ORIGINAL RESEARCH

Phosphoproteomics Study Based on In Vivo Inhibition Reveals Sites of Calmodulin-Dependent Protein Kinase II Regulation in the Heart

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***Background*—**The multifunctional Ca^{2+}- and calmodulin-dependent protein kinase II (CaMKII) is a crucial mediator of cardiac physiology and pathology. Increased expression and activation of CaMKII has been linked to elevated risk for arrhythmic events and is a hallmark of human heart failure. A useful approach to determining CaMKII's role therein is large-scale analysis of phosphorylation events by mass spectrometry. However, current large-scale phosphoproteomics approaches have proved inadequate for high-fidelity identification of kinase-specific roles. The purpose of this study was to develop a phosphoproteomics approach to specifically identify CaMKII's downstream effects in cardiac tissue.

***Methods and Results*—**To identify putative downstream CaMKII targets in cardiac tissue, animals with myocardial-delimited expression of the specific peptide inhibitor of CaMKII (AC3-I) or an inactive control (AC3-C) were compared using quantitative phosphoproteomics. The hearts were isolated after isoproterenol injection to induce CaMKII activation downstream of β-adrenergic receptor agonist stimulation. Enriched phosphopeptides from AC3-I and AC3-C mice were differentially quantified using stable isotope dimethyl labeling, strong cation exchange chromatography and high-resolution LC-MS/MS. Phosphorylation levels of several hundred sites could be profiled, including 39 phosphoproteins noticeably affected by AC3-I-mediated CaMKII inhibition.

***Conclusions*—**Our data set included known CaMKII substrates, as well as several new candidate proteins involved in functions not previously implicated in CaMKII signaling. **(*J Am Heart Assoc.* 2013;2:e000318 doi: 10.1161/JAHA.113.000318)**

Key Words: CaMKII • mass spectrometry • phosphorylation • proteomics • transgenic mouse model

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Accompanying Tables S1 and S2 are available at http://jaha.ahajournals.org/content/2/4/e000318/suppl/DC1

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A beating heart relies on the concerted action of many intertwined signaling networks that regulate cardiac contraction and relaxation at the molecular level. In cardiac myocytes (CMs) 1 of the best characterized signaling events is the ß-adrenergic receptor pathway, which directly regulates intracellular Ca^{2+} concentrations. First Ca^{2+} enters the CM through L-type calcium channels (LTCCs), which triggers release of Ca^{2+} from intracellular stores in the sarcoplasmic reticulum (SR) via the ryanodine receptor (Ryr). Reuptake of Ca^{2+} via sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) is regulated by phospholamban, and this, with outward transport via the sodium/calcium exchanger (NCX), completes the Ca^{2+} cycle of the beating heart.[1] The Ca^{2+} cycle is tunable by circulating β-adrenergic receptor agonists such as epinephrine and norepinephrine to accommodate requested changes in cardiac performance.

The second-messenger molecule cAMP and cAMP-dependent protein kinase (PKA) form the first intracellular response, which directly affects the adjustability of adrenergically driven intracellular Ca^{2+} levels (reviewed by references 1,2). A handful of cardiac PKA phosphorylation targets mediating this response have been identified. More recently, another kinase revealed itself as a key player in the adrenergic response: the

multifunctional Ca^{2+}- and calmodulin-dependent protein kinase II[3] (CaMKII). CaMKII is activated at elevated intracellular Ca^{2+} levels. Ca^{2+} binds calmodulin (CaM), and calcified CaM binds to the CaMKII regulatory domain.[4] Both PKA and CaMKII are among the most abundant kinases in heart.[5] There are 4 distinct *CaMKII* genes (α-δ) that encode multiple enzyme splice variants. CaMKIIγ and CaMKIIδ are present in heart, and excessive CaMKIIδ activity is most implicated in myocardial disease.[6,7] However, all CaMKII isoforms share highly conserved catalytic and regulatory domains and compete for overlapping substrates.

An adrenergically driven increase in CaMKII activity leads to direct phosphorylation of phospholamban (PLN) and the type II ryanodine receptor (Ryr2), at Thr17 and Ser2815, respectively, thereby directly affecting the Ca^{2+} cycle.[8,9] These events, together with CaMKII autophosphorylation at Thr287,[10] are considered hallmarks of cardiac CaMKII activity. Intriguingly, both these events are intertwined with cAMP action, as PLN and Ryr2 are also phosphorylated by PKA at nearby sites Ser16[11] and Ser2809,[12] respectively. In addition, other cAMP pathways acting through exchange proteins activated by cAMP, which are PKA independent, have also been reported.[13] Ca^{2+}/CaM-independent CaMKII activation also occurs via oxidation of a pair of regulatory domain methionines (Met281/282).[14]

Heart failure is characterized by activation of the sympathetic nervous system and subsequent overstimulation of cardiac ß-adrenergic signaling. As a consequence, CaMKII expression[15] and activity[16] were found to be increased in human heart failure. Mouse models with cardiac overexpression of CaMKIIδ serve as a model for heart failure,[17] whereas mice with myocardial CaMKII inhibition by transgenic expression of an inhibitory peptide[18] or gene deletion (CaMKII$\delta^{-/-}$)[7] are protected from various pathological stimuli leading to heart failure, including isoproterenol toxicity.[19] In contrast, CaMKII activity is increased in compensated hypertrophy and arrhythmia, whereas CaMKII expression remains at basal levels.[20] These findings support a view that CaMKII is a critical pathological signal transducer in myocardium for mediating the effects of chronic ß-adrenergic stimulation.

CaMKII signaling is heavily intertwined with other cardiac signaling events; therefore, understanding cardiac CaMKII signaling in more depth would benefit from a systems-wide method such as large-scale phosphoproteomics. Although such methodology can reveal tens of thousands of phosphorylation sites,[21–23] pinpointing the responsible kinase for each detected site is cumbersome because consensus sequences are often promiscuous. Ideally, a targeted phosphoproteomics approach, which only reveals phosphorylation sites affected by a single kinase, in this case CaMKII, is more appropriate. Here we present such a highly specific methodology to probe in vivo cardiac phosphorylation events, utilizing AC3-I transgenic mice, a highly validated model of myocardial-selective CaMKII inhibition.[18] As a control, we used transgenic mice from the same genetic background expressing AC3-C, a scrambled and inactive form of AC3-I (Figure 1). This novel approach led to the identification of a defined subset of direct and indirect cardiac CaMKII targets. These novel proteins were found in various cellular compartments not previously associated with CaMKII activity, such as proteins within the z-disc and a set of distinct sarcomeric proteins. Therefore, this study reveals interesting and novel roles for CaMKII in health and disease.

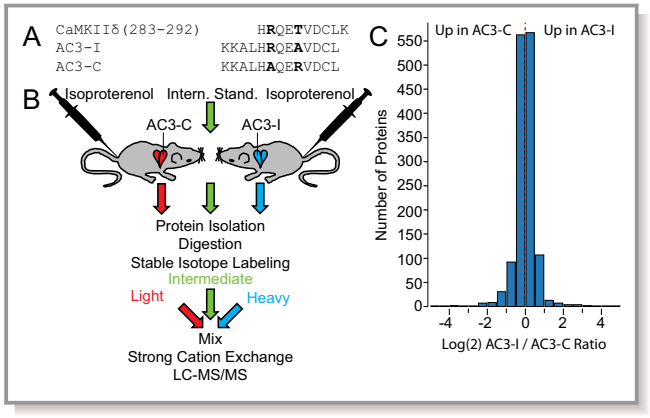

Figure 1. Work flow to determine CaMKII-regulated signaling nodes in vivo in mouse heart. A, Sequence of the mouse CaMKIIδ autoinhibitory domain (Uniprot Q6PHZ2), aligned with the sequence inhibitor AC3-I and the control peptide AC3-C. B, Work flow used to determine differential protein expression and phosphorylation between AC3-I (red) and AC3-C (blue) mice after stimulation with isoproterenol. C, Histogram of observed AC3-I/AC3-C dimethyl ratios of cardiac proteins (n=1423) reveals that differences in the cardiac proteome are marginal between AC3-I and AC3-C mice (see Table S1 for details). CaMKII indicates calcium- and calmodulin-dependent protein kinase II; MS, mass spectrometry.

Materials and Methods

Animals and Treatment Regime

All animal experiments were performed according to the ethical guidelines defined by the University of Iowa and the University Medical Centre Utrecht. All mice were bred in a C57BL/6 background. For proteomics, 2 AC3-I and 2 AC3-C mice were treated with a single bolus injection of 15 mg/kg isoproterenol (CAS# 5984-95-2; Sigma) for 30 minutes. Also 2 mice each with either transgenic peptide were treated with vehicle. The animals were euthanized, and their hearts isolated, flushed with ice-cold PBS, and immediately snap-frozen in liquid nitrogen, before storage at −80°C. For Western blots, mice were treated identically.

Tissue Lysis, Sample Preparation, and Western Blotting

Whole hearts were taken from the −80°C storage and further frozen in liquid nitrogen and subsequently pulverized in a

custom-made chilled steel mortar. For Western blotting, tissue was further lysed with RIPA lysis buffer (20 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, 10 mmol/L Na_2HPO_4, 1% [*v/v*] Triton X-100, 1% [*w/v*] Na-deoxycholate, 0.1% [*w/v*] SDS, 1 mmol/L EDTA, 50 mmol/L NaF, 2 mmol/L PMSF, and 14 μg/mL aprotinin). Isolated protein samples were loaded on SDS-PAGE for subsequent Western blotting. For detection, chemiluminescence was performed using an ECL-kit from Amersham. The following antibodies were used: anti-phospho-Cacnb2 antibodies made by Yenzyme, anti-Cacnb2 (Thr549; Neuromab), anti-phosphorylated-CaMKII (Thr 287; Upstate), anti-phospholamban (Upstate), and anti-phospho-PLN (Thr17; Santacruz).

Strong Cation Exchange and LC-MS/MS Analysis

Whole hearts were taken from the −80°C storage, pulverized as above, and taken up in 500 μL of lysis buffer (10 mmol/L sodium phosphate buffer [pH 7.8], 150 mmol/L NaCl, 8 mol/L urea, 1× PhosStop tablet, and complete min protease inhibitors [both Roche Diagnostics]) and left at room temperature for 10 minutes. Samples were sonicated 3 times on ice for 1 minute with 2 minutes of resting on ice and centrifuged at 20 000*g* for 15 minutes at 4°C. This procedure was repeated once, and the supernatants were combined. Protein concentrations were measured using a Bradford assay (BioRad). The individual samples were reduced with 10 mmol/L dithiothreitol for 30 minutes at 56°C and alkylated by the addition of 55 mmol/L iodoacetamide and incubation for 45 minutes at room temperature in the dark. Lysates were digested with Lys C (ratio 1:100 *w/w*) for 4 hours at 37°C. Samples were then diluted with 50 mmol/L ammoniumbicarbonate to a final concentration of 2 mol/L urea. Trypsin (ratio 1:100 *w/w*) was added, and the samples were digested overnight at 37°C. Proteolytically cleaved samples were desalted and dimethyl-labeled on a Sep-Pak C18 column (Waters) according to Boersema et al.[24] AC3-C samples were labeled light, AC3-I, heavy, and the internal standard (a mix of all samples, including non-stimulated saline mice) was tagged with the intermediate label. All samples were mixed in equal amounts prior to strong cation exchange and LC-MS/MS analysis. Strong cation exchange (SCX) separation was performed on 2 individual mouse sets consisting of AC3-I, AC3-C, and the internal standard according to Hennrich et al.[25] A total of 40 fractions were collected. Each mouse set was analyzed in duplicate (technical replicate) by LC-MS/MS as described below. The fractions enriched in phosphorylated peptides (1^+ fractions 7 to 17) were analyzed in quadruplicate Hennrich twice using CID as fragmentation method and twice with HCD. Nanoflow LC-MS/MS was performed by coupling an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) to a LTQ-Orbitrap Velos mass spectrometer (Thermo Electron, Bremen, Germany) as described previously.[26] The phospho-fractions were run with a 2-hour gradient, whereas the other fractions were analyzed using a 3-hour gradient elution. The early SCX fractions containing the phosphorylated peptides were run twice, once using fragmentation by CID in the ion trap and once using HCD fragmentation with fragment analysis in the Orbitrap.

Data Analysis

All raw data files of the individual SCX fractions of each of the 2 mouse experiments were imported into Proteome Discoverer v1.3.0.339 Hennrich and the combined peak list was split into CID and HCD data (where applicable) before database searching. Subsequently, CID and HCD peak lists were searched individually against an International Protein Index (IPI; http://www.ebi.ac.uk/ipi) database containing mouse sequences and common contaminants such as bovine serum albumin and human keratins (IPI-Mouse v3.84; 60 248 sequences) through a direct connection to our in-house Mascot server (Mascot v2.3.2, Matrix Science, London, UK). The following settings were used: carbamidomethylation on cysteines as static modification; light, intermediate, and heavy dimethylation of peptide N-termini and lysine side chains, as well as oxidation on methionine and phosphorylation on serine, threonine, or tyrosine as variable modifications; and precursor mass tolerance of 20 ppm and 0.8 Da on the fragment masses (for CID) but 20 ppm and 0.02 Da for HCD searching. The enzyme was specified as trypsin, and 2 missed cleavages were allowed. For both identification and quantitation, only spectra within the score limits of the 1% false discovery rate (FDR) were accepted, based on Mascot score thresholds. These were calculated independently for CID and HCD data in each data set using the built-in FDR calculator of Proteome Discoverer, which is based on Mascot's built-in FDR calculation. The *event detector* and *precursor ion quantifier* algorithms of Proteome Discoverer were used for quantitation using 2-ppm mass variability and 0.2-minute retention time tolerance on precursor ion pairs. Quantitation is based on the ratio of the summed areas of 3 matched isotope patterns (a feature) across the eluting chromatographic peak of that feature. The peptide ratios are calculated using the same number of isotopes. Protein ratios are based on the median peptide ratio, with exclusion of the identified phosphopeptides. At least 2 isotopic peaks were required for inclusion, as well as a minimal signal-to-noise level of 3. Protein identifications over all 4 analyses were combined and grouped by Proteome Discoverer. Each peptide spectral match (PSM) (Mascot peptide score >25) of a phosphorylated peptide was isolated from the data in Proteome Discoverer. Site localization was performed using the pRS algorithm.[27] Probability

scores >75% were considered localized. Phospho-PSMs were then grouped according to their sequence and site(s) of phosphorylation. If multiple quantitative data points for a unique phosphopeptide (including proper site localization) were available, these were averaged, and the standard deviation was calculated. All raw and annotated data are freely available through ProteomeXchange (http://www.proteomexchange.org) under accession number PXD000174 and ProteomeXchange submission title CaMKII Cardiac Phosphoproteome. Icelogos were generated using the Icelogo software package. All Icelogos were generated with a cutoff $P<0.01$.[28]

Immunohistochemistry

Frozen heart tissue from an AC3-I mouse was serially sectioned in 10-μm slices that were collected on aminopropyltriethoxysilane-coated glass slides. Immunohistochemistry was performed as described previously.[29] Primary antibodies against Csrp3 (Rabbit, Crp3 H-46 sc-98827, 1:100; SantaCruz) were used. Secondary labeling was performed with appropriate Alexa Fluor 594 (1:250) conjugated with whole IgG antibodies (Jackson Laboratories).

Results

To establish the downstream phosphorylation targets affected by CaMKII inhibition in vivo in cardiac tissue, the phosphorylation states of mice with transgenic expression of either AC3-I or AC3-C (Figure 1A) were compared using quantitative proteomics. We used SCX-based phosphopeptide enrichment,[30] dimethyl stable isotope labeling[24] and high-resolution LC-MS/MS analysis (Figure 1B). AC3-I is a potent, highly specific peptide inhibitor of CaMKII, whereas AC3-C is a scrambled nonfunctional homolog peptide[18,31] (Figure 1A). The AC3-I peptide mimics the autoinhibitory sequence of CaMKIIδ (mouse Q6PHZ2, amino acids 283 to 292) by posing as a pseudosubstrate with an intact docking site (Arg284), but a deficient phosphoacceptor (Thr287 substituted by an alanine; Figure 1A). Cardiomyocyte-specific expression of AC3-I leads to potent inhibition of CaMKII in vivo as demonstrated previously.[18,31] cDNA constructs of AC3-I and AC3-C fused to GFP were expressed under control of the cardiac-specific *myh6* promoter. This excludes any distant effects by inhibition of CaMKII in other organs or in nonmyocardial cardiac cells that may modulate cardiac performance. Chronic expression of AC3-I has no structural myocardial phenotype, as these mice age and function normally and have equal expression of CaMKII,[18] but show reduced chronotropic activation[32] and preserved myocardial function[18] after an isoproterenol challenge. To investigate this in more detail, we evaluated the differences in protein expression between both mouse models. Little variation was observed between AC3-I and AC3-C mouse hearts, as indicated from the 1410 quantified proteins, of which 1329 (>94%) presented a <2-fold difference between AC3-I and AC3-C (Figure 1C, Table S1), 90% of which were within 1.5-fold.

To identify downstream phosphorylation targets of CaMKII in the heart, the phosphoproteome of 2 AC3-I and 2 AC3-C mice were quantitatively compared after an intraperitoneal injection of isoproterenol (see Materials and Methods for details; Figure 1B). Prior to extensive LC-MS/MS analysis, we checked the phosphorylation of 2 known CaMKII target sites after identical isoproterenol treatment as described above: Thr17 of cardiac phospholamban (PLN)[9] and Thr287 autophosphorylation of CaMKII[10] (Figure 2A). Normalization to protein levels of PLN indicates that both phosphorylations were increased in the AC3-C mice. As expected, the response was blunted in the AC3-I mice. Similar trends were observed at the established Thr549 site of the ß-subunit of the voltage-gated calcium channel (Cacnb2)[33] (Figure 2B).

Subsequently, we enriched for cardiac phosphopeptides from the labeled mixture of AC3-C and AC3-I peptides. This led to the identification of 525 unique phosphopeptides on 282 phosphoproteins. Finally, using stringent filtering and the pRS phosphorylation site localization algorithm,[27] 310 confidently localized phosphorylation sites could be quantified (Table S2). Evaluation of their phosphorylation motif using Icelogo[28] showed a mixture of kinase motifs, including CaMKII's general motif RXX[pS/pT], but also the SP-directed motif and acidic-directed motifs (Figure 2C). We then isolated the putative CaMKII sites (84 in total) by selecting the motifs with an arginine or lysine residue on position −3 while disregarding those that contained a proline at position +1 ([R/K]XX[pS/pT][noP]) and made an additional motif (Figure 2D).

Quantitative analysis of the phosphorylated peptides revealed 36 sites that were downregulated on AC3-I expression, indicative of a direct inhibitory effect of AC3-I on these particular phosphorylation sites. In contrast, another 15 sites showed a negative AC3-I/AC3-C ratio, meaning that phosphorylation at these sites was increased when CaMKII was inhibited, presumably an indirect effect of myocardial CaMKII inhibition. Satisfactorily, an Icelogo of the downregulated sites showed clear enrichment of RXXpS-based phosphosites (Figure 2E).

To further structure our quantitative phosphorylation data, we cross-referenced published reports to verify the function of each regulated phosphoprotein in our screen (Figure 3). This showed several expected functional entities, such as ion handling, gene expression, and cytoskeletal proteins involved in contractility. We also found targets related to microtubule formation and upkeep that were not previously recognized as being affected by CaMKII. As expected, we identified other signaling proteins affected by CaMKII inhibition, including the myosin light chain kinase (Ser1798 and Ser1801

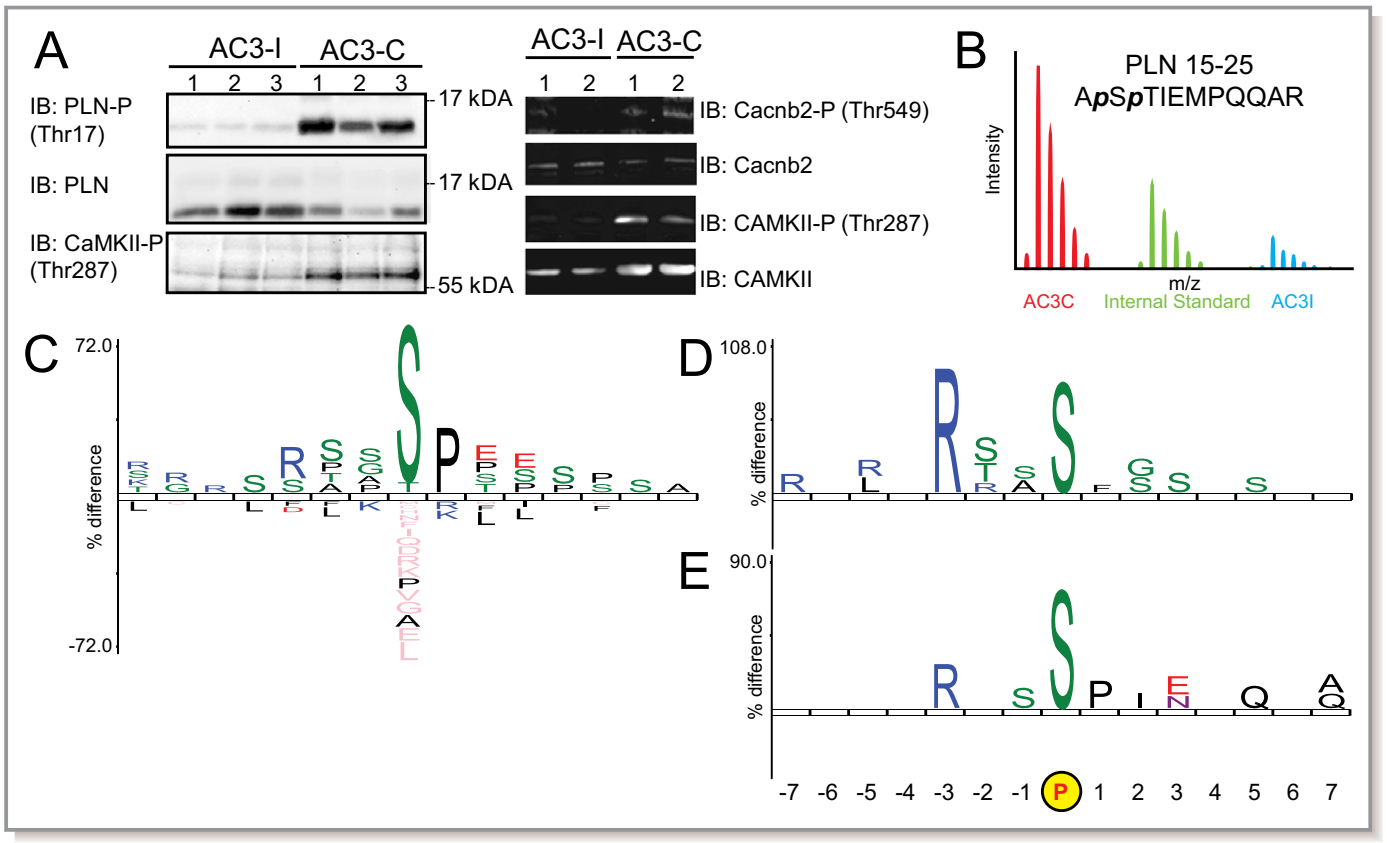

Figure 2. Benchmarking the CaMKII inhibitory mouse model. A, Western blots of cardiac phospholamban (PLN) Thr17 phosphorylation, CaMKIIδ autophosphorylation, and general PLN protein levels in set of 3 AC3-I and 3 AC3-C mice after isoproterenol stimulus (left). Western blots of Cacnb2 phosphorylation levels (Thr549, also referred to as Thr498[33]) in another set of 2 mice. B, Quantitative MS profile of PLN peptides 15 to 25 confirms PLN phosphorylation is blunted in AC3-I mice when compared with AC3-C mice. The internal standard, a 1:1 mixture of the 2, shows the expected intermediate level. C, Icelogo of all observed motifs surrounding the phosphorylation site (7 amino acids up- and downstream, only peptides with a defined localized phosphosite were used [see Materials and Methods for details]). Amino acid codes above the *x* axis show overrepresentation, whereas below indicates underrepresentation at a particular position. D, Icelogo of all motifs containing the minimal CaMKII consensus sequence [R/K]XX[pS/pT][notP]. E, Icelogo of all AC3-I downregulated sites. All Icelogos generated with a $P<0.01$. CaMKII indicates calcium- and calmodulin-dependent protein kinase II; Cacnb2, calcium channel beta 2.

downregulated). This is an interesting finding, as it suggests a direct connection between CaMKII inhibition and contractility.

Discussion

CaMKII in Health and Disease

Healthy CaMKII has thus far been implicated in 3 major physiological cardiac functions, that is, excitation-contraction coupling (ECC), excitation-transcription coupling (ETC), and "fight-or-flight" heart rate increases. As part of the ongoing efforts to understand CaMKII's function, research has mainly focused on 3 molecular nodes: (1) the regulation of ion channels (Ca^{2+}, Na^{+}, and K^{+} channels), (2) the regulation of SR-Ca^{2+} via the ryanodine receptor and the SERCA/PLN complex, and (3) HDAC5/NFAT/ MEF2-mediated transcription.[34] Over the past 2 decades an enormous body of work has also revealed that CaMKII is a potential driver of myocardial hypertrophy, arrhythmia, and heart failure. Both its activity and expression are increased in patients and animal models of these cardiac diseases.[15,20] Also, inhibition of cardiac CaMKII in various ways protects the heart against its damaging effects (reviewed in Anderson et al[35]). In pathological hypertrophy, patients suffer from defective ECC and ETC, leading to apoptosis and arrhythmias. However, the connection between CaMKII activity and expression and heart disease is not well understood. We reasoned that an important first step toward understanding the molecular pathways affected by CaMKII was to measure direct and indirect CaMKII target phosphorylation sites after isoproterenol stimulation.

Methodology to Probe CaMKII Action Directly in the Myocardium

Monitoring molecular pathways affected by CaMKII are not trivial because the field suffers from lack of a suitable,

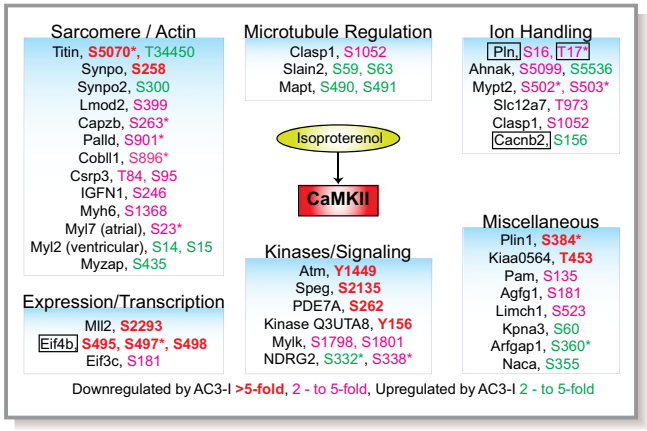

Figure 3. Phosphorylation sites affected by AC3-I-mediated CaMKII inhibition in mouse heart. Depicted are all upregulated (green) and downregulated (red and pink) sites categorized by specific function. Gene names are used. For specific ratios observed, see Table S2. *Means the mentioned site contains the minimum CaMKII motif (RXX[pS/pT]). Boxed proteins or sites are verified CaMKII targets. For acronyms, see Table S2. CaMKII indicates calcium- and calmodulin-dependent protein kinase II; Synpo2, synaptopodin-2; Lmod2, leiomodin-2; Capzb, capping protein subunit beta; Palldn, Palladin; Cobll1, Cordon-bleu protein-like 1; Csrp3, cysteine- and glycine-rich protein 3; IGFN, immunoglobulin-like and fibronectin type III domain-containing protein 1; Myh, myosin heavy chain; Myl, myosin light chain; Mll2, histone-lysine *N*-methyltransferase MLL2; Eif, eukaryotic translation initiation factor; Clasp1, CLIP-associating protein 1 isoform 2; Slain, isoform 1 of SLAIN motif-containing protein 2; Mapt, microtubule-associated protein tau; Speg, isoform 1 of striated muscle-specific serine/threonine-protein kinase; Pln, cardiac phospholamban; Ahnak, AHNAK nucleoprotein isoform 1; Mypt2, myosin phosphatase; Plin, isoform 1 of Periplin-1; Kiaa0564, isoform 1 of uncharacterized protein KIAA0564 homologue; Agfg1, isoform 3 of Arf-GAP domain and FG repeats-containing protein 1; Arfgap1, isoform 1 of ADP-ribosylation factor GTPase-activating protein 1; Naca, nascent polypeptide-associated complex subunit alpha.

contractile cardiac myocytes–resembling cell line. Therefore, such studies are ideally performed directly in cardiac tissue. When taking this route, several experimental designs could be used. The most obvious is to treat mice with a specific pharmacological inhibitor prior to investigating their altered cardiac phosphoproteome by quantitative mass spectrometry. Although KN-93 is used throughout the literature, its specificity for CaMKII is still debated,[36] and systemic administration may induce peripheral effects by inhibition of CaMKII at other sites in the body. In a more focused approach, one could use the established CaMKIIδ-null mouse and compare its phosphoproteome after isoproterenol stimulus with wild-type littermates using quantitative phosphoproteomics. Potential drawback of such an approach is that the CaMKIIδ-null phenotype is likely compensated through significant changes in cardiac protein expression. In addition, it is foreseen that, like PKA,[37] CaMKIIδ engages in higher-order signaling protein complexes, which may disintegrate on the absence of CaMKIIδ and cause signaling deviation far beyond CaMKIIδ action alone. Therefore, it is preferential to use a more subtle transgenic model that expresses a cardiac-specific kinase-dead version of CaMKII or a specific (peptide) inhibitor of CaMKII. The former has not been described, but for the latter 2 models are available: one based on the transgenic expression of the SR-targeted AIP_4-LSR,[38] the other on a general CaMKII inhibitory sequence derived from CaMKII's own autoinhibitory domain, called AC3-I.[18,31] Here, we probed the effect of AC3-I -mediated CaMKII inhibition on cardiac protein phosphorylation after a short ß-adrenergic stimulation (30 minutes of isoproterenol). As AC3-I mice have no apparent phenotype when not challenged into hypertrophy, we consider the observed changes as the functional outcome of healthy CaMKII action.

Established Targets of CaMKII

Only a handful direct phosphorylation targets of CaMKII are documented in the heart, with PLN-Thr17 being the best validated. Phosphorylation of PLN leads to aberration of its inhibitory function on SERCA2 and hence an increase in Ca^{2+} reuptake by the SR. As expected, transgenically expressed AC3-I negatively affects phosphorylation at this site, as shown previously,[18] and also in our screen (Figure 2B), thereby validating our approach. In addition, we found several of the known, or predicted, protein targets of cardiac CaMKII in our screen. In addition to Thr549 (Figure 2A), on Cacnb2 we identified a novel site at Ser156 (3.3-fold upregulated) that did not conform to the CaMKII motif, indicative of negative regulation at this site. Among the proteins involved in expression and translation (Figure 3) was Eif4B (eurkaryotic translation initiation factor 4B), a ribosomal RNA helicase. In vitro phosphorylation screens identified Eif4B as a CaMKII substrate, although the site was lacking.[39] Using our approach, 2 phosphorylation hot spots were detected on this protein (Figure 4), one containing Ser418, 420, 422, and 425 (Table S2) and another containing Ser495, 497, and 498. The former is functionally described as regulated at Ser422 by several kinases, including S6-kinase1 and Akt,[40] and we did not find these sites regulated (Table S2). The latter hot spot was represented by 4 different phosphopeptides, all of which were found to be downregulated between 3- to 5-fold in AC3-I mice. In addition, Ser497 is a putative CaMKII site with an RXXpS motif. Several other eukaryotic translation initiation factors were also observed, of which Eif3c also contained a downregulated site (Ser39, 2.5-fold; Figure 4). In addition to HDAC5, these reveal a putative novel site of action where CaMKII may influence gene expression. Interestingly, in light of hypertrophy developing over a long period because of altered gene expression, these sites already responded after 30 minutes of isoproterenol stimulation.

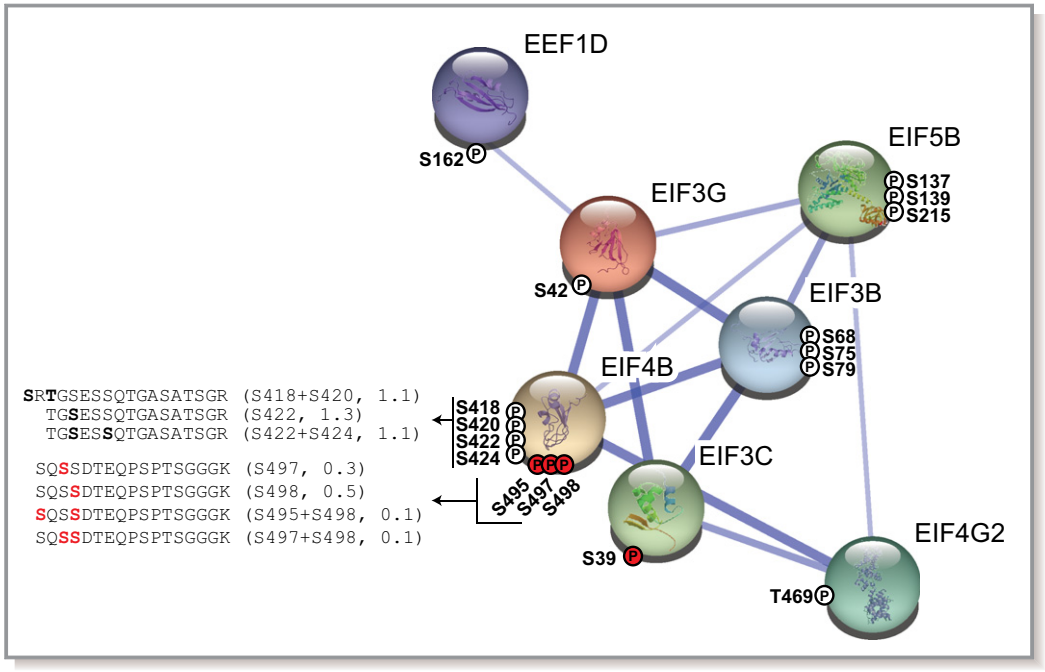

Figure 4. AC3-I inhibition of cardiac CaMKII influences elongation-initiation factors. String network of the detected phosphorylated translation-elongation proteins. Depicted are the observed phosphorylation sites (P). White phosphorylations mean unaffected sites, red is downregulated sites in AC3-I mice. In more detail the 2 phosphorylation hot spots in EIF4B are depicted, including the observed phosphopeptides with their localized sites in black boldface (not affected by AC3-I) or red boldface (downregulated in AC3-I mice) and the observed AC3-I/AC3-C ratios. CaMKII indicates calcium- and calmodulin-dependent protein kinase II; EEF1D, isoform 1 of elongation factor 1-delta; Eif, eukaryotic translation initiation factor.

CaMKII Regulates Phosphorylation of Many Myofibril Proteins in the z-Disc and a-Band

AC3-I regulated sites categorized in different cellular compartments and physiological functions (Figure 3). In our phosphoproteomics screen, the sarcomere category contained the most regulated sites. Even though our analysis is somewhat biased toward the more abundant myofilament proteins, many detailed observations could be made in this compartment, of which several are described below.

Scrutinizing the literature we could annotate the exact intracellular localization of each observed phosphoprotein. A set of z-disc proteins, a-band proteins, but also costamere proteins was observed (Figure 5, Table S2). The sarcomere of cardiac muscle is strictly organized in thick filaments that contain myosin and thin filaments consisting of filamentous actin. The thin filaments align and cross-link via α-actinin dimers at the z-disc and form a bridge to the thick filaments in the a-band (reviewed in Clark et al[41]; Figure 5A). The cardiac z-disc is an intricate network of many contractile (regulatory) proteins.[41] Costameric proteins link the z-disc with the sarcolemma to transmit force between these 2 regions.[42] When grouping the phosphoproteins by confirmed intracellular localization and known binding partners, a site-specific pattern became visible. Although the costameric proteins were found to be phosphorylated, none of them seemed affected by isoproterenol in AC3-I mice, whereas both the z-disc and a-band proteins were (Figure 5A). This suggests that isoproterenol-sensitive CaMKII is present or at least influences signaling nodes, specifically at these defined locations in the heart.

Titin is a major stability component of the cardiac sarcomere with its N-terminus embedded in the z-disc and its C-terminus extending into the a-band. We observed 5 different phosphopeptides on titin, harboring in total 7 quantified phosphorylation sites (Table S2). One site, Ser5070 at the z-disc part of titin, is a putative CaMKII site (RXXpS) only present in isoform 3 (A2ASS6-3), which was downregulated almost 20-fold in AC3-I mice. At the same time, at the C-terminal end, we found 2 adjacent phosphorylation sites Thr34450 (in isoform 1, A2ASS6-1) and Ser34451, of which the former was found to be 2.6-fold upregulated and the latter unchanged. These observations caused us to speculate that CaMKII directly influences the phosphorylation state of titin and thereby likely regulates sarcomere action. Further research should reveal more detailed information on the exact nature of these specific events.[43,44]

We found Csrp3 (cysteine and glycine-rich protein 3, or Crp3, also called muscle lim protein) less phosphorylated in

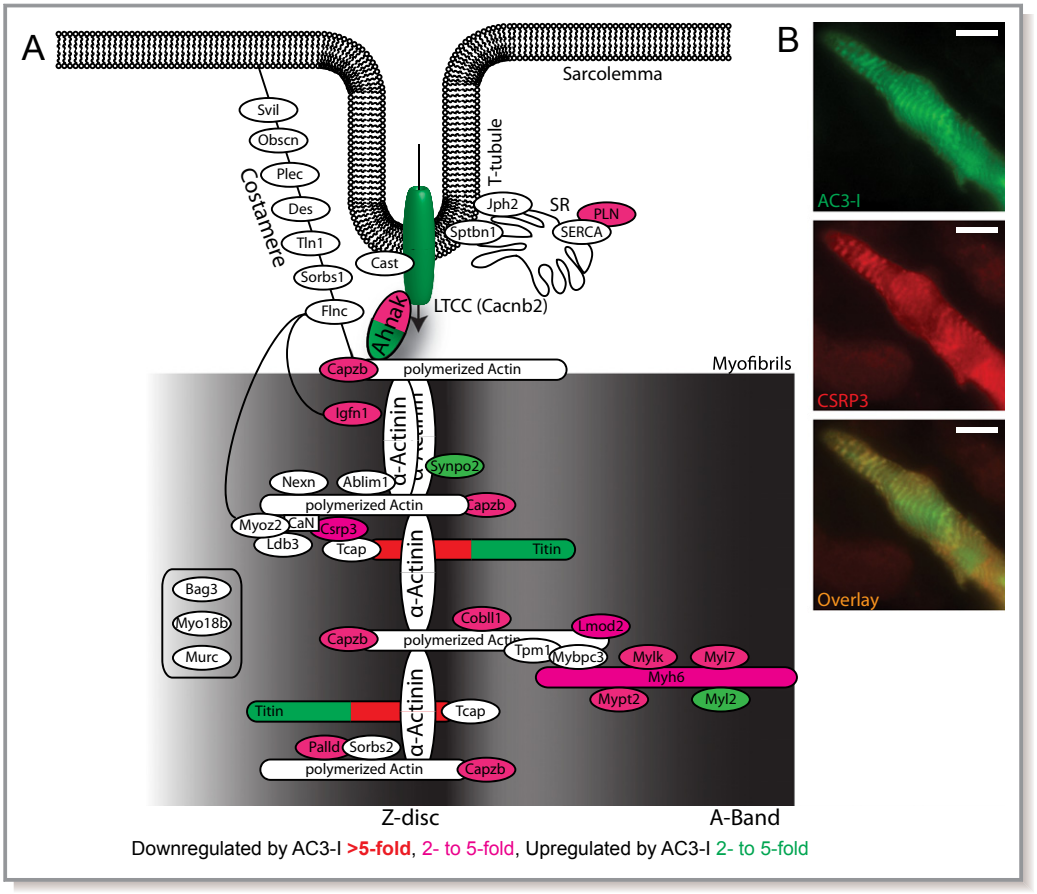

Figure 5. CaMKII affects the cardiac sarcomere at several distinct sites. A, Model of CaMKII-affected sites in the cardiac myofibrils (z-disc and a-band) displaying described links with the sarcolemma, the costamere and the sarcoplasmic reticulum (SR). All proteins depicted, except calcineurin (CaN), were observed with a phosphorylation site in this study. The color codes are the same as in Figure 3; double-colored proteins were observed with 2 oppositely regulated sites. The 3 boxed proteins (Bag3, Murc, and Myo18b) are established z-disc proteins; however, their interaction partners have currently not been described. LTCC is L-type calcium channel (Cacnb observed in our study); for other acronyms see Table S2. B, Immunolabeling of Csrp3 (red) revealed colocalization with the GFP-tagged transgenic AC3-I (green) at the sarcomere in left ventricle. Scale bar=10 μm. CaMKII indicates calcium- and calmodulin-dependent protein kinase II; Obscn, obscurin; Plec, plectin; Tln, talin-1; Sorbs1, sorbin and SH3 domain-containing protein 1; Flnc, filamin-C; Ahnak, AHNAK nucleoprotein isoform 1; Jph, junctophilin-1; PLN, phospholamban; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; Cacnb2, calcium channel beta 2; Capzb, isoform 1 of F-actin-capping protein subunit beta; Igfn1, immunoglobulin-like and fibronectin type III domain-containing protein 1; Nexn, nexilin; Ablim1, isoform 4 of actin-binding LIM protein 1; Myoz2, myozenin-2; Ldb3, isoform 4 of LIM domain-binding protein 3; Csrp3, cysteine- and glycine-rich protein 3; Tcap, telethonin; Cobll1, Cordon-bleu protein-like 1; Lmod2, leiomodin-2; Tpm, tropomyosin; Mybpc3, myosin-binding protein C; Myl, myosin light chain; Myh, myosin heavy chain; Palld, palladin; Murc, muscle-related coiled-coil protein.

AC3-I murine hearts at Thr84 and Ser95 (both ≈2.5-fold) than in AC3-C hearts. Csrp3 is crucial to the development and maintenance of cardiac cytoarchitectural organization. Mice deficient in this protein develop severe dilated cardiomyopathy within several weeks after birth.[45] Csrp3 localizes to the z-disc, where it binds telethonin (Tcap, phosphorylated at S161, unchanged; Figure 5) at the N-terminal end of the titin filaments calcineurin and alpha-actinin (Ser160, unchanged). Csrp3 localizes to the nucleus and does so in failing human heart in particular.[46] Whether this is also phosphorylation dependent is currently unknown, although it is tempting to speculate that the AC3-I inhibited sites found here combined with increased CaMKII expression and activity in failing hearts are somehow connected. Illustrative of this is the immunolabeling of Csrp3, which colocalized with the GFP-tagged transgenic AC3-I at the sarcomere in our model system (Figure 5B).

The a-band is another location where strong effects of AC3-I were observed, suggesting a direct effect of CaMKII in the contractile response (Figure 5). In addition to myosin itself (Ser1368 in isoform-6, Q02566, 2-fold downregulated), several of its master regulators were also affected. Interesting were the observations on 2 isoforms of the myosin regulatory light chain, types 2 and 7 (Myl2 and Myl7). The former is the ventricular isoform, which had an intricate phosphorylation pattern concerning Ser14, Ser15, and Ser19;

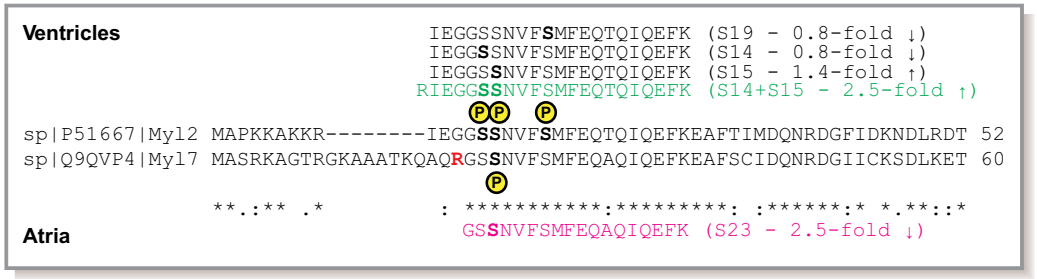

Figure 6. Compartment-specific regulation of myosin regulatory light chain. Alignment of the N-termini of Myl2 (1 to 52, ventricle) and Myl7 (1 to 60, atrium) reveals conservation of the 3 observed phosphorylation sites (yellow P and boldface) at Ser14, Ser15, and Ser19 in Myl2 and Ser22, Ser23 and Ser27 in Myl7. When comparing the quantitative data, we observed opposite regulation in both compartments as well as different consensus motifs for putative kinases at this site, suggesting that different kinases regulate these proteins in ventricles and atria. Arg20 of Myl7 (red R) is a putative CaMKII site, of which the phosphorylated form was found downregulated 2.5-fold. This arginine, and thus consensus site, is absent in Myl2. Myl indicates myosin light chain; CaMKII indicates calcium- and calmodulin-dependent protein kinase II.

dual phosphorylation at Ser14 and Ser15 was upregulated 2.5-fold and at Ser15 alone 1.7-fold (Figure 6). Myl7, the atrial isoform, showed a 2.5-fold downregulation on Ser23. When Myl2 and Myl7 were aligned (Figure 6), it became clear that Ser15 and Ser23 are each other's equivalent; however, Ser23 on Myl7 is a putative CaMKII site (RGSpS) and downregulated, Ser15 in Myl2 is not (GGSpS) and was also not found to be downregulated. This is an interesting observation in light of heart compartment–specific regulation of contractility.

In addition to these examples, many other interesting putative CaMKII connections were detected, such as synaptopodin-2 (synpo2, myopodin), the capping proteins Lmod2 and Capzb, but also Palladin (Palldn) and Cordon-bleu protein-like 1 (Cobll1) as well as NDRG2. These proteins seem interesting novel CaMKII-regulated phosphoproteins that deserve a closer investigation in the future.

Links to Pathophysiology; Future Directions

Increased CaMKII activity and expression are a hallmark of human heart failure, hypertrophy, and arrhythmia. Much research has been focused on the role of CaMKII in Ca^{2+} handling. However, the role of CaMKII on the myofibrils has thus far been largely unreported. Our data reveal that CaMKII inhibition has effects on many myofibrillar proteins in both the z-disc and the a-band. Interestingly, several of the AC3-I regulated (myofibril) phosphoproteins found in this study (Myh6/7, Myl2, Csrp3, PLN) are also known as genetic factors in hereditary cardiomyopathy,[47] which often leads to heart failure. We found several more of such factors with documented hereditary mutations to be phosphorylated, although not regulated by AC3-I; these include Tpm1, MyBPC3, Tcap, Jph2, Obscn, Myoz2, Des, and LDB3. Based on our data, future research should focus on the interplay of CaMKII and perhaps other important cardiac kinases in their regulation of the sarcomere. Also, our data suggest putative novel genes to be investigated for causative cardiomyopathic mutations, for instance, in Lmod2 (Leiomodin-2), an actin capping gene involved in regulation of the thin filament length.[48]

Conclusions

The study described here is the first of its kind and highlights the feasibility of performing kinase-specific quantitative phosphoproteomics directly in cardiac mouse tissue. Using the transgenic model allowed us to isolate a small subset of 39 CaMKII-regulated phosphoproteins, including exact site localization, which provides a valuable resource for future research into CaMKII's role in the healthy but also the diseased heart.

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Disclosures

None.

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