquitous and pleiotropic serine threonine kinase. The predominant isoform of CaMKII in the heart is CaMKII delta (CaMKII δ). CaMKII phosphorylates and regulates a number of cardiac proteins involved in excitation-contraction coupling and Ca⁺⁺ handling including the cardiac ryanodine receptor (RyR2), phospholamban (PLN), the L-type Ca⁺⁺ channel α_{1C} - and β_{2a} -subunits, and the Na channel (1). Studies using animal models have increasingly linked CaMKII activation and its effects on these targets to the development of heart failure and arrhythmogenesis. There is also considerable evidence that CaMKII expression and activity are increased in human heart failure (1).

Cardiac β -adrenergic receptors (β -AR) respond to input from the sympathetic nervous system by increasing cyclic AMP and activating another serine threonine kinase, protein kinase A (PKA). PKA has many targets in common with CaMKII, although the sites of phosphorylation, for example those on PLN and RyR2, are distinct for the two protein kinases. There is an extensive literature documenting the involvement of increased sympathetic tone and β -AR stimulation in heart failure. Indeed β -AR blockade is amongst the most common treatments for patients with heart failure. Theories about the basis for efficacy of β -AR blockade in heart failure include improvement of contractile function, reduction of myocardial oxygen consumption, reduction of cardiac afterload, normalization of myocardial gene expression, and beneficial effects on left ventricular remodeling. In addition, β -AR blockers decrease the incidence of sudden cardiac death.

Is there reason to consider that the β -AR and CaMKII pathways, both clearly implicated in cardiac remodeling and heart failure, are in some way coupled? Surprising evidence from the Xiao and Cheng labs demonstrated that CaMKII (rather than PKA) mediates sustained (hrs) vs. acute (minutes) contractile responses of rat cardiomyocytes to β_1 -AR stimulation. Subsequent studies implicated CaMKII as the mediator of a variety of cardiac responses to β -AR signaling, including induction of hypertrophic genes, phospholamban (PLN) phosphorylation and apoptosis (Table 1).

What is the evidence that β -AR responses occur through CaMKII? Table 1 summarizes the endpoints measured and experimental proof implicating CaMKII in these β -AR responses. These findings have been discussed in more detail in a recent review (2). It is notable that in many cases the proof for CaMKII involvement is blockade by a pharmacological inhibitor

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(KN-93) which appears to be selective for CaMKII but may have additional kinase targets, especially at high concentrations. Phosphorylation of PLN at the CaMKII site is a very commonly used, albeit indirect, readout for CaMKII activation; while the CaMKII site on PLN is indeed distinct from that for PKA, there is evidence that it may be a target for other protein kinases such as Akt. Only a few studies have used gene deletion or genetic inhibition of CaMKII function to examine CaMKII involvement in physiologic or pathologic responses to β -AR stimulation.

CaMKII is activated by its interaction with calcium bound calmodulin ($\text{Ca}^{++}/\text{CaM}$) and thus by elevation of intracellular Ca^{++} . It is not actually known whether CaMKII is activated in parallel with the increases in cytosolic Ca^{++} that occur on a beat to beat, but if it is this activation likely reverses as Ca^{++} levels fall. At higher frequencies or with more prolonged Ca^{++} elevation, however, the $\text{Ca}^{++}/\text{CaM}$ bound enzyme can autophosphorylate on its Thr 286/287 site. Autophosphorylation renders the enzyme Ca^{++} independent, allowing maintenance of enhanced activity even when Ca^{++} returns to resting levels. Autophosphorylation has been widely used as a measure of CaMKII activation and can be detected using a commercially available antibody to the phospho-Thr 286/287 site. One caveat is that this antibody is not specific for the predominant cardiac CaMKII δ isoform, and would also detect phosphorylated CaMKII sequences on other CaMKII isoforms (e.g. CaMKII γ) or possibly CaMKII consensus sequences on undefined proteins of similar molecular weight. Indeed our lab has noted, as has Johannes Back's laboratory (personal communication), that a signal is detected by the phospho-CaMKII antibody that can still be increased in CaMKII δ knockout mice. Another important and newly discovered mechanism by which CaMKII can be constitutively activated is by methionine oxidation which, like autophosphorylation, maintains CaMKII in a chronic state of activation, independent of the need for elevated Ca^{++} (4). This has been demonstrated to occur in response to angiotensin, but could also be considered to occur when the heart is exposed to sustained high doses of catecholamines which induce oxidative stress.

How can β -AR signaling lead to the activation of CaMKII? There is no consensus, in fact there seem to be rather disparate conclusions on the mechanism by which β -AR stimulation increases the activity of CaMKII, as recently reviewed (2). Potential mechanisms are depicted in Fig 1. The most straightforward, but not routinely substantiated, model would be that cAMP activates PKA, which increases L-type Ca^{++} channel activity, SR Ca^{++} stores and Ca^{++} release. Alternatives include cAMP mediated (but PKA independent) activation of the exchange protein directly activated by cAMP (Epac), which Smrcka's group showed to subsequently activate CaMKII through PKC, or targeting of Epac and CaMKII through interaction with β -arrestin, as suggested by the Rockman laboratory. There is suggestive evidence that NO synthase (NOS) can mediate CaMKII activation in response to β -AR stimulation, and, as indicated above ROS could also activate CaMKII via oxidation. The reason that there is no mechanistic consensus from studies in this area may be because of differences in the endpoint and evidence used to implicate CaMKII.

The generation of CaMKII δ knockout mice in our laboratory provided us with the opportunity to directly test the involvement of CaMKII in cardiac responses to β -AR stimulation. We used these knockout mice to examine acute effects of ISO on cardiac function and protein phosphorylation, as well as the ability of *in vivo* ISO treatment to induce cardiac hypertrophy and heart failure. Our findings, which are currently being submitted for publication, were surprising. In studies using Langendorff perfused hearts, we determined that acute effects of ISO on contractility and heart rate were unaffected by CaMKII δ deletion. Cardiomyocytes isolated from CaMKII δ KO mice also responded to ISO with identical sarcomere shortening and had Ca^{++} transient kinetics indistinguishable from those of WT mice. The phosphorylation of PLN and RyR at CaMKII sites was examined

and found to be increased by ISO in WT mouse cardiomyocytes, as others have observed (Table 1). Remarkably however, ISO still increased phosphorylation of PLN and RyR at these putative CaMKII sites even when CaMKII δ was deleted. Finally *in vivo* studies in which WT and KO mice were treated by injection (3) or infusion of ISO for 3-14 days demonstrated that ISO induced hypertrophy (increased HW/BW ratio) was not different in WT vs. CaMKII δ KO mice. These data indicate, most surprisingly, that the predominant cardiac isoform, CaMKII δ , does not contribute significantly to the acute effects of isoproterenol on excitation-contraction coupling, to ISO induced phosphorylation of PLN or RyR2, or to cardiac hypertrophy induced by β -AR stimulation. While we cannot rule out the involvement of other more minor cardiac CaMKII isoforms (e.g. CaMKII γ or β) in the aforementioned responses to β -AR stimulation, there is an 80% decrease in total CaMKII enzymatic activity in the KO mice. Thus one would expect to see some diminution of CaMKII dependent β -AR responses, unless they are specifically regulated by compartmentalized effects of a small pool of CaMKII. It is also worth noting that more minor CaMKII isoforms present in the heart may well be inhibited in experiments using KN-93 or the in TG mice expressing the CaMKII inhibitor peptide AC3-I, which also blocks PKD activity.

A very different situation is seen when WT and CaMKII δ KO mice are treated by chronic infusion or daily injection of high doses (10-30 mg/kg/day) of ISO. This treatment leads to development of cardiac dysfunction and ventricular dilation, characteristics of heart failure. Anderson's lab showed diminished cardiac remodeling in response to long term ISO injection in mice expressing the CaMKII inhibitor peptide AC3-I. We likewise find that the induction of fibrosis and collagen gene expression following infusion or chronic injection of ISO are markedly attenuated in CaMKII δ KO mice. Moreover 4 wks of daily ISO injection leads to marked increases in lung weight and impaired contractile function, both of which are greatly attenuated in mice that do not express CaMKII δ . The mechanisms by which chronic ISO treatment leads to development of heart failure and fibrosis are not well understood. However, many would consider this a form of ISO toxicity as it requires high doses and long times of treatment. Colucci and others have linked ISO toxicity in cardiomyocytes to generation of ROS and induction of mitochondrial death pathways. Other studies have linked ISO induced apoptosis to CaMKII δ effects on mitochondrial death pathways (see Table 1). Our finding that ISO-induced heart failure requires CaMKII δ is consistent with the hypothesis that ISO treatment leads to generation of ROS and to oxidation and chronic activation of CaMKII δ , resulting in induction of apoptosis, maladaptive remodeling and heart failure development.

Of additional interest, we observed significant mortality in mice treated daily with high doses of ISO but noted that survival was improved in the CaMKII δ KO mice. While we did not establish the cause of death in these mice, ISO treatment can induce lethal arrhythmias, and the ability of ISO to induce arrhythmias has been shown to increase in CaMKII δ TG mice (5). ISO induced arrhythmias have also been shown to be decreased when CaMKII is inhibited by AC3-I expression. Together these data indicate that there is an interaction between ISO and CaMKII signaling in promoting arrhythmogenesis. There are a number of sites at which increased sympathetic stimulation and increased CaMKII activity could synergize to dysregulate Ca^{++} handling and enhance arrhythmias including the combined effects of PKA to increase SR Ca^{++} and of CaMKII phosphorylation of RyR2 to increase SR Ca^{++} leak. Whether or not CaMKII acts in a signaling cascade downstream of the β -AR, coincident increases in sympathetic tone and CaMKII signaling in heart failure can be markedly synergistic in inducing lethal arrhythmias.

From our viewpoint, the canonical cAMP/PKA pathway is more than sufficient to mediate the acute effects of β -AR signaling on cardiac E-C coupling. If there is a role for CaMKII δ

in acute physiological β -AR signaling it would be a subtle modulatory one (e.g. on diastolic Ca^{++} leak). Involvement of CaMKII as a mediator of ISO induced cardiac hypertrophy is also uncertain, and in fact we demonstrated that pressure overload (TAC) induced hypertrophy does not require CaMKII δ (2). On the other hand, it appears likely that chronic increases in sympathetic tone, as occur in HF, activate CaMKII to stimulate cardiomyocyte cell death, as well as to enhance the propensity for fatal arrhythmias. Thus in heart failure the β -AR transduces signals not only through the classic cAMP and PKA pathways but also by engaging CaMKII as an executor of cardiomyocyte death and maladaptive remodeling. Current strategies aimed at blocking β -AR signaling may indeed ultimately be beneficial due in part to blockade of CaMKII activation. More direct targeting of the CaMKII pathway is now being considered as a viable therapeutic strategy for treatment of heart failure, and particularly the associated arrhythmias.

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Fig 1. Schematic of proposed pathways for β -AR signaling to CaMKII in cardiac myocytes
 Pathways are color coded. In blue the canonical pathway in which cAMP activates PKA, phosphorylating the L-type Ca^{++} channel (LTCC) and RyR2, is shown to activate Ca^{++} /CaM and CaMKII. The pathway shown in orange utilizes the exchange protein directly activated by cAMP (Epac) rather than PKA, activating CaMKII through the small GTP'ase Rap, phospholipase C epsilon (PLC ϵ), and protein kinase C epsilon (PKC ϵ). The purple pathway utilizes reactive oxygen species (ROS) signaling to effect CaMKII activation at known sites; the green pathway utilizes NO synthase (NOS) signaling to effect CaMKII activation through more hypothetical mechanisms.

Table 1
Responses elicited by β -AR signaling and mediated through CaMKII

Selected papers, listed in chronological order, use various means of activating β -AR (“stimulus”) to measure selected responses (“endpoints”). Involvement of CaMKII in transducing signals from β -AR to these endpoints is inferred by using various interventions (“proof”). W-7 and KN-93 are pharmacological inhibitors of CaMKII; AIP and AC3-1 are peptide inhibitors of CaMKII.

Stimulus	Endpoint	Proof	Reference
Isoproterenol	PLN phosphorylation	Ca channel blockers	Kuschel, Am J Physiol 1999
Isoproterenol	PLN phosphorylation, contractility, relaxation	W-7, Ca channel blocker, okadaic acid	Said, Pflugers Arch 2002
Isoproterenol, norepinephrine, prazosin, Adv- β 1AR,	Apoptosis	KN-93, AIP	Zhu, J Clin Invest 2003
Norepinephrine + prazosin	Contractility, acute vs. chronic, PLN phosphorylation	KN-93, AIP, Adv-DN CaMKII	Wang, Circ Res 2004
Isoproterenol	Maladaptive remodeling	AC3-I transgenic	Zhang, Nat Med 2005
Isoproterenol	Hypertrophic gene expression	KN-93, Ca channel blocker	Sucharov, Am J Physiol Heart Circ Physiol 2006
Isoproterenol	Apoptosis	AC3-I transgenic	Yang, Am J Physiol Heart Circ Physiol 2006
Isoproterenol, forskolin	SR Ca leak	KN-93, AIP	Curran, Circ Res 2007
Isoproterenol	RyR phosphorylation	KN-93, Ca channel blockers, low Ca	Ferrero, J Mol Cell Cardiol 2007
8-CPT (Epac activator)	Ca transients, Ca sparks, RyR phosphorylation	KN-93, CaMKII phosphorylation and activity	Pereira, J Physiol 2007
	Hypertrophy		Métrich, Circ Res. 2008
Isoproterenol	ISO-inducible arrhythmias	KN-93, AIP, CaMKII δ KO	Sag, Circ Heart Fail 2009
Isoproterenol	Heart rate	AC3-I transgenic	Wu, PNAS 2009
Isoproterenol β AR-KOs	Contractility, apoptosis, PLN phosphorylation	CaMKII phosphorylation and activity	Yoo, Am J Physiol Heart Circ Physiol 2009
Isoproterenol, forskolin	PLN phosphorylation	KN-93, CaMKII phosphorylation, CaMKII activity,	Mangmool, J Cell Biol 2010
Isoproterenol	L-type calcium current, left ventricular function.	CaMKII δ KO	Xu, Circ Res 2010