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ORIGINAL RESEARCH

AKAP18δ Anchors and Regulates CaMKII Activity at Phospholamban-SERCA2 and RYR

Cathrine R. Carlson, Jan Magnus Aronsen, Anna Bergan-Dahl, Marie Christine Moutty, Marianne Lunde, Per Kristian Lunde, Hilde Jarstadmarken, Pimthanya Wanichawan, Laetitia Pereira, Terje R.S. Kolstad, Bjørn Dalhus, Hariharan Subramanian, Susanne Hille, Geir Christensen, Oliver J. Müller, Viacheslav Nikolaev, Donald M. Bers, Ivar Sjaastad, Xin Shen, William E. Louch, Enno Klussmann, Ole M. Sejersted

BACKGROUND: The sarcoplasmic reticulum (SR) Ca^{2+}-ATPase 2 (SERCA2) mediates Ca^{2+} reuptake into SR and thereby promotes cardiomyocyte relaxation, whereas the ryanodine receptor (RYR) mediates Ca^{2+} release from SR and triggers contraction. Ca^{2+}/CaMKII (CaM [calmodulin]-dependent protein kinase II) regulates activities of SERCA2 through phosphorylation of PLN (phospholamban) and RYR through direct phosphorylation. However, the mechanisms for CaMKIIδ anchoring to SERCA2-PLN and RYR and its regulation by local Ca^{2+} signals remain elusive. The objective of this study was to investigate CaMKIIδ anchoring and regulation at SERCA2-PLN and RYR.

METHODS: A role for AKAP18δ (A-kinase anchoring protein 18δ) in CaMKIIδ anchoring and regulation was analyzed by bioinformatics, peptide arrays, cell-permeant peptide technology, immunoprecipitations, pull downs, transfections, immunoblotting, proximity ligation, FRET-based CaMKII activity and ELISA-based assays, whole cell and SR vesicle fluorescence imaging, high-resolution microscopy, adenovirus transduction, adenoassociated virus injection, structural modeling, surface plasmon resonance, and alpha screen technology.

RESULTS: Our results show that AKAP18δ anchors and directly regulates CaMKIIδ activity at SERCA2-PLN and RYR, via 2 distinct AKAP18δ regions. An N-terminal region (AKAP18δ-N) inhibited CaMKIIδ through binding of a region homologous to the natural CaMKII inhibitor peptide and the Thr17-PLN region. AKAP18δ-N also bound CaM, introducing a second level of control. Conversely, AKAP18δ-C, which shares homology to neuronal CaMKIIα activator peptide (N2B-s), activated CaMKIIδ by lowering the apparent Ca^{2+} threshold for kinase activation and inducing CaM trapping. While AKAP18δ-C facilitated faster Ca^{2+} reuptake by SERCA2 and Ca^{2+} release through RYR, AKAP18δ-N had opposite effects. We propose a model where the 2 unique AKAP18δ regions fine-tune Ca^{2+}-frequency-dependent activation of CaMKIIδ at SERCA2-PLN and RYR.

CONCLUSIONS: AKAP18δ anchors and functionally regulates CaMKII activity at PLN-SERCA2 and RYR, indicating a crucial role of AKAP18δ in regulation of the heartbeat. To our knowledge, this is the first protein shown to enhance CaMKII activity in heart and also the first AKAP (A-kinase anchoring protein) reported to anchor a CaMKII isoform, defining AKAP18δ also as a CaM-KAP.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: calmodulin ■ calcium-calmodulin-dependent protein kinase type 2 ■ myocytes, cardiac ■ phospholamban ■ ryanodine receptor ■ sarcoplasmic reticulum calcium-transporting ATPases

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In cardiac myocytes, Ca^{2+} cycling is centrally involved in excitation-contraction coupling (ECC).[1] In this process, Ca^{2+} enters the cell through L-type Ca^{2+} channels leading to the opening of ryanodine receptors (RYRs) in the sarcoplasmic reticulum (SR), and release of Ca^{2+} (Ca^{2+}-induced Ca^{2+} release). The resulting

Correspondence to: Cathrine R. Carlson, Institute for Experimental Medical Research, Oslo University Hospital, Kirkeveien 166, N-0407 Oslo, Norway. Email cathrine.carlson@medisin.uio.no

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For Sources of Funding and Disclosures, see page 42.

Novelty and Significance

What Is Known?

- Sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 2 (SERCA2) and ryanodine receptor (RYR) are essential for cardiac excitation-contraction coupling.
- Ca^{2+}/CaMKII (CaM [calmodulin]-dependent protein kinase II) modulates SERCA2 and RYR activities through indirect and direct phosphorylation events, respectively, but CaMKII anchoring and local regulation mechanisms remain elusive.
- AKAP18δ (A-kinase anchoring protein 18 delta) anchors protein kinase A to SERCA2-PLN (phospholamban).

What New Information Does This Article Contribute?

- AKAP18δ anchors and functionally regulates CaMKII activity at SERCA2-PLN and RYR, indicating a crucial role of AKAP18δ in heartbeat regulation.
- AKAP18δ also anchors CaM, inducing a second level of control.
- AKAP18δ is the first AKAP reported to anchor a CaMKII isoform, defining AKAP18δ as a CaM-Kinase Anchoring Proteins (CaM-KAP).

SERCA2 mediates Ca^{2+} reuptake into SR and thereby promotes cardiomyocyte relaxation, whereas RYR mediates Ca^{2+} release from SR and triggers contraction. CaMKIIδ regulates activities of SERCA2, through phosphorylation of PLN, and RYR by direct phosphorylation. However, the mechanisms for CaMKIIδ anchoring to SERCA2-PLN and RYR and its regulation by local Ca^{2+} signals remain unclear. Here, we provide mechanistic insight into the anchoring and regulation of CaMKIIδ activity by AKAP18δ at SERCA2-PLN and RYR. We identified 2 unique regions in AKAP18δ that inversely regulate CaMKIIδ activity (CaMKIIδ-catalyzed phosphorylation of Thr17-PLN and Ser2814-RYR) and SERCA2 and RYR function. We specifically showed that an inhibitory domain (AKAP18δ-N) also binds calcified CaM, while an activating domain (AKAP18δ-C) wedges CaMKIIδ open, trapping CaM within the kinase, and lowering the Ca^{2+} threshold for its activation. Based on our data, we propose a working model where the 2 unique AKAP18δ regions fine-tune Ca^{2+}-frequency-dependent activation of CaMKIIδ at SERCA2-PLN and RYR. The peptide sequences derived from the 2 AKAP18δ regions should be viewed as novel reagents that may help identify new CaMKII targets and approaches to therapeutically modify CaMKII activity and cardiomyocyte Ca^{2+} cycling.

Nonstandard Abbreviations and Acronyms

AKAP	A-kinase anchoring protein
AKAP18δ	A-kinase anchoring protein 18δ
CaM	calmodulin
CaM-KAP	CaM-kinase anchoring protein
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
ECC	excitation-contraction coupling
GST	glutathione S-transferase
PDE3A1	phosphodiesterase 3A1
PKA	protein kinase A
PLN	phospholamban
RYR	ryanodine receptor
SERCA2	sarco/endoplasmic reticulum Ca^{2+}-ATPase 2
SPR	surface plasmon resonance
SR	sarcoplasmic reticulum

increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) causes Ca^{2+} binding to troponin C and activation of the myofilaments leading to contraction. For diastolic relaxation to occur, Ca^{2+} is removed from cytoplasm by the SR Ca^{2+}-ATPase 2 (SERCA2), and to a lesser extent by the Na^+/Ca^{2+} exchanger. Alterations in this Ca^{2+} cycling are associated with decreased contractility and arrhythmia during heart failure.[2]

Ca^{2+}/CaMKIIδ (CaM [calmodulin]-dependent protein kinase δ), which is the predominant CaMKII isoform expressed in heart, regulates ECC by phosphorylating several Ca^{2+} handling proteins, including RYR and PLN (phospholamban).[3] PLN is a key modulator of SERCA2, and thus SR Ca^{2+} reuptake, SR Ca^{2+} load, and cardiomyocyte relaxation. Dephosphorylated PLN inhibits SERCA2 activity, whereas PLN phosphorylation at Thr17 by CaMKIIδ (or Ser16 by PKA [protein kinase A]) reduces PLN interaction with SERCA2 and relieves this inhibition.[4] Likewise, phosphorylation at Ser2814-RYR by CaMKIIδ increases RYR Ca^{2+} sensitivity, leading to augmented SR Ca^{2+} release and cardiomyocyte contraction. Inhibition of SR CaMKIIδ activity results in decreased phosphorylation of RYR and PLN, and associated changes in Ca^{2+} homeostasis and cardiac contractility.[5] These data support a pivotal role of CaMKIIδ in fine-tuning ECC.

CaMKII forms a dodecamer that comprises 2 stacked 6-fold symmetrical rings.[6] The CaMKII monomer contains an N-terminal ATP-binding pocket, catalytic and autoregulatory domains, and a C-terminal association

domain mediating its oligomerization.[7] When CaMKIIδ is in its inactive state, the Thr287 segment (Thr286 in CaMKIIα) in the autoregulatory domain binds to the so-called T-site in catalytic domain. This positions the adjacent sequence (pseudosubstrate) in the substrate-binding site (S-site) and ATP-binding pocket[7] (Figure S1A). Upon activation, Ca^{2+}/CaM binds to the CaM-binding site in the autoregulatory domain, displacing the Thr287 segment from the T-site (Figure S1B) and enabling the kinase to be phosphorylated by a neighboring active (open) CaMKIIδ molecule. Thus, binding of at least 2 CaM molecules is required for CaMKII autophosphorylation. Autophosphorylation of Thr287 increases CaMKII affinity for CaM,[8] maintains the kinase in an autonomously active state[9] (Figure S1C), and permits the kinase to translate the frequency of Ca^{2+} spikes into kinase activity in vitro,[10] a form of molecular memory or integration.

Although CaMKIIδ regulates several aspects of ECC, it remains unclear how the kinase is functionally regulated within the distinct nanodomains where these Ca^{2+} handling proteins are localized. For example, local [Ca^{2+}] near the PLN-SERCA2 complex is expected to be too low to appreciably activate CaMKII, while CaMKII near RYRs in the dyadic cleft should be activated by the much higher local [Ca^{2+}] levels.[11] Although activated CaMKIIδ in myocytes is more mobile than traditionally thought,[12] another potential explanation for PLN phosphorylation by CaMKIIδ is that as yet unidentified CaMKAPs (CaM-kinase anchoring proteins) could enable locally higher Ca^{2+} sensitivity of the kinase.

We postulated that the A-kinase anchoring protein, AKAP18δ (also known as AKAP7δ), could perform such a CaMKIIδ anchoring and regulatory role at PLN-SERCA2 and RYR. Originally identified in rat kidney,[13] AKAP18δ has been shown to interact directly with PKA and PLN, thereby enabling PKA-dependent phosphorylation at Ser16-PLN and augmentation of SERCA2 activity.[14] In human myocardium, the AKAP18δ orthologue, AKAP18γ, similarly complexes with PKA, PLN-SERCA2, and PDE3A1 (phosphodiesterase 3A1) to control Ser16-PLN phosphorylation and SR Ca^{2+} reuptake.[15] In the present work, we show that AKAP18δ also anchors CaMKIIδ to PLN-SERCA2 and RYR, defining AKAP18δ as the first CaMKII anchoring protein. We further identify 2 unique regions in AKAP18δ that inversely regulate CaMKIIδ activity, CaMKIIδ-catalyzed phosphorylation of Thr17-PLN and Ser2814-RYR, and thus SERCA2 and RYR functional activities. This capability is enabled by one region of AKAP18δ, which binds CaM and inhibits CaMKII activation, and a second region which lowers the Ca^{2+} threshold for CaMKIIδ activation and induces CaM trapping. Based on our results, we propose a model in which the 2 AKAP18δ regions fine-tune the Ca^{2+}-frequency-dependent activation of CaMKIIδ at PLN-SERCA2 and RYR.

METHODS

Data Availability

The authors declare that all supporting data are available within the article and in its Supplemental Material. Detailed methods are provided in the Supplemental Material. The data that support the findings of this study and analytical tools are available from the corresponding author upon reasonable request.

Statistics

All data sets with a small n (n<8) were tested for normal distribution using Kolmogorov-Smirnov, Shapiro-Wilk, or D'Agostino & Pearson normality test (GraphPad Prism 8.0.1 or 9.1.0). Differences between groups with normally distributed data were analyzed using ordinary 1-way ANOVA with Dunnett, Holm-Sidak, or Tukey multiple comparisons test, or unpaired *t* test for simple 2-group comparison. Non-normal distributions were examined by Wilcoxon matched-pairs signed-rank test, Mann-Whitney test, or Kruskal-Wallis with Dunn's multiple comparisons test. When multiple measures were drawn from individual animals, nested *t* test, nested 1-way ANOVA with Tukey or Dunnett multiple comparisons test or the linear mixed effect model from the R nlme package (https://CRAN.R-project.org/package=nlme) with Tukey post hoc correction was used. Only within-test corrections were made. $P<0.05$ were considered statistically significant. Outliers were removed using the ROUT method (Q=1%) (GraphPad Prism) (Figure 6A, Figure S3B and S3C and Figure S6A and S6B). Power analysis was performed a priori to determine anticipated optimal sample size and number of adenoassociated virus-injected animals. All experiments were performed in a randomized manner, and data analysis was performed blinded using name and allocation concealment. Representative immunoblots were selected to represent the means of the quantified data. Representative images were selected by eye and based on good signal/noise ratios.

Other methods are given in details in the Supplemental Material.

RESULTS

AKAP18δ-Associated CaMKIIδ Controls Thr17-PLN Phosphorylation

First, we tested whether CaMKIIδ and AKAP18δ interact at SERCA2-PLN. Immunoprecipitation analysis revealed co-precipitation of CaMKIIδ and SERCA2 with AKAP18δ in adult cardiomyocyte lysate (Figure 1A). High-resolution imaging of adult cardiomyocytes further demonstrated that AKAP18δ and CaMKIIδ (Figure 1B, blue, upper and lower respectively) co-localized with SERCA2 (red) at Z-line. AKAP18δ also co-localized with SERCA2 at M-line where little CaMKIIδ was observed, indicating a role for AKAP18δ also within the sarcomere center (Figure 1B). Wheat germ agglutinin staining of t-tubules was used as marker for Z-lines, since these structures co-localize with α-actinin (Figure S1D). Secondary antibody controls and specificity of CaMKIIδ and AKAP18δ antibodies are shown in Figure S1E through S1G.

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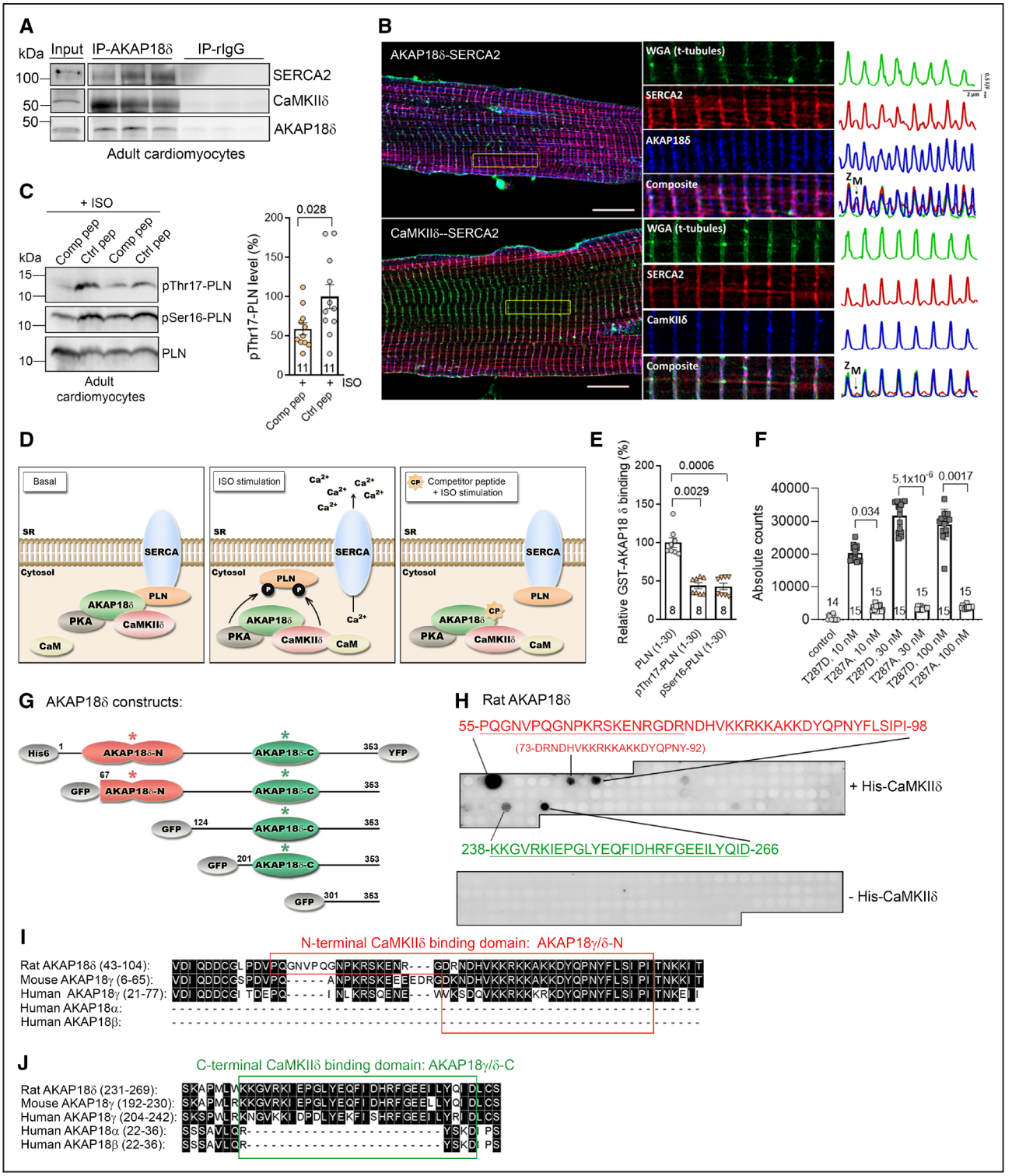

Figure 1. AKAP18δ (A-kinase anchoring protein 18δ) anchors CaMKIIδ to the PLN (phospholamban)-sarcoplasmic reticulum (SR) Ca^{2+}-ATPase 2 (SERCA2) complex and controls Thr17-PLN phosphorylation.

A, Immunoprecipitation of CaMKIIδ-AKAP18δ from adult cardiomyocyte lysate detected by immunoblotting. Rabbit IgG was used as control. **B**, High-resolution imaging of SERCA2-AKAP18δ (upper) and SERCA2-CaMKIIδ (lower) in adult mouse cardiomyocytes using anti-SERCA2, anti-AKAP18δ, and anti-CaMKIIδ. Single images and corresponding traces are shown in middle and at right. Z/M-lines are indicated. Scale bars=10 μm. **C**, pThr17-PLN, pSer16-PLN, and PLN levels in ISO-stimulated adult cardiomyocytes pretreated with cell-permeant AKAP18δ-PLN competitor or control peptide. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by the linear mixed effect model from the R nlme package with Tukey post hoc correction (n=11, 4 rats). **D**, Illustration of the AKAP18δ-PLN competitor experiment. Left: without ISO, PLN is dephosphorylated and inhibits SERCA2 activity. Middle: during ISO-stimulation, AKAP18δ-associated CaMKIIδ (and PKA) phosphorylates PLN, leading to SERCA2 activation and Ca^{2+} uptake into SR. (*Continued*)

To analyze whether AKAP18δ-associated CaMKIIδ controls pThr17-PLN phosphorylation, AKAP18δ was displaced from PLN using a cell-permeant AKAP18δ-PLN competitor peptide.[14] Adult and neonatal cardiomyocytes treated with this peptide before isoproterenol stimulation exhibited reduced pThr17-PLN (and pSer16-PLN as previously reported[14]) compared with control (Figure 1C and Figure S1H, respectively), indicating that AKAP18δ-associated CaMKIIδ phosphorylates Thr17-PLN (illustrated in Figure 1D). The peptides showed no changes at basal level or cytotoxicity (Figure S1I and S1J, respectively). Using biotin-labeled PLN peptides, we found that Thr17 phosphorylation reduced GST (glutathione S-transferase)-AKAP18δ binding (Figure 1E), closely paralleling reported effects of Ser16 phosphorylation.[14] Thus, Thr17 phosphorylation seems to provide an on/off mechanism for the AKAP18δ-PLN interaction.

Taken together, the data strongly support that AKAP18δ anchors CaMKIIδ to PLN-SERCA2, and thereby controls CaMKIIδ-mediated Thr17-PLN phosphorylation.

CaMKIIδ Binds Directly to 2 Unique Regions in AKAP18δ

The CaMKIIδ-AKAP18δ interaction was further investigated using AlphaScreen technology. Only recombinant CaMKIIδ-T287D (mimicking active kinase)[16] and not CaMKIIδ-T287A (mimicking inactive kinase) (mutated proteins are validated in Figure S1K and S1L) was found to bind to AKAP18δ (Figure 1F), indicating that AKAP18δ binds to autophosphorylated CaMKIIδ.

To identify CaMKIIδ-binding sites, AKAP18δ-YFP variants (Figure 1G) were co-expressed with CaMKIIδ-T287D. CaMKIIδ precipitated all AKAP18δ variants, except for GFP-AKAP18δ (301-353) (Figure S1M), indicating that CaMKIIδ binds between amino acids 201-301 (Figure 1G, green region). This region is located C-terminally from the PLN binding domain (amino acids 124-220[14]) and N-terminally from the PKA binding domain (amino acids 301-314[13]). In addition, AKAP18δ-YFP and GFP-AKAP18δ (67-353) precipitated more strongly with CaMKIIδ compared with other AKAP18δ variants, consistent with a second CaMKIIδ-binding site toward the N-terminus of AKAP18δ (Figure 1G, red).

To more precisely identify CaMKIIδ binding, rat AKAP18δ was spot-synthesized as 20-mer overlapping peptides on membranes and incubated with active His-CaMKIIδ. Immunoblotting identified CaMKIIδ binding to 2 regions; amino acids 55-98 (AKAP18δ-N, in red) and 238-266 (AKAP18δ-C, in green) (Figure 1H) and 2 homologous regions in human AKAP18γ (Figure S1N). CaMKIIδ-T287D (coated in wells) binding was confirmed by an ELISA-based method using biotinylated peptides spanning the 2 AKAP18δ regions (Figure S1O and S1P). Sequence alignments showed that the 2 CaMKIIδ-binding regions were only present in AKAP18δ and AKAP18γ and not in the shorter AKAP18α and AKAP18β isoforms[13] (Figure 1I and 1J).

AKAP18δ Binds CaMKIIδ Through Multiple Sites

We next sought to define AKAP18δ binding in CaMKIIδ$_{C/2}$, which is largely cytoplasmic and regulates ECC. CaMKIIδ was spot-synthesized as 20-mer overlapping peptides on membranes, which were overlaid with GST-AKAP18δ (Figure 2A). AKAP18δ bound to the ATP-binding region[7] (amino acids 19-68), S-site (amino acids 130-164) and T-site regions (amino acids 241-269) and a sequence within the autoregulatory domain (amino acids 280-317)[7] (Figure 2A, GST only as negative control is shown in Figure S2A, bottom). Notably, the S- and T-site regions within the catalytic domain have not been clearly defined but are reported to contain at least residues Glu97 (S-site), Glu140 (S-site), Ile206 (T-site), and Trp238 (T-site)[19] (Figure 2A, boxed regions in upper panel). Consistent with the above results, a biotinylated peptide covering the N-terminal region of AKAP18δ (55-98; Figure S2B) showed an almost identical CaMKIIδ binding pattern as GST-AKAP18δ.

CaMKIIδ binding of biotin-ahx-AKAP18δ-C was too weak or dynamic to be detected by peptide arrays. However, overlaying a larger recombinant GST-AKAP18δ-C fragment (amino acids 201-301), revealed a binding site residing centrally within the T-site region (amino acids 205-233) (Figure 2B). This binding site was not detected using GST-AKAP18δ full length protein, suggesting that GST-AKAP18δ (201-301) exhibits differential folding. These data are consistent with AKAP18δ-C

Figure 1 Continued. Right: in the presence of the cell-permeant AKAP18δ-PLN competitor peptide (CP),[14] AKAP18δ displaces from PLN-SERCA2. AKAP18δ-associated CaMKIIδ is no longer able to phosphorylate PLN. Both AKAP18δ[76] and PLN locate to membrane, but this is not shown for simplicity. **E**, Analyses of biotin-ahx-PLN (1-30), biotin-ahx-pSer16-PLN (1-30), and biotin-ahx-pThr17-PLN (1-30) binding to GST-AKAP18δ (coated in wells) by an ELISA-based assay. Binding was detected with a biotin-HRP conjugated antibody and incubation with Ultra TMB. Significant differences were examined by Kruskal-Wallis with Dunn's multiple comparisons test (n=8). **F**, Analysis of AKAP18δ-CaMKIIδ interaction by AlphaScreen™. GST-AKAP18δ was incubated with increasing concentrations of recombinant CaMKIIδ-T287D or CaMKIIδ-T287A. Significant differences were examined by Kruskal-Wallis with Dunn multiple comparisons test (n=14–15). **G**, The 2 CaMKIIδ binding regions in AKAP18δ are illustrated in red and green. **H**, Residues important for CaMKIIδ binding were identified by overlaying 20-mer overlapping AKAP18δ peptides spot-synthesized on membranes with active His-CaMKIIδ and immunoblotting. Immunoblotting without His-CaMKIIδ was used as control (lower). Underlined sequences were synthesized as soluble peptides for further experiments. **I** and **J**, The 2 CaMKIIδ-binding regions (red and green) are indicated in the alignment of rat AKAP18δ, human and mouse AKAP18γ, and the smaller AKAP18α and AKAP18β. Black boxes indicate identical amino acids (DNA Star).

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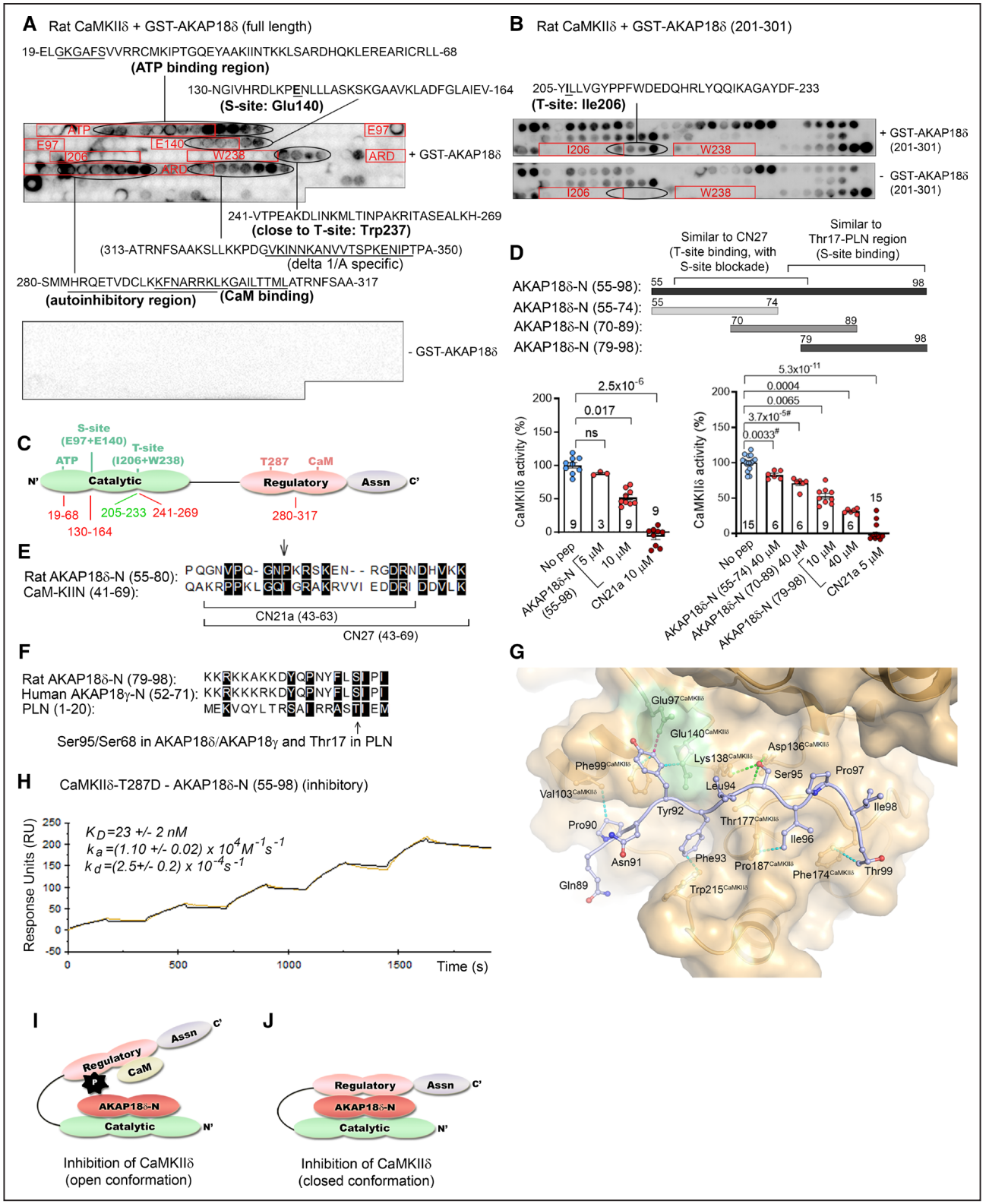

Figure 2. AKAP18δ (A-kinase anchoring protein 18δ) binds CaMKIIδ through multiple sites and inhibits CaMKIIδ through sequences similar to the natural CaMKII inhibitor protein and Thr17-PLN (phospholamban) region.
AKAP18δ binding was identified by overlaying 20-mer overlapping CaMKIIδ peptides with (**A**) GST-AKAP18δ or (**B**) GST-AKAP18δ (201-301) and immunoblotting with anti-GST-HRP. Immunoblotting without recombinant protein (lower) or GST (Figure S2A, lower) was used as control. Peptides containing ATP-binding region[7] (ATP-binding motif underlined), S-site (E97 and E140-containing sequences), T-site (I206 and W238-containing sequences) or autoregulatory domain (ARD) are boxed. Stretches of spots that were not apparent in negative controls were regarded as potential-binding sites. Sequences for spots with strongest signal are given. Autoinhibitory region and (*Continued*)

and AKAP18δ-N binding to distinct T-site sequences. ELISA-based experiments confirmed AKAP18δ binding to the autoregulatory domain, ATP binding region, S-site and 2 T-site regions of CaMKIIδ (Figure S2C through S2E), supporting the interpretation of the overlay data. The 5 AKAP18δ binding sites in CaMKIIδ$_{C/2}$ are indicated in Figure 2C (19-68, 130-164, 205-233, 241-269, and 280-317, inhibitory ones in red and activating in green). The fact that AKAP18δ-N binds to several different regions in these critical CaMKII domains may serve to stabilize (or rigidify) CaMKII in the closed inactivated state. Multiple CaMKII-binding sites have also been identified in other proteins,[17,18,20] for example, densin, which binds to several T-site sequences.[18]

AKAP18δ-N Inhibits CaMKIIδ Through Sequences Similar to the Natural CaMKII Inhibitor Protein and Thr17-PLN Region

AKAP18δ-N (55-98) interacted only with activated CaMKIIδ (Figure S2F). The effect of AKAP18δ-N on CaMKIIδ activity was analyzed in an in vitro kinase assay. AKAP18δ-N (55-98) reduced CaMKIIδ-catalyzed phosphorylation of syntide (a CaMKII substrate) by 50% under high [Ca^{2+}] and [CaM], conditions that should maximally activate CaMKII (Figure 2D, left panel). Closer examination showed that amino acids 55-74 were less inhibitory (right panel). Bioinformatics revealed that this sequence exhibited similarities to CN27, a natural CaMKII inhibitory peptide[21] (Figure 2E). In a crystal structure of CaMKII, a shorter variant of CN27 (CN21a in Figure 2E) has been shown to bind to the T-site, while being sufficiently long to prevent access for substrate binding to the adjacent S-site.[21] In an ELISA-based assay, CN27 outcompeted the AKAP18δ-N (55-74)-CaMKIIδ interaction, indicating that CN27 and AKAP18δ-N (55-74) may bind to the same site in CaMKIIδ (Figure S2G). In addition, AKAP18δ-N (55-74) contains a proline (Pro64) in its central region (Figure 2E, arrow); a feature also reported for the inhibitory T-site binding sequence in densin,[18] which might explain its weaker effect on CaMKIIδ activity.

CaMKIIδ binding of the more inhibitory amino acids 79-98 of AKAP18δ (Figure 2D) were not outcompeted by CN27 (Figure S2H). Bioinformatics revealed that these amino acids rather showed some sequence similarity to the Thr17-PLN region, with Ser95^{AKAP18δ} corresponding to Thr17^{PLN} (Figure 2F). Modeled placement of AKAP18δ (89-99) into the peptide binding groove of CaMKIIδ centered on Ser95 in AKAP18δ (Figure 2G) and suggested several favorable interactions between CaMKIIδ and AKAP18δ. Hydrophobic interactions between Pro90-Val103^{CaMKIIδ}, Tyr92-Lys138^{CaMKIIδ}, Phe93-Trp215^{CaMKIIδ}, Ile96-Pro187^{CaMKIIδ}, and Thr99-Phe174^{CaMKIIδ} were suggested (cyan dashed lines). The model indicated that Ser95 could interact with both Asp136^{CaMKIIδ}, Lys138^{CaMKIIδ} and Thr177^{CaMKIIδ} (green dashed lines) via hydrogen bonds. A possible unfavorable repulsive interaction between Tyr92 and Glu97^{CaMKIIδ} was also indicated (red dashed line). Model predictions were validated by mutating key residues in AKAP18δ, and subsequent ELISA analysis (Figure S2I, see legend for detailed description).

Kinetics of AKAP18δ-N (55-98) binding to CaMKIIδ were analyzed by surface plasmon resonance (SPR). A range of concentrations of recombinant CaMKIIδ-T287D (47.6–500 nmol/L) was injected over immobilized biotin-AKAP18δ-N on a streptavidin affinity chip and analyzed by fitting with a 1:1 interaction model (Langmuir). The dissociation equilibrium constant (K_D) was 23±2 nM, with an association rate constant (k_a)=(1.10±0.02)×10^4 M^{-1} s^{-1} and a dissociation rate constant (k_d)=(2.5±0.2)×10^{-4} s^{-1} (Figure 2H). These findings indicate a strong CaMKIIδ-T287D interaction with AKAP18δ-N with medium association and dissociation rates. We also performed SPR analyses of AKAP18δ-N (55-74) and AKAP18δ-N (79-98). The inhibitory AKAP18δ-N (79-98) bound strongly to CaMKIIδ-T287D with similar SPR values as identified above (Figure S2J). AKAP18δ-N (55-74), which was less inhibitory and contained a proline in its central region, bound more weakly and exhibited higher association and dissociation rate constants (faster on/off) (Figure S2K).

Taken together, our data indicate that AKAP18δ-N (55-98) is a potent inhibitor of the open CaMKIIδ

Figure 2 Continued. CaM-binding site are underlined.[7] The CaMKIIδ1/A-specific sequence is only present in neonatal mouse hearts.[77] Amino acids 205-233 (upper in **B**) was regarded as potential-binding site, since it was absent in negative control (lower). **C**, The 5 AKAP18δ-binding regions identified by peptide arrays (in **A** and **B**) are indicated in CaMKIIδ2/C (inhibitory ones in red and activating in green). **D**, Effect of different AKAP18δ-N sequences on CaMKIIδ-T287D activity (^{32}P incorporation into syntide). CN21a derived from natural CaMKII inhibitor protein (CaM-KIIN)[21] was used as control. Significant differences were examined by Kruskal-Wallis with Dunn's multiple comparisons test (n=3–9 in left, n=6–15 in right). #Significant differences were detected by Mann-Whitney *U* test. Upper panel illustrates the different AKAP18δ-N peptides. **E**, Alignment of rat AKAP18δ (55-80) with the natural CaMKII inhibitor protein (amino acids 41-69). CN27 and CN21a are indicated.[21] Arrow denotes a proline in AKAP18δ. **F**, Alignment of rat AKAP18δ (79-98) and human AKAP18γ (52-71) with PLN (1-20). Arrow indicates Ser68/Ser95 in AKAP18γ/AKAP18δ and Thr17 in PLN. Black boxes indicate identical or functionally similar amino acids (DNA Star). **G**, Structural model of the AKAP18δ (89-QPNYFLSIPIT-99) binding to CaMKIIδ centered at Ser95 (corresponds to Thr17 in PLN). AKAP18δ is shown as a ball-and-stick model, while CaMKIIδ peptide backbone is shown as a cartoon with central residues as a ball-and-stick motif. The negatively charged patch around S-site residues Glu97 and Glu140 is colored light green. Rest of the transparent CaMKIIδ surface is shown in yellow. **H**, SPR analysis of immobilized biotin-ahx-AKAP18δ (55-98) on an SA chip and recombinant CaMKIIδ-T287D injected at a range of concentrations (47.6–500 nmol/L) (n=3). **I** and **J**, Illustration of CaMKIIδ inhibition by AKAP18δ-N. AKAP18δ-N (red) binds to catalytic region (light green) and inhibits CaMKIIδ in both an (**I**) open and (**J**) closed conformation after pThr287 dephosphorylation and CaM dissociation.

with strong affinity. Inhibition is effected by sequences with similarities to CN27 derived from natural CaMKII inhibitor protein (T-site binding, with associated S-site blockade) and Thr17-PLN region (S-site binding) (summarized in Figure 2D, upper panel). CaMKIIδ inhibition by AKAP18δ-N is illustrated in Figure 2I (open conformation). Since AKAP18δ-N also bound to the autoregulatory domain, it is likely that AKAP18δ-N also continues to bind and inhibit CaMKIIδ after pThr287 dephosphorylation and CaM dissociation (Figure 2J, closed conformation).

AKAP18δ-N Reduces pThr17-PLN and Inhibits SR Ca^{2+} Reuptake

Analyses performed using biotin-ahx-PLN (1-30) as a substrate showed that AKAP18δ-N reduced CaMKIIδ-T287D-catalyzed phosphorylation of Thr17 in PLN (Figure 3A, full immunoblots in Figure S3A). We further examined whether AKAP18δ-N also reduced Thr17-PLN phosphorylation in cardiomyocytes. Compared with the scrambled control peptide, cell-permeant AKAP18δ-N (55-74) reduced the pThr17-PLN level in both isoproterenol treated (15 minutes) adult (Figure 3B) and neonatal cardiomyocytes (Figure S3B), whereas pSer16, which is a PKA substrate, was hardly affected. Neither AKAP18δ-N (55-74) nor control peptide showed any changes at basal level or cytotoxicity (Figure S3C and S3D, respectively).

Effects of AKAP18δ-N on Ca^{2+} fluxes in adult cardiomyocytes were also investigated. Rat adult cardiomyocytes were treated with the cell-permeant version of AKAP18δ-N (55-74) or a scrambled control peptide, with or without isoproterenol stimulation. Cell-permeant TAT-AKAP18δ-N (55-74) treatment prolonged decay time of Ca^{2+} transients at 4 Hz (Figure 3C, representative tracings are shown in Figure S3E), where Thr17-PLN phosphorylation is increased[40] and SERCA2 plays a proportionally larger role in controlling Ca^{2+} removal from the cytosol.[41] In the presence of isoproterenol, which augments SERCA2 activity via PLN phosphorylation, TAT-AKAP18δ-N (55-74) continued to slow Ca^{2+} transient decay at high stimulation frequencies (4 and 6 Hz) (Figure 3D, representative tracings are shown in Figure S3F). Compared with the scrambled control peptide, AKAP18δ-N (79-98) also reduced SERCA2 Ca^{2+} reuptake rate in isolated mouse cardiac SR vesicles (Figure 3E). These findings are consistent with reduction of pThr17-PLN levels by AKAP18δ-N (Figure 3A and 3B).

Finally, compared with control mice, adult mice injected with adenoassociated virus encoding AKAP18δ-N (55-74) exhibited reduced pThr17-PLN levels after isoproterenol stimulation, suggesting that AKAP18δ-N (55-74) also inhibits CaMKIIδ-catalyzed phosphorylation of Thr17 in PLN in vivo (Figure 3F, upper panel). No effect of adenoassociated virus-AKAP18δ-N (55-74) on pSer16-PLN was observed (Figure 3F, middle panel).

Calcified CaM Binds to AKAP18δ-N in Competition With CaMKIIδ

Rat AKAP18δ-N and human AKAP18γ-N contain stretches of alternating hydrophobic and basic residues, including valine (V), lysine (K), arginine (R), phenylalanine (F), and proline (P), which are characteristic for CaM-binding motifs.[23] Closer inspection revealed that both AKAPs showed sequence similarities to the CaM-binding motif in AKAP79[24] (Figure 4A) and CaMKIIδ (Figure S4A). We therefore analyzed whether AKAP18δ-N also bound calcified CaM. Immunoblotting demonstrated that the inhibitory AKAP18δ-N (79-98) peptide, the corresponding sequence in human AKAP18γ-N (52-71) and GFP-AKAP18δ (67-353) precipitated with CaM-agarose in the presence of Ca^{2+} (Figure S4B through S4D, respectively). CaM binding to AKAP18δ-N (79-98) was also confirmed in an ELISA-based assay where CaM-coated wells were incubated with biotinylated AKAP18δ-N (79-98) or the scrambled control (Figure 4B). Interestingly, in further experiments, the presence of CaM attenuated both the inhibitory AKAP18δ-N (79-98)-CaMKIIδ-T287D (Figure 4C) and AKAP18δ-N (79-98)-CaMKIIδ (1-282) (Figure 4D) interactions. Since CaMKIIδ (1-282) lacks the CaM-binding site, this finding indicates that CaM also outcompetes AKAP18δ-N (79-98) binding to the CaMKIIδ catalytic site.

Kinetics of the CaM-AKAP18δ-N (79-98) interaction were analyzed by SPR. In the presence of 1 mM Ca^{2+}, the dissociation equilibrium constant (K_D) was 4.7±0.7 μM, with an association rate constant (k_a)=(1.3±0.3)×10^2 M^{-1} s^{-1} and a dissociation rate constant (k_d)=(5.3±0.3)×10^{-4} s^{-1} (Figure 4E). These findings indicate that the CaM-AKAP18δ-N (79-98) interaction is quite slow and weak.

In summary, our data indicate that calcified CaM binds directly to AKAP18δ-N and outcompetes the inhibitory AKAP18δ-N-CaMKIIδ interaction (illustrated in Figure 4F). By this mechanism, rising Ca^{2+} levels, and resulting calcification of CaM, relieve the inhibition of CaMKII.

AKAP18δ-C Is Homologous to the Neuronal CaMKIIα Activator N2B-s and Lowers the Ca^{2+} Threshold for CaMKIIδ Activation

As shown in Figure 1J, both AKAP18δ and AKAP18γ contain a second CaMKIIδ interaction site positioned toward the C-terminus. Bioinformatics revealed that this region showed sequence similarity to the N2B-s sequence derived from the neuronal N-methyl-D-aspartate receptor NR2B subunit (Figure 5A). N2B-s is homologous to the Thr286 autoinhibitory region in CaMKIIα and linked to autonomous CaMKIIα activation in brain via T-site binding.[17,25,26] As demonstrated

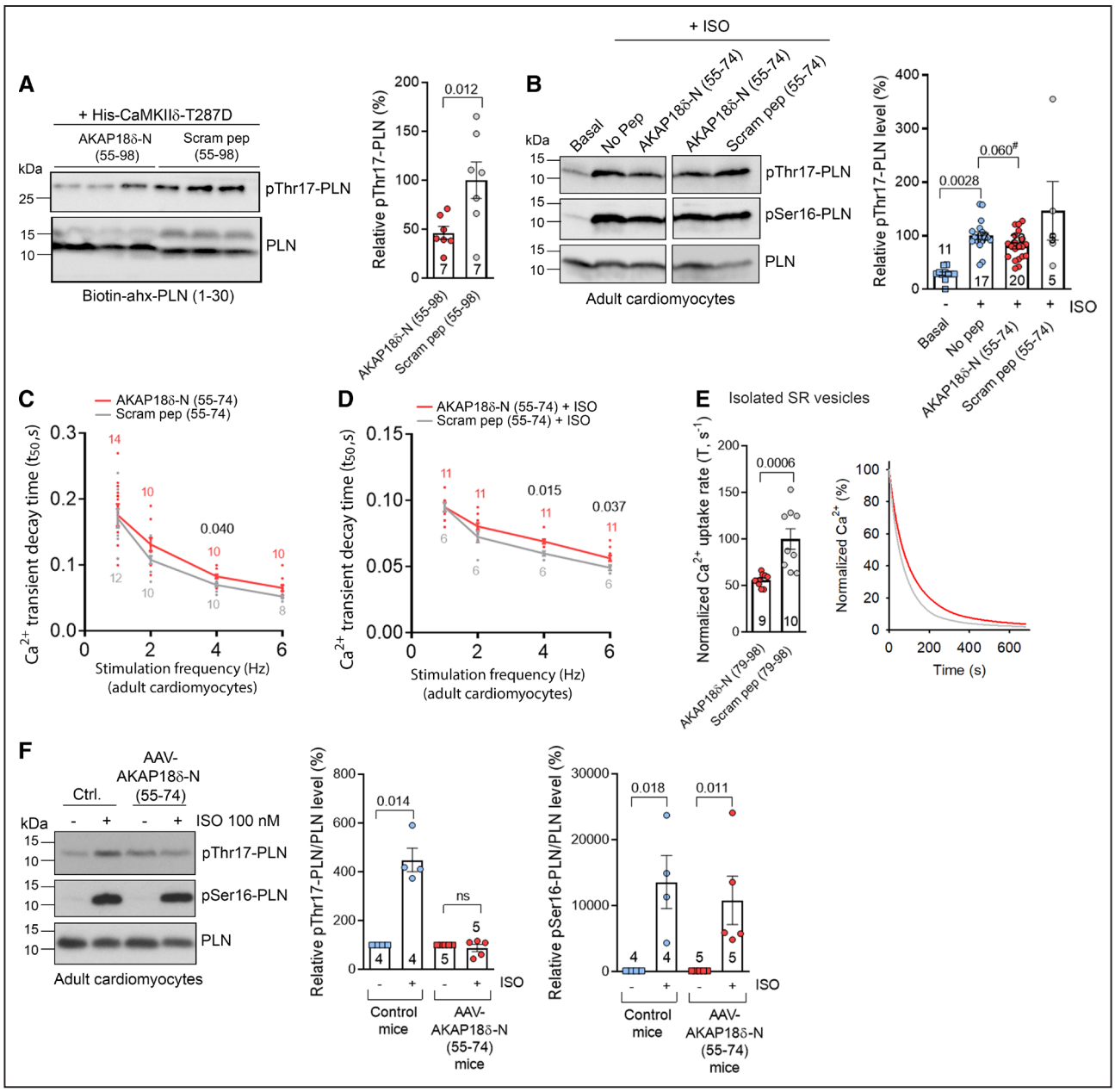

Figure 3. AKAP18δ (A-kinase anchoring protein 18δ)-N reduces pThr17-PLN (phospholamban) and inhibits Ca^{2+} reuptake into sarcoplasmic reticulum (SR).

A, CaMKIIδ phosphorylation of biotin-ahx-PLN (1-30), in the presence of AKAP18δ-N (55-98) or a scrambled control peptide. Phosphorylated Thr17-PLN was observed at 25 kDa consistent with induced oligomerization[78] (Figure S3A, complete immunoblots). Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by unpaired *t* test (n=7). **B**, Adult cardiomyocytes were treated with TAT-AKAP18δ-N (55-74) or the scrambled control for 45 min before ISO-stimulation (15 min) and immunoblotted with pThr17-PLN, pSer16-PLN, and PLN antibodies. pThr17-PLN was quantified against PLN or GAPDH. Normal distribution was confirmed by Shapiro-Wilk test (not tested for scram pep with n<6). Significant differences were examined by nested 1-way ANOVA with Tukey's multiple comparisons test (n=5–20, 2–6 rats). #Significant differences were examined by nested *t* test. The effect of TAT-AKAP18δ-N (55-74) on decay time of Ca^{2+} transients at different stimulation frequencies of adult cardiomyocytes in the (**C**) absence or (**D**) presence of ISO. Scrambled TAT-AKAP18δ-N (55-74) peptide was used as control. Normal distribution was confirmed by Shapiro-Wilk test (in **C**) and Kolmogorov-Smirnov or D'Agostino and Pearson test (in **D**, except for the scrambled peptide at 1 Hz). Significant difference was examined by nested *t* test, n=8–14 in **C** (2–4 rats) and n=6–11 in **D** (3–4 rats). Representative Ca^{2+} tracings are shown in Figure S3E and S3F. **E**, The effect of TAT-AKAP18δ-N (79-98) or a scrambled control on SERCA2 Ca^{2+} reuptake rate in isolated mouse SR vesicles (left ventricle crude homogenate). Normal distribution was confirmed by D'Agostino and Pearson test. Significant differences were examined by unpaired *t* test, n=9–10. **F**, Levels of pThr17-PLN, pSer16-PLN, and PLN in adult cardiomyocytes isolated from control (WT) and AAV-AKAP18δ-N (79-98) mice, treated with or without ISO. Significant differences were examined by Kruskal-Wallis with Dunn multiple comparisons test (n=4–5, 3–4 mice in each group).

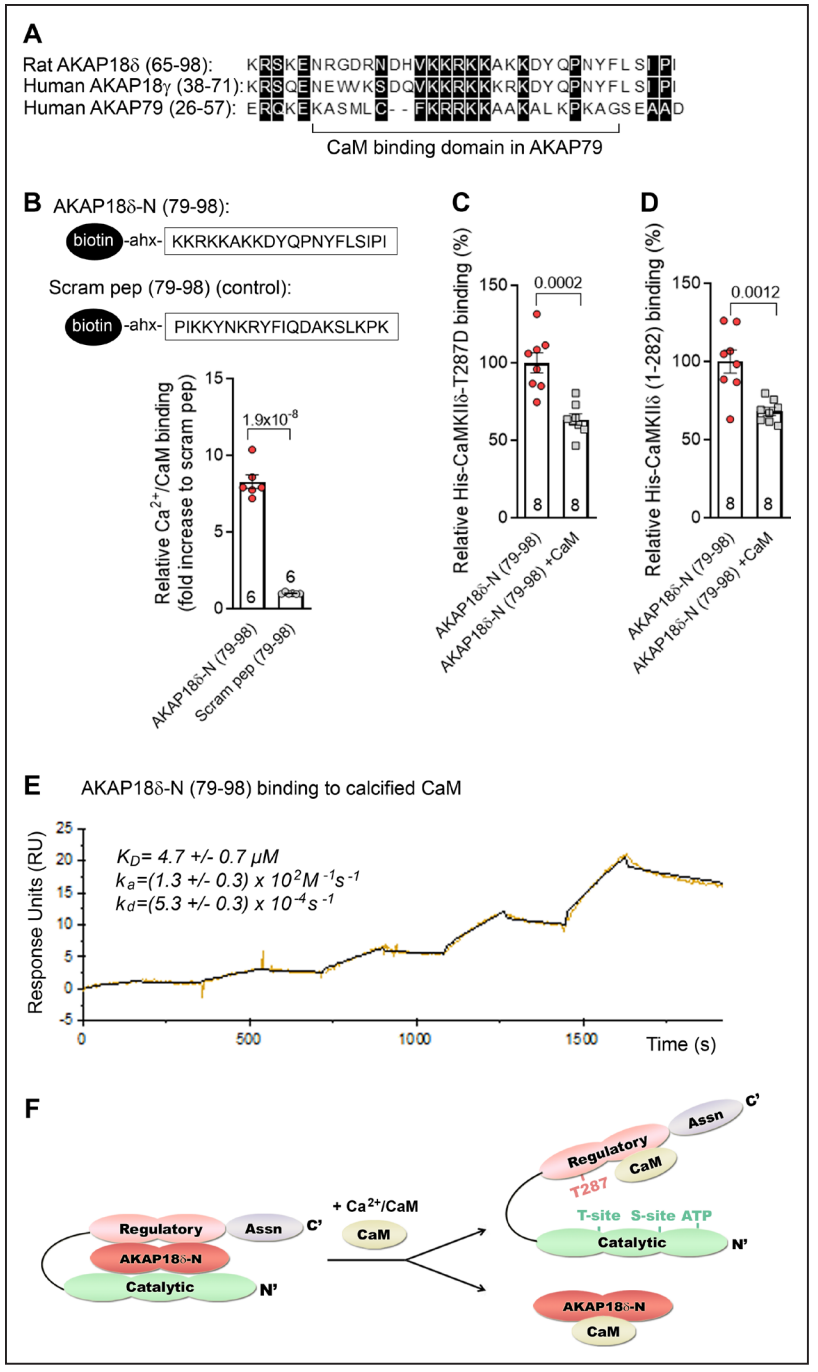

Figure 4. Calcified CaM (calmodulin) binds to AKAP18δ (A-kinase anchoring protein 18δ)-N and outcompetes the inhibitory AKAP18δ-N-CaMKIIδ interaction.
A, Alignment of rat AKAP18δ-N (65-98) and human AKAP18γ (38-71) with the CaM-binding domain in AKAP79.[24] Black boxes indicate identical or functional similar amino acids (DNA Star). **B**, Binding of biotin-ahx-AKAP18δ-N (79-98) or a scrambled control to Ca^{2+}/CaM (coated in wells) analyzed by an ELISA-based method. Normal distribution was confirmed by Kolmogorov-Smirnov test. Significant differences were examined by unpaired *t* test (n=6). Biotinylated AKAP18δ-N (79-98) peptide was incubated with or without CaM in wells coated with recombinant (**C**) CaMKIIδ-T287D or (**D**) CaMKIIδ (1-282). Binding was detected with anti-biotin-HRP. Normal distribution was confirmed by Shapiro-Wilk test (**C** and **D**). Significant differences were examined by unpaired *t* test (n=8 in **C** and **D**). **E**, SPR analysis of immobilized biotin-ahx-AKAP18δ (79-98) and recombinant CaM injected (952.6–10 000 nmol/L) (n=3). **F**, CaM (beige) outcompetes the inhibitory AKAP18δ-N-CaMKIIδ interaction upon binding to the CaMKIIδ regulatory region (pink) and AKAP18δ-N (red).

in Figure 2B, AKAP18δ-C also bound to T site (amino acids 205-233).

In similarity to effects of N2B-s, we observed that AKAP18δ-C increased substrate phosphorylation by CaMKIIδ-T287A in an in vitro kinase assay where CaM was omitted (Figure 5B). This finding suggests that AKAP18δ-C augments CaMKIIδ activity independently of Thr287 autophosphorylation and CaM binding. Consistently, cell-permeant TAT-AKAP18δ-C also increased Camui activity,[27] a fluorescence resonance energy transfer-based CaMKII activation state sensor, transduced into adult rabbit ventricular myocytes (Figure 5C). As CaMKII requires relatively high intracellular Ca^{2+} to achieve activation, we hypothesized that AKAP18δ-C was able to decrease the Ca^{2+} threshold for CaMKIIδ activation. Indeed, at 0.5 μM free Ca^{2+}, which does not fully activate CaMKIIδ-T287A to phosphorylate Thr17-PLN, further increased Thr17-PLN phosphorylation in the presence of either AKAP18δ-C (tendency) or the N2B-s positive control peptide (Figure 5D and 5E, respectively, complete immunoblots in Figure S5A and S5B). These observations suggest that AKAP18δ-C is able to potentiate CaMKIIδ by keeping the inhibitory gate open as a wedge, thereby sensitizing CaMKII to Ca^{2+}-dependent activation.

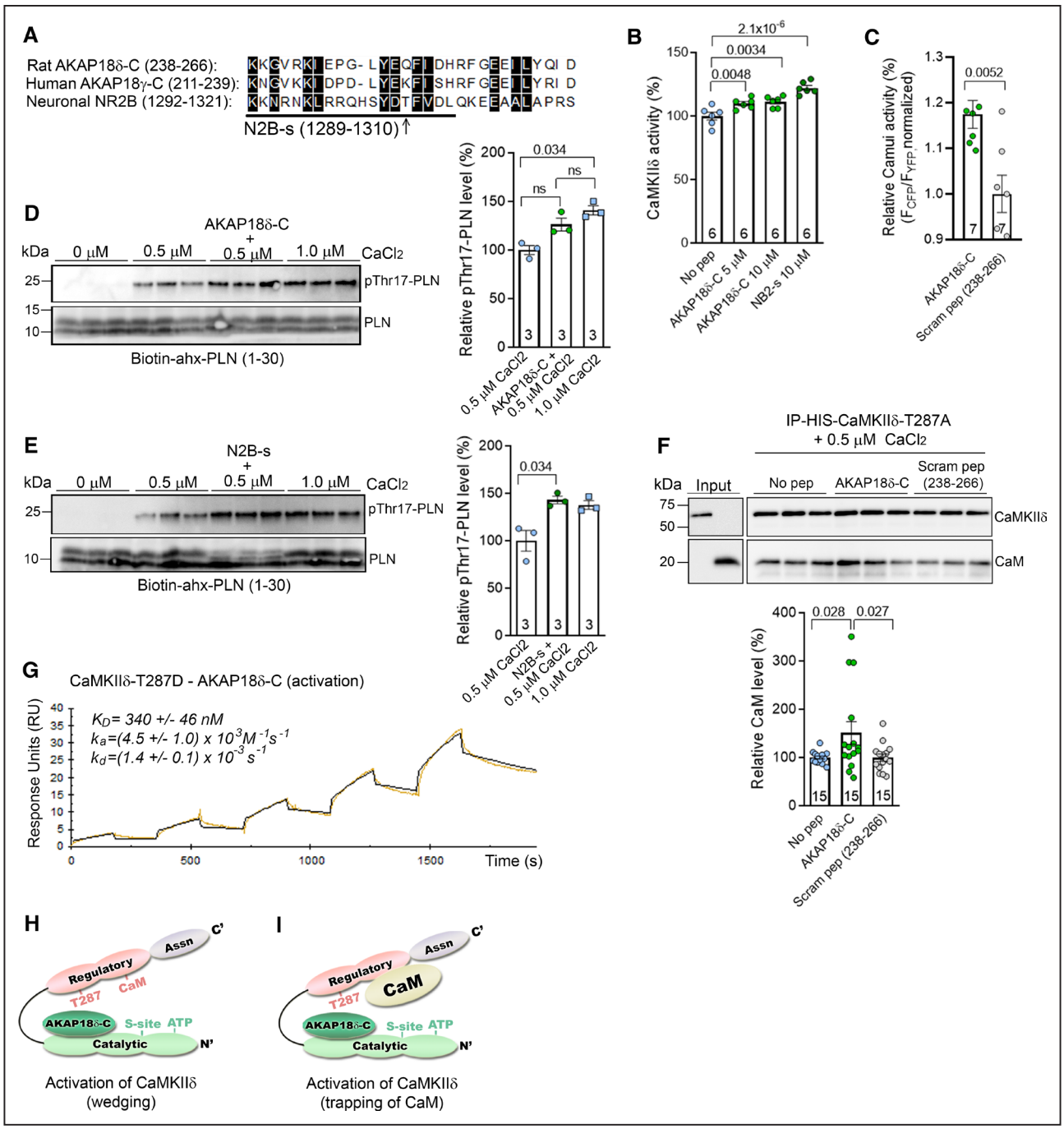

Figure 5. AKAP18δ (A-kinase anchoring protein 18δ)-C is homologous to the neuronal CaMKIIα activator N2B-s and lowers the Ca^{2+} threshold for CaMKIIδ activation.

A, Alignment of AKAP18δ-C (238-266) and AKAP18γ-C (211-239) with CaMKIIα binding site in neuronal NMDA receptor NR2B subunit (amino acids 1289-1321).[17] N2B-s (1292-1310) is underlined. Arrow denotes CaMKIIα phosphorylation site in NR2B (Thr1306). Black boxes indicate identical or functionally similar amino acids. **B**, Effect of AKAP18δ-C on CaMKIIδ-T287A activity analyzed in a CaMKII kinase assay in the absence of CaM (^{32}P incorporation into syntide). N2B-s[17] and CN21a[21] were used as controls. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by ordinary 1-way ANOVA with Holm-Sidak multiple comparisons test (n=6). **C**, Effect of TAT-AKAP18δ-C on a Camui FRET-based biosensor,[27] transduced into adult rabbit ventricular myocytes. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by unpaired *t* test (n=7). Immunoblot analysis of CaMKIIδ-catalyzed phosphorylation of Thr17-PLN (phospholamban) at 0.5 μM CaCl2 with or without the presence of (**D**) AKAP18δ-C or (**E**) N2B-s. Significant differences were examined by Kruskal-Wallis with Dunn's multiple comparisons test (**D** and **E**). **F**, Immunoblot analyses of immunoprecipitations of CaM with CaMKIIδ-T287A in the absence or presence of AKAP18δ-C (n=15). Normal distribution was confirmed by D'Agostino & Pearson test, and significant differences were examined by ordinary 1-way ANOVA with Dunnett multiple comparisons test. **G**, SPR analysis of immobilized biotin-ahx-AKAP18δ-C and recombinant CaMKIIδ-T287D injected (95.2–1000 nmol/L) (n=3). **H** and **I**, AKAP18δ-C (dark green) binds to catalytic region of CaMKIIδ (light green) and activates CaMKIIδ by (**H**) lowering the Ca^{2+} threshold for activation by keeping the inhibitory gate open and (**I**) trapping CaM (beige) within the kinase.

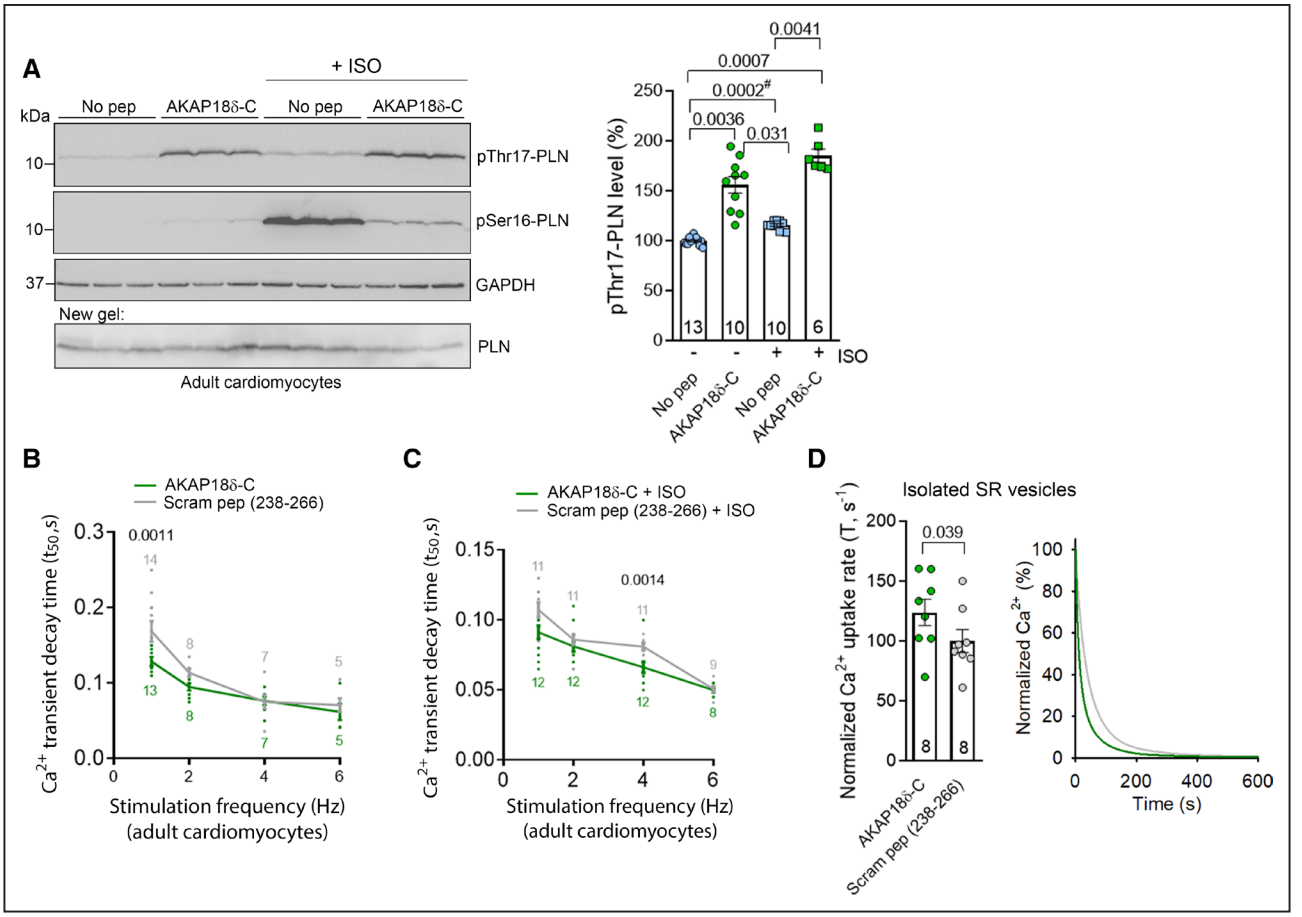

Figure 6. AKAP18δ (A-kinase anchoring protein 18δ)-C increases pThr17-PLN (phospholamban) and facilitates faster Ca^{2+} reuptake into sarcoplasmic reticulum (SR).

A, Effect of TAT-AKAP18δ-C on pThr17-PLN in basal and during ISO-stimulation (5 min) in adult cardiomyocytes. pThr17-PLN, pSer16-PLN and PLN levels were detected by immunoblotting. Normal distribution was confirmed by Shapiro-Wilk test. Significant differences were examined by nested 1-way ANOVA with Tukey's multiple comparisons test (n=6–13, 4 rats). Effect of TAT-AKAP18δ-C or a scramble control on decay time of Ca^{2+} transients across a range of stimulation frequencies in adult cardiomyocytes in (**B**) absence or (**C**) presence of ISO. Normal distribution was confirmed by Shapiro-Wilk test (not tested for the 6 Hz data set with n<6). Significant difference was examined by nested *t*-test, n=5–14 in **B** (3–4 rats) and n=8–12 in **C** (5–6 rats). Representative tracings are shown in Figure S6C and S6D. **D**, Effect of TAT-AKAP18δ-C or a scrambled control on Ca^{2+} reuptake rate in isolated mouse SR vesicles (left ventricle crude homogenate). Significant differences were examined by Wilcoxon matched-pairs signed rank test.

Immunoprecipitations employing CaMKIIδ-T287A in the presence of CaM showed that AKAP18δ-C also induced trapping of CaM to CaMKIIδ-T287A (Figure 5F); a feature which has been similarly described for N2B-s.[17]

Kinetics of the AKAP18δ-C (238-266)-CaMKIIδ interaction analyzed by SPR revealed a dissociation equilibrium constant (K_D)=340±46 nM, association rate constant (k_a)=(4.5±1.0)×10^3 M^{-1} s^{-1} and dissociation rate constant (k_d)=(1.4±0.1)×10^{-3} s^{-1} (Figure 5G). This indicated that the CaMKIIδ-T287D-AKAP18δ-C interaction is weaker than the CaMKIIδ-T287D-AKAP18δ-N interaction, has a slower on rate, and a faster off rate.

Taken together, our data indicate that AKAP18δ-C is a CaMKIIδ activator that lowers the Ca^{2+} threshold for CaMKIIδ activation (perhaps by keeping the inhibitory gate open) and also allows CaM trapping by the kinase (Figure 5H and 5I).

AKAP18δ-C Increases pThr17-PLN and Facilitates Faster SR Ca^{2+} Reuptake

When introduced into adult rat cardiomyocytes, TAT-AKAP18δ-C increased pThr17-PLN compared with both basal and isoproterenol-stimulated cardiomyocytes, and when combined with isoproterenol (5 minutes), the pThr17-PLN was further increased (tendency) (Figure 6A). pThr17-PLN was also augmented in neonatal rat cardiomyocytes treated with AKAP18δ-C or N2B-s[17] (Figure S6A and S6B). No cytotoxicity was observed for AKAP18δ-C or control peptide (Figure S3D).

Isolated adult rat cardiomyocytes were treated with cell-permeant TAT-AKAP18δ-C or scrambled control peptides, in the presence and absence of isoproterenol. Ca^{2+} transient recordings revealed findings opposite to those observed for AKAP18δ-N, as TAT-AKAP18δ-C accelerated Ca^{2+} decline, both in the absence and

presence of isoproterenol (Figure 6B and 6C, representative tracings are shown in Figure S6C and S6D). This effect was most marked at low frequencies (1 and 4 Hz), as rising resting Ca^{2+} levels, CaMKIIδ activation, and increasing pThr17-PLN at higher pacing rates likely overpowered the effects of AKAP18δ-C observed at baseline. Consistent with the data above, TAT-AKAP18δ-C also increased the SERCA2 Ca^{2+} uptake rate in isolated mouse SR vesicles (Figure 6D).

In summary, these data support the notion that by activating CaMKIIδ, AKAP18δ-C increases pThr17-PLN, and thereby accelerates Ca^{2+} reuptake into the SR.

AKAP18δ Also Anchors and Functionally Regulates CaMKIIδ Activity at RYR

RYR has been identified together with PLN-SERCA2 in SR nanodomains.[28] We therefore analyzed whether AKAP18δ also regulates CaMKIIδ activity at RYR. Colocalization and immunoprecipitation of CaMKIIδ and RYR with AKAP18δ in adult rat cardiomyocytes was shown using proximity ligation assay (Figure 7A and 7B, yellow spots) and Western blotting (Figure 7C). In similarity to effects on pThr17-PLN (Figure 3A), AKAP18δ-N (55-98) also reduced CaMKIIδ phosphorylation of Ser2814-RYR using biotin-ahx-RYR (2797-2827) as substrate (Figure 7D). When introduced into adult cardiomyocytes, cell-permeant TAT-AKAP18δ-N (79-98) and TAT-AKAP18δ-N (55-74) reduced Ser2814-RYR phosphorylation (Figure 7E) and RYR functional activity after isoproterenol stimulation (Figure 7G), whereas TAT-AKAP18δ-C in the absence of isoproterenol exhibited opposite effects (Figure 7F and 7G). TAT-AKAP18δ-N (79-98) exhibited no effect on pSer2814-RYR at basal level (Figure S7B) or at pSer2808-RYR after isoproterenol stimulation (Figure S7C), consistent with Ser2814 being the main CaMKII phosphorylation site in RYR.[29] Finally, we tested the effect of the AKAP18δ-PLN competitor peptide on Ser2814-RYR phosphorylation. Contrary to pThr17-PLN (Figure 1C), no significant changes in the pSer2814-RYR level in cardiomyocytes treated with the AKAP18δ-PLN competitor peptide were observed (Figure 7H). This finding indicate that RYR and PLN-SERCA2 might not compete for the same AKAP18δ-CaMKIIδ pool.

Taken together, our data indicate that in addition to effects at PLN-SERCA2, AKAP18δ also anchors and regulates CaMKIIδ activity at RYR.

DISCUSSION

Here, we have provided mechanistic insight into the anchoring and regulation of CaMKIIδ activity by AKAP18δ at PLN-SERCA2 and RYR. We identified 2 unique regions in AKAP18δ that inversely regulate CaMKIIδ activity, CaMKIIδ-catalyzed phosphorylation of Thr17-PLN and Ser2814-RYR, and SERCA2 and RYR functional activities. We specifically showed that an inhibitory domain (AKAP18δ-N) also binds calcified CaM, while an activating domain (AKAP18δ-C) wedges CaMKIIδ open, trapping CaM within the kinase, and lowering the Ca^{2+} threshold for its activation.

Working Model to Explain AKAP18δ Effects on CaMKIIδ in Myocytes

CaMKII activity has been shown to be sensitive to the frequency of Ca^{2+} oscillations in vitro[10] and in intact adult cardiomyocytes.[27] Based on our data, we propose a working hypothesis whereby AKAP18δ fine-tunes this frequency-dependent activation of CaMKIIδ. At low stimulation frequency (Figure 8A), AKAP18δ-N (in red) binds the CaMKIIδ autoregulatory domain (pink), the ATP binding pocket, T- and S-site in the catalytic domain (green). This multipoint contact may stabilize the closed, inactive CaMKII conformation. Inhibition of the kinase persists. However, when the frequency of the Ca^{2+} transients increases (Figure 8B), accumulated calcified CaM (beige) may outcompete the inhibitory AKAP18δ-N-CaMKIIδ interaction by binding to the CaMKIIδ autoregulatory domain (pink) and AKAP18δ-N (red). These events lead to displacement of the S-site from AKAP18δ-N. CaM binding to AKAP18δ-N may alter its conformation, leading to its release also from the ATP-binding region and T-site (Figure 8B), augmented access of Thr17-PLN to the S-site, and greater CaMKIIδ potency. Concomitantly, AKAP18δ-C (in green) binds to the released T-site, keeping the inhibitory gate open as a wedge. This potentiates CaMKIIδ by lowering the Ca^{2+} threshold for its activation and by trapping CaM, leading to substrate phosphorylation (eg, Thr287 in the neighboring CaMKIIδ subunit, Thr17-PLN or Ser2814-RYR) during subsequent Ca^{2+} transients in a feed-forward manner: molecular memory of CaMKIIδ results. Thereafter, when the frequency of Ca^{2+} transients declines, CaM dissociates from CaMKIIδ and AKAP18δ-N, making the latter accessible to bind the CaMKIIδ autoregulatory domain, ATP binding region, and S- and T-sites, leading to re-inhibition of CaMKIIδ (Figure 8A). While plausible, it is unknown whether in the intact continuously beating heart the activating effect of AKAP18δ-C is dominant over the inhibitory stabilization caused by AKAP18δ-N.

In contrast to the CaMKIIδ autoregulatory domain, AKAP18δ-N does not contain the cluster of Phe293, Asn294, and Arg296, which is critical for CaM trapping by CaMKIIδ,[22] suggesting that AKAP18δ-N does not trap calcified CaM as the Ca^{2+} transient frequency declines. This may allow AKAP18δ-N to inhibit CaMKIIδ before complete dissociation of CaM from CaMKIIδ. In fact, since AKAP18δ-C and AKAP18δ-N (55-74) bind differently within the T-site region, there might not be any need for AKAP18δ-C to dissociate from CaMKIIδ

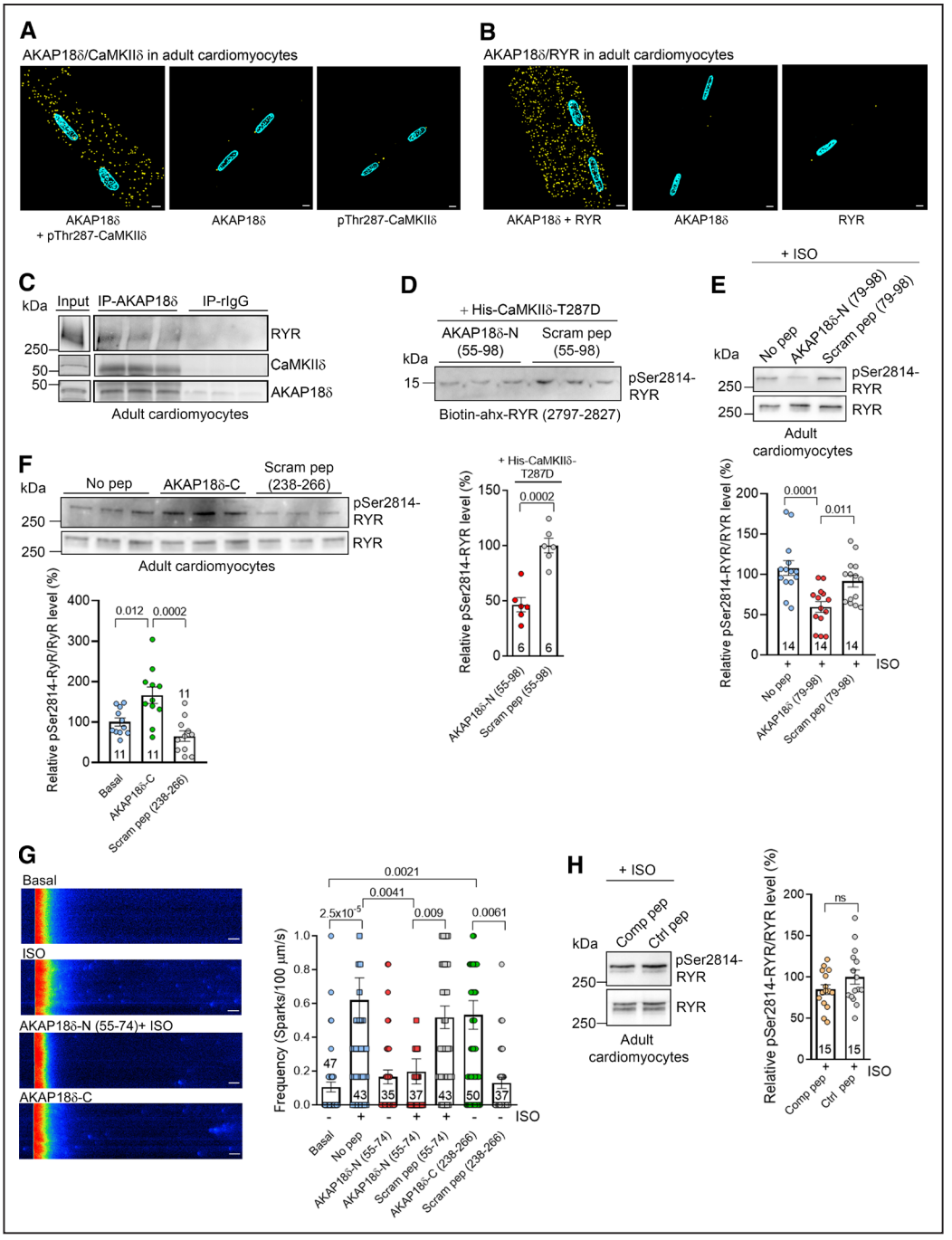

Figure 7. AKAP18δ (A-kinase anchoring protein 18δ) anchors and functionally regulates CaMKIIδ activity also at ryanodine receptor (RYR).

A, In situ proximity ligation assay of (**A**) AKAP18δ-pThr287-CaMKIIδ and (**B**) AKAP18δ-RYR (yellow dots in left panels) in adult cardiomyocytes (see Materials and Methods section for detailed description). Incubations with only anti-AKAP18δ, anti-pThr287-CaMKIIδ, or anti-RYR were used as negative controls (middle and right panels). Positive control for the assay is shown in Figure S7A. Scale bars=5 µm. **C**, Immunoprecipitation of AKAP18δ-CaMKIIδ-RYR in adult cardiomyocyte lysate detected by immunoblotting. **D**, CaMKIIδ phosphorylation of biotin-ahx-RYR (2797-2827), with or without presence of AKAP18δ-N (55-98) or the scrambled control. Biotin-ahx-pSer2814-RYR was detected by immunoblotting. Normal distribution was confirmed by Shapiro-Wilk test, and significant differences were examined by unpaired *t* test n=6. pSer2814-RYR and RYR levels in adult cardiomyocytes treated with (**E**) TAT-AKAP18δ-N (79-98) in presence of ISO or (**F**) AKAP18δ-C (238-266) in absence of ISO. The respective scrambled peptides were used as controls. Normal distribution was confirmed by Shapiro-Wilk test (in **E** and **F**). Significant differences examined by the linear mixed effect model from the R nlme package with Tukey post hoc correction (n=14, 5 rats in **E**, and n=11, 4 rats in **F**). **G**, Line scans and Ca^{2+} sparks of cardiomyocytes treated with or without ISO, TAT-AKAP18δ-N (55-74), AKAP18δ-C (238-266) or the respective scrambled control peptides. Significant differences examined by the linear mixed effect model from the R nlme package with Tukey post hoc correction (n=35–50, 5 rats). Scale bars=150 ms. **H**, Immunoblotting of pSer2814-RYR and RYR in ISO-stimulated cardiomyocytes pretreated with the cell-permeant AKAP18δ-PLN competitor or control peptide. Normal distribution was confirmed by Shapiro-Wilk test. Ns, not significant, examined by nested *t* test (n=12, 5 rats).

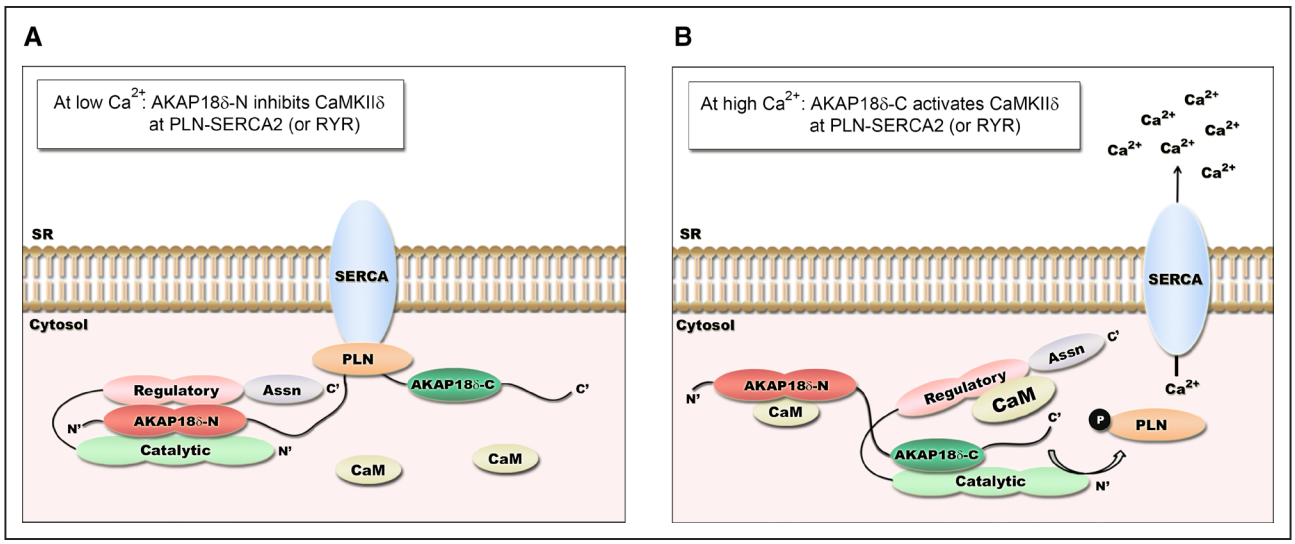

Figure 8. A model where the 2 unique regions in AKAP18δ (A-kinase anchoring protein 18δ) fine-tune CaMKIIδ activation at PLN (phospholamban)-sarcoplasmic reticulum (SR) Ca^{2+}-ATPase 2 (SERCA2 or ryanodine receptor [RYR]).
A, At low Ca^{2+} transient frequency, AKAP18δ-N (red) inhibits CaMKIIδ by binding to the regulatory domain (pink), and ATP-binding pocket, T- and S-sites in the catalytic domain (green). No Thr17-PLN (or Ser2814-RYR) phosphorylation results. **B**, When the Ca^{2+} transient frequency increases, accumulated calcified CaM (beige) outcompetes the inhibitory AKAP18δ-CaMKIIδ interaction by binding to the regulatory domain (pink) and AKAP18δ-N (red). AKAP18δ-C (dark green) binds to the released T-site in CaMKIIδ and lowers the Ca^{2+} threshold for kinase activation, by keeping the inhibitory gate open and trapping CaM within CaMKIIδ. CaMKIIδ catalyzes further Thr17-PLN (or Ser2814-RYR) phosphorylation, leading to reduced PLN association and faster SR Ca^{2+} reuptake by SERCA2 (or Ca^{2+} release by RYR). It is also possible that AKAP18δ-N and AKAP18δ-C regulate 2 different CaMKII molecules. Assn indicates association domain.

when the frequency declines. With this positioning, AKAP18δ-C might rapidly potentiate kinase activity during a subsequent rise in Ca^{2+} transient frequency since this could occur as soon as AKAP18δ-N dissociates, and independently of the Thr287 autophosphorylation state or other posttranslational modifications reported to promote autonomous activation of CaMKII.[30–33] In the presence of posttranslational modifications, AKAP18δ-C may increase CaMKIIδ activation to an even higher level, whereas AKAP18δ-N is expected to still be able to inhibit CaMKIIδ (illustrated in Figure 2I), even if its binding to the autoregulatory domain is abolished by oxidation (Met281/282)[30] or S-nitrosylation (Cys290).[33] The stoichiometry of the AKAP18δ-CaMKIIδ interaction is not known, but since AKAP18δ has been shown to oligomerize,[34] it is plausible that several AKAP18δ molecules anchor and regulate different CaMKIIδ monomers within the same CaMKII oligomer. Bioinformatics revealed that the AKAP18δ-binding regions are well conserved across CaMKII isoforms (Figure S8), suggesting that AKAP18δ may be able to anchor and regulate different CaMKII isoforms, and might provide an explanation for some Thr17-PLN phosphorylation observed in the CaMKIIδ knock out mouse model.[35]

Additional studies are needed to further interrogate the proposed AKAP18δ-CaMKIIδ-SERCA2-PLN/RYR model (Figure 8). Both PLN and RYR are thought to be important targets for CaMKIIδ effects during the force frequency relationship.[29,36–38] While the effects of CaMKII and PLN on frequency-dependent acceleration of relaxation have been more controversial,[39,44,45] recent works employing transgenic CaMKII inhibition in PLN-deficient mice have indicated that CaMKII-dependent regulation of PLN is critical to achieve frequency-dependent acceleration of relaxation, but that as yet unidentified CaMKII targets may also contribute.[38] Our data support an important role of CaMKII during frequency-dependent acceleration of relaxation, as we also observed that AKAP18δ-C-dependent CaMKII stimulation accelerated Ca^{2+} transient decline at low stimulation frequency (Figure 6B).

AKAP18δ Is a Novel CaMKII Anchoring Protein

To our knowledge, this is the first AKAP (A-kinase anchoring protein) reported to anchor a CaMKII isoform, defining AKAP18δ also as a CaM-KAP. From the crystal structures of fragments of AKAP18δ[46] and AKAP18γ,[42] it appears as though the 2 CaMKIIδ-binding sites are mostly accessible and do not overlap with the PLN[14] or PKA[13] binding sites. Thus, both CaMKIIδ and PKA may bind to AKAP18δ and phosphorylate PLN at the same time. AKAP18δ also coordinates phosphorylation of inhibitor-1,[47] which in turn inhibits protein phosphatase 1, the major phosphatase responsible for dephosphorylating PLN.[43,48,49]

It remains to be determined whether the opposing effects of the 2 AKAP18δ regions on CaMKIIδ activity are unique to AKAP18δ, or whether a similar arrangement exists in other proteins. CaMKIIα interacts with

2 sites in the N-methyl-D-aspartate receptor subunit NR2B.[17] One site (N2B-s) is similar to AKAP18δ-C, both at sequence level, as well as functionally, as it generates autonomous activation of CaMKIIα.[17] The second-binding site is reported to be CaMKIIα-Thr286 dependent.[17] Densin, located in neuronal postsynaptic densities, also inhibits CaMKII-mediated phosphorylation through T-site binding[18] but binds to the CaMKII association domain through a second site.[50–52] Furthermore, GTPase Rem2, a critical regulator of dendritic branching and homeostatic plasticity, binds also to the association domain, but inhibits CaMKII rather through the S-site.[20] Interestingly, Rem2 also interacts with CaM.[53,54]

Pathophysiological Relevance

Understanding the molecular mechanisms of CaMKII regulation is important in the context of various heart diseases and the development of new treatment strategies. Sustained CaMKII activation is linked to impaired cardiomyocyte Ca^{2+} homeostasis, cardiac dysfunction, and arrhythmias in diseases spanning atrial fibrillation, heart failure, and diabetic cardiomyopathy. Although CaMKII activity and RYR functional activity are often increased in these conditions,[4] decreased SERCA2 activity is often reported. During heart failure, reduction in SERCA2 activity is likely a result of both lowered protein expression[55] and hypophosphorylation of PLN following increased phosphatase activity.[56,57] Restoration of SR Ca^{2+} re-uptake through increasing PLN phosphorylation levels and/or SERCA2 activity is therefore considered to be a potential therapeutic strategy.[58,59] Future studies are needed to test whether the CaMKII activator peptide identified in this study (AKAP18δ-C) can be used to increase pThr17-PLN levels and thus SERCA2 activity in vivo. One strategy may be to target AKAP18δ-C to longitudinal SR, using a strategy similar to that described for the AIP_4-longitudinal SR transgenic mice.[5,60] To this end, the peptide sequences presently derived from the 2 AKAP18δ regions should be viewed as novel reagents that may help identify new CaMKII targets and approaches to therapeutically modify CaMKII activity and cardiomyocyte Ca^{2+} cycling.

ARTICLE INFORMATION

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Affiliations

Institute for Experimental Medical Research, Oslo University Hospital and University of Oslo, Norway (C.R.C., J.M.A., A.B.-D., M.L., P.K.L., H.J., P.W., T.R.S.K., G.C., I.S., X.S., W.E.L., O.M.S.). The KG Jebsen Cardiac Research Center, University of Oslo, Norway (A.B.-D., M.L., P.K.L., T.R.S.K., G.C., I.S., X.S., W.E.L., O.M.S.). Department of Molecular Medicine, Institute of Basic Medical Sciences, University of Oslo Norway (J.M.A.). Department of Pharmacology, Oslo University Hospital, Norway (J.M.A.). Max-Delbrück-Center for Molecular Medicine in the Helmholtz Association (MDC), Berlin, Germany (M.C.M., E.K.). Department of Pharmacology, University of California at Davis (L.P., D.M.B.). Department of Microbiology, Oslo University Hospital, Norway (B.D.). Department of Medical Biochemistry, Institute for Clinical Medicine, University of Oslo, Norway (B.D.). Institute of Experimental Cardiovascular Research, University Medical Center Hamburg-Eppendorf, Hamburg, Germany (H.S., V.N.). German Centre for Cardiovascular Research, Partner Site Hamburg/Kiel/Lübeck, Germany (H.S., S.H., O.J.M., V.N.). Department of Internal Medicine III, University of Kiel, Kiel, Germany (S.H., O.J.M.). German Centre for Cardiovascular Research (DZHK), Partner Site Berlin, Germany (E.K.).

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Disclosures

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Supplemental Material

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