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Cardiac Troponin T, a Sarcomeric AKAP, Tethers Protein Kinase A at the Myofilaments*

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Efficient and specific phosphorylation of PKA substrates, elicited in response to β-adrenergic stimulation, require spatially confined pools of PKA anchored in proximity of its substrates. PKA-dependent phosphorylation of cardiac sarcomeric proteins has been the subject of intense investigations. Yet, the identity, composition, and function of PKA complexes at the sarcomeres have remained elusive. Here we report the identification and characterization of a novel sarcomeric AKAP (A-kinase anchoring protein), cardiac troponin T (cTnT). Using yeast two-hybrid technology in screening two adult human heart cDNA libraries, we identified the regulatory subunit of PKA as interacting with human cTnT bait. Immunoprecipitation studies show that cTnT is a dual specificity AKAP, interacting with both PKA-regulatory subunits type I and II. The disruptor peptide Ht31, but not Ht31P (control), abolished cTnT/PKA-R association. Truncations and point mutations identified an amphipathic helix domain in cTnT as the PKA binding site. This was confirmed by a peptide SPOT assay in the presence of Ht31 or Ht31P (control). Gelsolin-dependent removal of thin filament proteins also reduced myofilament-bound PKA-type II. Using a cTn exchange procedure that substitutes the endogenous cTn complex with a recombinant cTn complex we show that PKA-type II is troponin-bound in the myofilament lattice. Displacement of PKA-cTnT complexes correlates with a significant decrease in myofibrillar PKA activity. Taken together, our data propose a novel role for cTnT as a dual-specificity sarcomeric AKAP.

Activation of β-adrenergic (β-AR)[2] receptors in the heart is a major mechanism responsible for enhancing myocardial contractility. β-AR receptor stimulation is quickly followed by a sharp rise in cyclic AMP levels evoking a plethora of physiological and biochemical reactions, such as positive chronotropic, inotropic or lusitropic effects (1, 2). The effector arm of cAMP signaling is PKA, which translates the extracellular stimuli into timely and specific responses.

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[2] The abbreviations used are: β-AR, β-adrenergic; cTnT, cardiac troponin T; PKA, protein kinase A; PKA-R, regulatory domain of PKA; AKAP, A-kinase anchoring protein; Y2H, yeast two-hybrid; IP, immunoprecipitation; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

PKA is a heterotetramer formed of two catalytic subunits kept in an inactive conformation by two regulatory subunits (either type I or II) (3). Another function of the regulatory (R) subunits is to facilitate tethering of the holoenzyme in the vicinity of relevant substrates thereby ensuring the fidelity of cAMP signaling (4, 5). cAMP binds cooperatively to two sites termed A and B, located at the interface between a regulatory and catalytic subunit, releasing the catalytic subunit, which become catalytically active.

Cardiomyocyte contraction-relaxation cycles are regulated by fluctuating intracellular Ca^{2+} levels (6, 7). Contraction is initiated by the opening of voltage-sensitive L-type Ca^{2+} channels (LTCC) as a result of sarcolemmal depolarization. This causes a small influx of Ca^{2+} that activates ryanodine receptors (RyR) promoting a massive release of Ca^{2+} from the sarcoplasmic reticulum (SR) stores into the cytosol (8). The rise of intracellular Ca^{2+} levels is sensed by troponin C (cTnC), which upon Ca^{2+} binding undergoes a conformational change inducing dissociation of troponin I (cTnI) from actin. This movement is transmitted through cTnT to tropomyosin (Tm) exposing myosin binding sites on actin and promoting contraction. Relaxation occurs when Ca^{2+} is pumped out of the cytosol by SERCA (sarcoplasmic reticulum calcium ATPase) or by Na^{+}/Ca^{2+} exchanger.

PKA regulates the strength and frequency of contraction through phosphorylation of key proteins, such as LTCC, RYR, phospholamban, cTnI, myosin binding protein C (MyBP-C), and titin (9). Considering the extent and diversity of PKA targets within the cardiomyocyte, a tightly regulated and spatially segregated activity of the cAMP/PKA pathways is critical for the specificity of the response to the inciting stimulus (10, 11). Both, speed and precision are achieved through: (i) targeting and pre-assembly of PKA signaling components in clusters, at discrete subcellular locations, through the help of protein kinase A anchoring proteins (12, 13); and (ii) generation of local gradients of cAMP through spatially confined, phosphodiesterases (14). Phosphodiesterases, by degrading cAMP, regulate the amplitude, duration, and compartmentation of intracellular cyclic nucleotide signaling (10). The ingeniously assembled macromolecular complexes, containing AKAP, PKA, and other factors, confer efficient and specific transduction of cAMP signal.

Growing data over the last decade have shed new light on the role of AKAP-PKA complexes bound to sarcoplasmic reticulum (regulating SERCA and RyR), *t*-tubules (regulating LTCC), and nuclear envelope (15). Despite the fact that cardiac myofilaments consist of >50% of the cellular volume (16)

TABLE 1
Oligonucleotide primers used for cloning, truncations, point mutations of hcTnT, and cloning of PKA-RI and -RII

Gene	Vector	Primer sequence 5′-3′	Restriction sites
hcTnT	pGBKT7	GAAGGAGATATCATATGTCTGACATAGAAGAGGTG	NdeI
		CTTTGTTAGCAGCCGGATCCCTATTTCCAGCGCCC	BamHI
hcTnT	pCMV6-AN-Myc	GTACCAGATGCGATCGCCATGTCTGACATAGAAGAG	SgfI
		CTGCAGCTCAAGCTTGATGGATCCCTATTTCCAGCGC	HindIII
hcTnT-(1–117)	pGBKT7	GAGGAGGACCTGCATATGTCTGACATAGAAGAGGTG	NdeI
		CTTTGAGAGAAACGAATTCCTACTCCTCTTTCTTCC	EcoRI
hcTnT-(1–180)	pGBKT7	GAGGAGGACCTGCATATGTCTGACATAGAAGAGGTG	NdeI
		ATGTAACCCCCAGAATTCATCTAGTTGGACAAAGCC	EcoRI
hcTnT-(118–228)	pGBKT7	ACAGGAAGAAACATATGGAGGAGCTCGTTTCTCTCA	NdeI
		CAGGTCGACGGATCCCTATTTCCAGCGC	BamHI
hcTnT-(181–228)	pGBKT7	AAGAAGGCTTTGTCCCATATGATGCATTTTGGGGGT	NdeI
		CAGGTCGACGGATCCCTATTTCCAGCGC	BamHI
hcTnT-(225–228)	pGBKT7	GTGCTGGCCATTGACCATATGAATGAAGATCAGCTG	NdeI
		CAGGTCGACGGATCCCTATTTCCAGCGC	BamHI
hcTnT-K210-del	pGBKT7	GGGAAAAGAAGAAGATTCTGGCTGAGAGG	NdeI
		CCTCTCAGCCAGAATCTTCTTCTTTTCCC	BamHI
hcTnT-V218P	pGBKT7	GCTGAGAGGAGGAAGCCACTGGCCATTGACCAC	NdeI
		GTGGTCAATGGCCAGTGGCTTCCTCCTCTCAGC	BamHI
hcTnT-R216I	pGBKT7	ATTCTGGCTGAGAGGATCAAGGTGCTGGCCATT	NdeI
		AATGGCCAGCACCTTGATCCTCTCAGCCAGAAT	BamHI
hcTnT-A220N	pGBKT7	GAGGAGGAAGGTGCTGAACATTGACCACCTGAATG	NdeI
		CATTCAGGTGGTCAATGTTCAGCACCTTCCTCCTC	BamHI
hPKA-RI	pGADT7	CGTCCCCAGAGACATATGGAGTCTGGCAGTACCGCC	NdeI
		AGGCACAGGAGGGGATCCTCAGACAGACAGTGACAC	BamHI
hPKA-RI	pCMV6-AN-HA	GTACCAGATGCGATCGCCATGGAGTCTGGCAGTACCG	SgfI
		TCTGCAGCTCAAGCTTGATGGATCCTCAGACAGACAGT	HindIII
hPKA-RI	pCMV6-AC-HA	CATACGACGTACAAGCTTACGCTCATATGGAGTC	HindIII
		GCTCGAGCTCGATGGACTCGAGGACAGACAGTG	XhoI
hPKA-RII	pGADT7	CCTTCGGCCGCCGCCCATATGAGCCACATCCAGATC	NdeI
		CTCTGGGGTGTGGGATCCCTACTGCCCGAGGTTGCC	BamHI
hPKA-RII	pCMV6-AN-HA	GTACCAGATGCGATCGCCATGAGCCACATCCAG	SgfI
		GCAGCTCGAGCTCGAGGGATCCCTACTGCCCGAG	XhoI
hPKA-RII	pCMV6-AC-HA	TACCAGATTACGCGTATATGAGCCACATCCAGAT	MluI
		GCTCGATGGACTCGAGCTGCCCGAGGTTGCCCAG	XhoI

and that PKA-dependent phosphorylation of sarcomeric proteins cTnI, MyBP-C, and titin appear to be dominant in control of myofilament function by β-AR stimulation (9), very little is known about the identity, composition, or function of AKAP-PKA complexes at the sarcomeres. Here we report the identification and characterization of a novel AKAP, cardiac TnT, which docks PKA at the thin filaments in proximity of its main sarcomeric substrates.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening of Human Heart cDNA Libraries—Human heart cDNA libraries (catalog numbers 638865 and HL4042AH) were from Clontech. For the library 1 screen, MATα yeast strain Y187, pretransformed with the cDNA library in pGADT7 vector, was mated with MATa yeast strain AH109 containing human cTnT subcloned into pGBKT7 vector, at the NdeI and BamHI restriction endonuclease sites (Table 1). For the library 2 screen, AH109 yeast cells were co-transformed with the pACT2-cDNA library and pGBKT7-cTnT using the Yeastmaker Yeast transformation System 2 (Clontech) according to the manufacturer's instructions. Resulting diploid colonies from both screens were evaluated for positive interactions on quadruple selective medium (-Ade, -His, -Leu, -Trp). Positive colonies were subjected to three rounds of additional selection on quadruple selective medium plates. Only yeast cells carrying both plasmids grew on selective medium plates. Protein-protein interactions were further confirmed by both α- and β-galactosidase colorimetric assays. Once a positive colony was identified, the plasmid carrying the library clone was isolated from yeast, transformed in *Escherichia coli* strain XL10-Gold (Stratagene) for amplification, and subjected to DNA sequencing. The confirmed cDNA clones were identified from human genomic databases using the BLAST search engine. Positive hits were re-tested a minimum of 3 times in separate experiments by co-transforming with pGBKT7-cTnT in AH109 yeast cells, plated on quadruple selective medium, and verified for blue color development by α- and β-gal colorimetric assays.

α- and β-Gal Colorimetric Assays—For the α-gal assays, yeast transformants were spread on quadruple selective plates containing α-X-gal (40 μg/ml, Sigma), incubated at 30 °C and then analyzed for color development. For the β-gal (or colony-lift) assays, freshly transformed yeast cells were replica-plated to filter papers, immersed into liquid nitrogen to lyse the cells, placed on filters pre-soaked in 49 mM β-X-gal solution, and incubated at 30 °C for up to 2 h. Colonies that turned blue indicated positive protein-protein interactions.

Co-expression and Immunoprecipitation of cTnT/PKA-R from HEK293 Cells— cTnT was subcloned into pCMV6-AN-MYC vector (N-terminal Myc tag). PKA-RI and -RII were subcloned into both pCMV6-AN-HA (N-terminal HA tag) and pCMV6-AC-HA (C-terminal HA tag) mammalian expression vectors from Origene (see Table 1). Both amino-terminal and carboxyl-terminal tags were used for PKA to rule out the role of the free carboxyl terminus. PKA constructs were individually co-transfected with the cTnT construct into HEK293 cells using the MBS mammalian transfection kit (Stratagene), following the manufacturer's instructions. To determine specificity of the PKA-TnT inter-

action some HEK293 cells were incubated with 30 μM membrane-permeable PKA/AKAP disruptor peptide stearate-Ht31 (St-Ht31) or St-Ht31P (inactive peptide, control) (Promega). 48 h after transfection cells were lysed in an ice-cold modified RIPA buffer. To reduce nonspecific protein binding, the cell lysate was first incubated with protein A/G-agarose beads (Santa Cruz) for 4 h at 4 °C. After removal of the protein A/G beads by centrifugation (1,000 × *g*), the cleared supernatant was incubated with 4 μg of anti-Myc antibody (clone 9E10, Santa Cruz Biotechnology) or anti-HA antibody (clone F-7, Santa Cruz Biotechnology). Immunocomplexes were allowed to form overnight on a rotating platform at 4 °C, and then precipitated following incubation with protein A/G-agarose for 1–2 h. Pelleted beads were washed (5 times) with TBST, resolved by SDS-PAGE, and analyzed by immunoblotting. Anti-PKA antibodies were from BD Transduction Labs, and anti-cTnT antibodies were either from Sigma (clone JLT-12) or the University of Iowa Hybridoma Bank (clone CT3).

In Vitro Translation and Immunoprecipitation—Human cTnT and PKA-regulatory subunits (RI and RII) interactions were confirmed by immunoprecipitation (IP). Epitope-tagged myc-cTnT and HA-PKA-R proteins were expressed individually *in vitro* using a rabbit reticulocyte lysate system in the presence of a modified lysine tRNA labeled with the fluorophore BODIPY®-FL (Promega). After expression, the two protein mixtures (*i.e.* cTnT and PKA-R) were mixed and interaction was determined by IP with anti-Myc and anti-HA antibodies (Santa Cruz Biotechnology), as detailed above. Using this system, fluorescently labeled lysine residues were incorporated into nascent proteins during translation and detected in-gel after SDS-PAGE on a Typhoon 9410 molecular imager. Specificity of cTnT-PKA-R binding was assessed in the presence of 20 μM Ht31 or Ht31P (inactive peptide).

Gluathione S-Transferase (GST)-cTnT Expression and Purification— cTnT was subcloned into the GST vector pGEX 5X-1 (GE Healthcare) to generate the construct GST-cTnT (see Table 1, primer), which was sequence verified. GST-cTnT protein expression in *E. coli* and purification were performed as described (17).

GST Pulldown Assays—Heart lysates were prepared from freshly excised hearts of 6-month-old Sprague-Dawley rats from Harlan. Hearts were quickly removed under deep anesthesia (sodium pentobarbital; 100 mg/kg IP) and rinsed free of blood in ice-cold saline (0.9% NaCl) or Tyrodes buffer. Left ventricular tissue was minced into small pieces and homogenized in a Dounce homogenizer in ice-cold homogenization buffer consisting of 20 mM HEPES, pH 7.4, 150 mM NaCl, 15% glycerol, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM Na_3VO_4, 10 mM sodium pyrophosphate, 50 mM NaF, 1% Triton X-100, 1% sodium deoxycholate, 1 mM DTT, 0.1% SDS, 50 μg/ml of leupeptin, 50 μg/ml of aprotinin, 50 μg/ml of pepstatin A, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride. Lysates were cleared by centrifugation prior to use. For GST pulldown assays, 10 μg of GST-cTnT protein was added to 200 μg of cell lysate (pre-cleared with 50% glutathione-agarose slurry for 1 h) and incubated overnight at 4 °C. Then 50 μl of 50% glutathione-agarose slurry (GE Healthcare) was added and incubated for 1–2 h at 4 °C. Glutathione-agarose containing bound GST-cTnT complexes were collected by centrifugation and washed five times with ice-cold TBST buffer. Specificity of interaction was assessed in the presence of 20 μM Ht31 or Ht31P. The resulting pellets were resolved by SDS-PAGE and analyzed by immunoblotting. Anti-GST antibody used in control experiments was from Santa Cruz Biotechnology.

Immunoprecipitation of Endogenous cTnT-PKA-R Complexes from Rat Hearts—Heart lysates were prepared as above. The IP protocol was essentially as described above (HEK293 cells).

Mapping PKA Binding Region— cTnT truncations or mutations (see Table 1, oligonucleotide) were generated by PCR and cloned into pGBKT7 vector. pGBKT7-cTnTs (WT or truncation/mutation) were co-transformed with pGADT7-hcTnI (positive control), pGADT7-PKA-RI, or pGADT7-PKA-RII followed by selective medium plating and α-gal and β-gal assays. To determine specificity of PKA-TnT interaction, co-transformed yeast cells were incubated with 50 μM membrane permeable stearate-Ht31 (St-Ht31) or St-Ht31P (inactive peptide, control) (Promega).

PKA-RI and -RII Bacterial Expression and Purification— cDNA for PKA-RI (BC036285) and -RII (BC002763) was subcloned into the pGADT7 dual-purpose vector (see Table 1, primer) and transformed into the *E. coli* BL21(DE3) strain for protein expression. PKA-RI and -RII (regulatory subunits) were purified by affinity chromatography on a resin prepared by cross-linking 8-AEA-cAMP (BioLog) to *N*-hydroxysuccimide (NHS)-activated Sepharose (GE Healthcare), according to a modified procedure from Ref. 18. Purified PKA proteins were used for the peptide SPOTS assays (see below) at concentrations of 10–20 μg/ml.

SPOTS Blot Solid Phase Assay—139 overlapping 13-mer peptides frame shifted by two residues, representing the entire cTnT sequence, were synthesized on a membrane by SPOT technology (Jerini Peptide Technology). Binding of PKA-RI and -RII to cTnT spots was determined as recommended by the manufacturer. Briefly, the membrane was rehydrated in methanol, washed three times for 10 min with TBS, and blocked for 2 h. PKA-R (10–20 μg/ml in TBS) was incubated with the blocked membrane overnight at 4 °C. Unbound protein was removed by three washes with TBS-T. Spot-bound PKA reacted with anti-PKA-RI or -RII antibodies (BD Transduction Labs) and was detected using the ECL kit (GE Healthcare).

Gelsolin Treatment of Isolated Myofibrils—Purified bovine gelsolin (Sigma) was solubilized in ice-cold Reaction Buffer (20 mM MOPS, pH 7.3, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT) containing a mixture of protease inhibitors (1 mM leupeptin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 mM E-64, 2 μg/ml of pepstatin A, 2 μg/ml of aprotinin). Soluble gelsolin fraction was dialyzed overnight at 4 °C in reaction buffer supplemented with 2 mM leupeptin and used immediately to treat isolated myofibrils (19). Myofibrils were washed three times at 4 °C with fresh reaction buffer containing a mixture of protease inhibitors (see above) supplemented with 4 mM ATP and 0.2 mM $CaCl_2$. Washed myofibrils were incubated ± gelsolin at 25 °C for 5 h with gentle shaking in a Vor-

Temp 56^{TM} Shaking Incubator (Alpco Diagnostics) to maintain myofibril suspension. Following centrifugation (10 min, 14,000 × *g*, 4 °C) the pellet and supernatant were carefully separated. Samples were then resolved on 8–16% SDS-PAGE gels (Bio-Rad) and either stained with Sypro Ruby as previously reported (20) or transferred to a PVDF membrane for immunoblotting.

Cardiac Troponin Expression, Purification, and Complex Reconstitution—Recombinant cTnT, cTnI, and cTnC were expressed in *E. coli* and purified as previously described (20) (21). Recombinant heterotrimeric cTn complex was reconstituted by mixing equimolar amounts of cTnT, cTnI, and cTnC in a solubilization buffer containing 6 M urea and 1 M KCl as previously reported (20, 21).

Troponin Exchange Experiments—Fiber bundles were prepared from hearts of 3–4-month-old C57BL6 mice purchased from Harlan or Charles Rivers laboratories. Hearts were quickly removed under deep anesthesia (sodium pentobarbital; 100 mg/kg IP) and rinsed free of blood in ice-cold saline (0.9% NaCl) or Tyrodes buffer. Muscle strips (200–300-μm wide and 3–4-mm long) were dissected from left ventricular papillary muscle. Fiber bundles were then detergent treated at 4 °C, in a relax buffer (20). Following detergent extraction the fiber bundles were transferred to a bath containing a recombinant cTn complex (20–30 μM) in exchange buffer (20 mM MOPS, pH 6.5, 190 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 1 mM DTT) and incubated overnight at 4 °C. The extent of native cTn substitution by recombinant cTn was determined by immunoblot analysis of control *versus* exchanged fiber bundles, as previously described (20, 21).

PKA Activity Assay—Permeabilized papillary muscle strips (native or exchanged) were resuspended in ice-cold PKA assay buffer containing 50 mM MOPS, pH 7.0, 25 mM β-glycerophosphate, 1 mM EGTA, 10 mM $MgCl_2$, 0.25 mg/ml of BSA, 20 μM cAMP, 1 mM DTT, and a mixture of protease and phosphatase inhibitors. The mixtures were briefly sonicated in a chilled water bath and centrifuged at 16,000 × *g* at 4 °C to remove all myofilaments. The clarified supernatant containing active PKA (catalytic domain) was then supplemented with 0.25 mM Kemptide (Sigma) and 0.25 mM [γ-^{32}P]ATP, in the presence or absence of 15 μM PKA inhibitor fragment 6-22 amide (PKI, Sigma). Reactions were incubated for 15–30 min at 30 °C and stopped by the addition of 75 mM phosphoric acid. PKA activity was determined by measuring the initial rate of radiolabeled phosphate incorporation in the Kemptide substrate (filter paper assay, modified from Ref. 21).

Statistical Analysis—All values are presented as mean ± S.E. Differences among means were considered significant at $p < 0.05$. Statistical evaluation was by Student's *t* test (KaleidaGraph, Synergy Software).

Animal Care—All animals were handled in accordance with the guidelines of the University of Kentucky Animal Care Committee.

RESULTS

Yeast Two-hybrid Screens Show Human Cardiac PKA Interacts with Cardiac TnT—To identify signaling proteins that bind and post-translationally modify human cardiac troponin, we used a yeast two-hybrid approach to screen two separate adult human heart cDNA libraries (Clontech) with human cardiac TnT as "bait." The yeast two-hybrid system (Y2H) is a sensitive eukaryotic assay widely and successfully used to detect novel interactions between proteins (22). The Y2H is also suited to map the specific interacting domains of two proteins known to form a complex in which the strength of the interaction can be correlated with the level of expression of an appropriate reporter gene (23). Y2H screens of more than 4 × 10^6 cDNA clones identified over 1000 colonies that grew on high-stringency plates. 375 colonies turned blue in the presence of α-gal, indicating positive interactions with cTnT (Fig. 1). DNA sequencing led to the identification of 19 gene products (see Table 2), including multiple hits for known binding partners of cTnT, cTnI, and Tm.

Among the positive clones interacting with cTnT, we identified cAMP-dependent protein kinase A regulatory subunit type I (Table 2). A characteristic feature of PKA regulatory subunits is the presence of docking domains that tether the holoenzyme in the vicinity of substrates through interaction with an amphipathic helix of an anchoring protein (24). In general, PKA-R subunits interact with small aliphatic (Ala, Val, Leu, and Ile) and basic amino acids (Arg, Lys) that decorate one side of the AKAP helix (25, 26). *In silico* analysis of the cTnT amino acid sequence using the ClustalW multiple alignment algorithm (part of the MacVector 11 sequence analysis suite) identified a fragment of an amphipathic α-helix (spanning residues 212–224) as a putative PKA-R binding site (Fig. 2).

Human cTnT Interacts Directly with PKA-R—To establish whether the association between cTnT and PKA-R occurs through direct interaction or is mediated through another protein, we assessed the ability of recombinant human cTnT to bind recombinant human PKA-RI and PKA-RII *in vitro*. Using a previously validated cell-free expression method, epitope-tagged Myc-cTnT and HA-PKA-RI or HA-PKA-RII were expressed *in vitro* using a rabbit reticulocyte lysate system in the presence of a modified lysine tRNA labeled with the fluorophore BODIPY®-FL. As a result, fluorescently labeled lysines (accounting for 11.4% of cTnT amino acids, 5.8% of PKA-RI and 5.5% of PKA-RII) were incorporated into the nascent proteins during translation, allowing for convenient "in-gel" detection. Newly expressed, fluorescent Myc-cTnT was incubated with fluorescent HA-PKA-RI or -RII and the resulting complexes were immunoprecipitated using anti-Myc or anti-HA antibodies. Fig. 3 shows data from a representative experiment, confirming the Y2H results that cTnT and PKA-R interact directly with each other. Alignment of cTnT-(212–230) and Ht31 (PKA/AKAP disruptor peptide) shows high homology between the two regions (Fig. 2*B*), signifying that the Ht31 peptide might be an efficient cTnT-PKA-R disruptor. Indeed, the addition of Ht31 peptide (Promega) completely abolished both cTnT-RI and cTnT-RII interactions. On the other hand, the inactive peptide Ht31P had no effect on the cTnT-PKA-R interaction (Fig. 3).

Human cTnT Associates with PKA-R in a Cellular Environment—To ascertain whether human cTnT can interact with human PKA-R in a cellular environment, we co-ex-

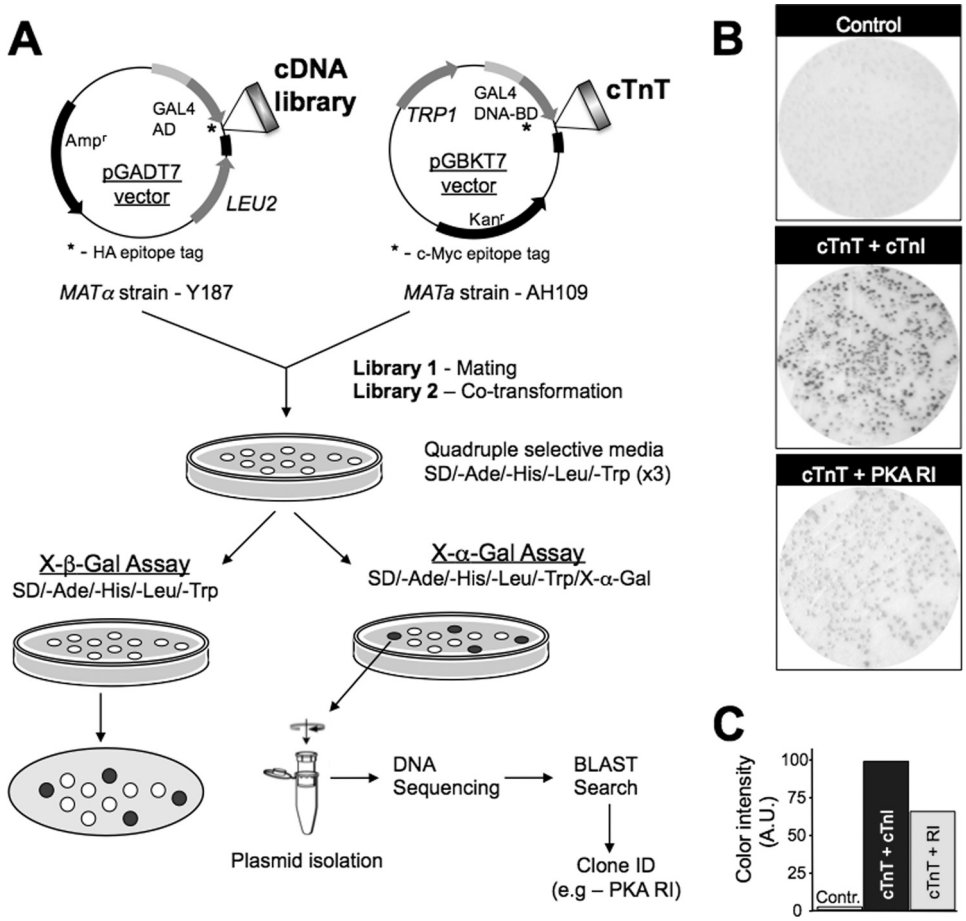

FIGURE 1. **Yeast two-hybrid library screens identify cTnT-PKA-R novel interaction.** *A*, schematic representation of the main steps of the two yeast two-hybrid assays including high stringency screening on quadruple selective medium. *B*, β-galactosidase assay shows the interaction between cTnT and Laminin C (negative control, *top panel*), cTnI (positive control, *middle panel*), and PKA-RI (*bottom panel*) as monitored by *shades of blue*. The *intense blue color* developed by yeast cells harboring cTnT-cTnI indicates a stronger interaction between these two proteins compared with cTnT-PKA RI (*lighter blue*). *C*, quantitative analysis of the interaction was determined using Image J software and normalized to that of control. Data shown are from a single experiment and are representative of 4 separate experiments.

pressed Myc-cTnT and HA-PKA-RI or -RII in the heterologous cell line HEK293 (Fig. 4). Immunoprecipitation with anti-Myc and anti-HA antibodies revealed that human cTnT specifically interacts with both PKA-RII and -RI (data not shown). The addition of membrane-permeable St-Ht31 peptide disrupts both cTnT-RI and cTnT-RII interactions. Collectively, these data strongly suggest that cTnT and PKA-R form stable complexes in a cellular environment.

Characterization of PKA Binding Site on cTnT—To investigate the identity of the PKA binding site on cTnT, Y2H assays were set up with human cTnT as bait and human PKA regulatory subunits RI or RII as "prey" (Fig. 5). Various cTnT truncations were generated to the left or right of the putative PKA binding site shown in Fig. 2. These fragments of cTnT (Fig. 5*A*) were previously confirmed as good functional representations of that particular fragment of the protein (27). Interactions between cTnT constructs and PKA-RI, or -RII, were assessed by two different colorimetric assays, using either α-gal (on selective medium plates) or β-gal (in a colony lift assay) (see Fig. 1, *B* and *C*). Our data from β-gal assays are summarized in Fig. 5, *B* and *D*. Identical results were obtained with the α-gal assays (data not shown). Truncation analysis of cTnT demonstrate that the region spanning amino acids 212–224 is required for PKA binding. St-Ht31 peptide, but not St-Ht31P, obstructs the cTnT-PKA-R interaction (Fig. 5*D*).

To verify that the cTnT fragment spanning residues 212 and 224 is essential to PKA-R binding we designed point mutations to either disrupt the amphipathic helix (V218P) or remove an essential hydrophobic residue (A220N) conserved in all AKAPs (Fig. 5, *C* and *D*). Our data demonstrate that both mutants, V218P and A220N, successfully abolished cTnT-PKA-R association. Interestingly, a mutation that replaced Arg^{216} with an Ile (R216I) residue, found in other AKAPs at that position, seemed to be a gain-of-function mutant as it significantly enhanced interaction of cTnT with both PKA-Rs.

In addition to the loss- or gain-of-function mutants, we also investigated the effect of a cardiomyopathy-causing deletion in cTnT at residue 210 (in the vicinity of PKA binding domain). Data from our recent study (19) indicates that deletion of Lys^{210} (ΔK210) affects PKA-dependent phosphorylation of MyBP-C and cTnI (decreased by over 40% in knock-in ΔK210 mouse hearts). Consistent with our previous findings, ΔK210 induces a ~65% decrease of PKA binding to cTnT (Fig. 5, *C* and *D*).

TABLE 2

Summary of human heart cDNA prey clones interacting with human cTnT bait

Y2H screens of more than 4×10^6 cDNA clones identified over 1000 colonies that grew on high-stringency plates, of which 375 turned blue in the presence of α-galactosidase, indicating positive interactions with hcTnT. DNA sequencing led to the identification of 19 gene products, including multiple hits for cTnI and tropomyosin, known binding partners of cTnT.

Clone ID	Accession	Gene	Library
Sarcomeric			
Multiple	NM_000363	Troponin I, type 3 (TNNI3)	1; 2
Multiple	NM_001018005	Tropomyosin 1 α (TPM1)	2
63	EU106177	Tropomyosin β (TPM2b)	2
42	BC015821	Myosin light chain 2, regulatory, slow (MYL2)	1
43	NM_0011011515	PDZ and LIM domain 5 (PDLIM5)	1
1	NM_001824	Creatine kinase, muscle (CKM)	1
Sarcoplasmic reticulum			
142	NM_002667	Phospholamban (PLN)	2
Nuclear			
38	BC005893	Atrial natriuretic factor, (ANF)	1
144	NM_012068	Activating transcription factor 5 (ATF5)	2
22, 26	NM_003074	SWI/SNF rel. matrix assoc. actin reg. of chromatin C1 (SMARCC1)	2
76, 92	BC000898	Myeloid leukemia factor 2 (MLF2)	2
88	AK223404	Death-associated protein 6 (DAXX)	2
165	AF151852	UTP11-like ribonucleoprotein (UTP11L)	1
Cytoskeleton			
106	NM_006289	Talin 1 (TLN1)	2
194	BC111836	Dystrophin (DMD)	1
Membrane/cytoplasm			
6,7	AF036536	Mitofusin 2 (MFN2)	1
58	NM_002734	Protein kinase type I-α regulatory sub., (PRKAR1A)	1
Unknown			
123	AC114489	Chromosome 1 clone RP11-114B7	1
143	BC007531	Chimeric clone	2

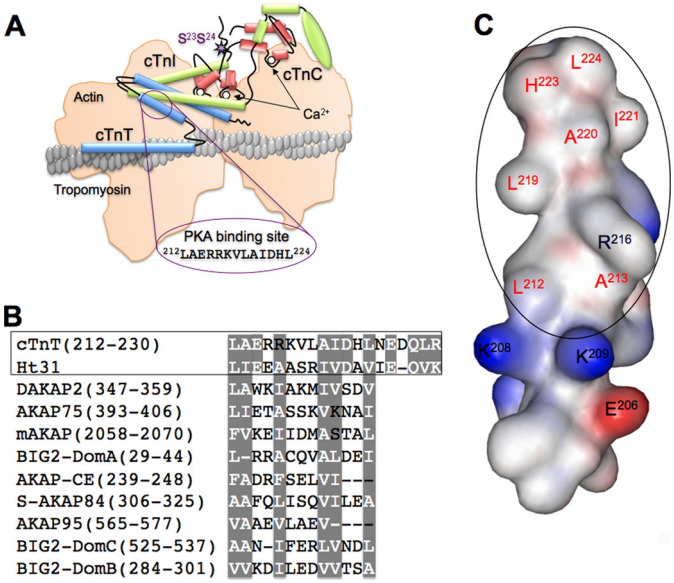

FIGURE 2. **Cardiac TnT contains a highly conserved PKA docking site.** *A*, schematic illustration shows the location of the PKA binding site and cardiac-specific cTnI-Ser^{23}-Ser^{24} phosphorylation sites (*star*). Drawing of the troponin complex is based on the crystal structure of the troponin core domain (50). N-terminal region of cTnT (residues 1–204) was not solved in the crystal structure. *Rectangles* represent helical structures. cTnC is colored *red*, cTnI is *green*, and cTnT is *blue*. *B*, ClustalW multiple sequence alignment of cTnT with nine other AKAPs. Conserved residues responsible for tethering PKA are shown in *white*. The high homology between cTnT and Ht31 is also shown (*boxed*). *C*, surface representation of cTnT (PDB 1J1D) helix 203–224 shows the position of hydrophobic residues (*red*) involved in PKA docking.

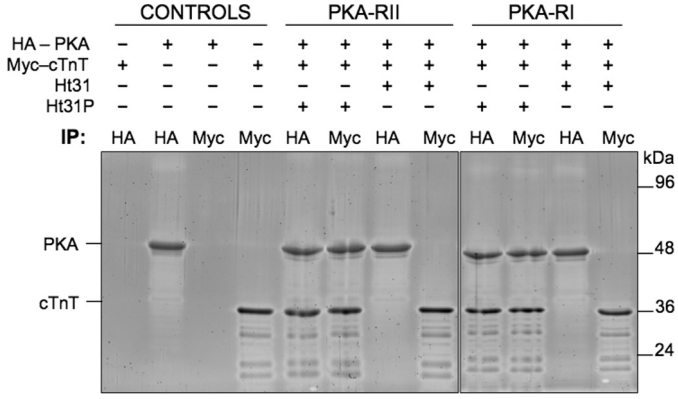

FIGURE 3. **Human cTnT interacts directly with both PKA-RI and PKA-RII.** Interaction between epitope-tagged Myc-cTnT and HA-PKA-RI or HA-PKA-RII was determined by IP. Fluorescently labeled, recombinant proteins were expressed in a cell-free system (Promega). Proteins were mixed and the interaction was verified by IP using anti-c-Myc and anti-HA antibodies. Content of each lane is shown on *top*. 20 μM Ht31 or Ht31P (control) peptides were used as shown to assess the specificity of the cTnT-PKA interactions. Similar data were obtained in 3 separate experiments. Molecular mass markers are shown on the *left*.

Solid Phase Peptide Scan Binding Assays Corroborate PKA Binding Site on cTnT—PKA binding residues were confirmed using peptide SPOTS blot technology (Jerini Peptide Technology). The SPOTS blot displays the 288-amino acid sequence of cTnT as a set of 139 overlapping 13-mer peptides frameshifted by two residues (Fig. 6). Spot-bound PKA reacted with an anti-PKA-R antibody (BD Biosciences) and was detected using the ECL kit (GE Healthcare). Our results indicate that the PKA-RII interaction was strongest with cTnT peptides (numbers 104–108) spanning the proposed PKA binding region (*i.e.* amino acids 212–224). Other, weaker interactions were due to nonspecific binding (Fig. 6, *top panel*). The inhibitory peptide Ht31 selectively prevented PKA-cTnT peptide interaction (numbers 104–108) (Fig. 6, *bottom panel*).

Endogenously Expressed PKA-R Interacts Directly and Specifically with cTnT—To determine whether endogenously expressed, rat heart PKA-R specifically interacts with cTnT,

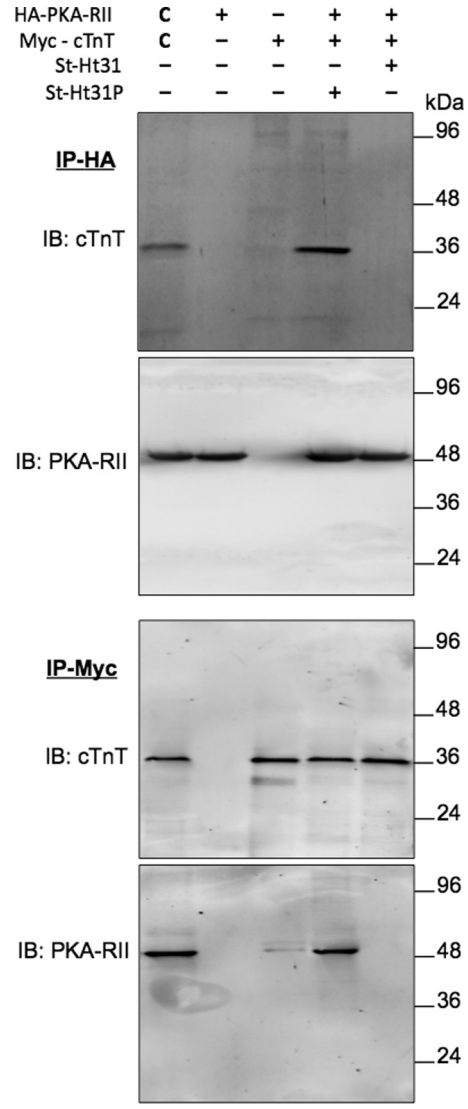

FIGURE 4. **Characterization of cTnT-PKA interaction in a cellular environment.** Co-expression of cTnT and PKA-RII in HEK293 cells, in the presence or absence of membrane-permeable St-Ht31 or St-Ht31P, was followed by IP with agarose-conjugated anti-Myc or anti-HA antibodies. Co-precipitation was detected with anti-cTnT antibody and anti-PKA-RII antibody. Molecular mass markers are shown on the *left*. Data shown are from a single experiment and are representative of 4 separate experiments. *IB,* immunoblot.

we used a GST-cTnT pulldown assay. We monitored the ability of purified GST-tagged cTnT to associate with endogenous cardiac PKA-RI and -RII from adult rat heart lysates (Fig. 7*A*). PKA types I and II are localized, in cardiomyocytes, in a variety of functionally and spatially distinct compartments such as nuclear envelope, sarcolemma, SR, cytoplasm, mitochondria, sarcomere, etc. (28, 29). Heart lysates contain PKA-R released from all these compartments (especially from membranous domains disrupted by detergents present in lysis buffer). A recent report indicates that homogenization of rodent hearts leads to augmentation of "free" PKA-R (non-AKAP bound), likely due to mechanical/chemical disruption (11). Therefore, rat heart lysates contain enough unbound PKA-R to associate with GST-cTnT (as shown in Fig. 7*A*). Our results indicate that endogenously expressed rat heart, PKA-RI and -RII interact with GST-cTnT. The specificity of the interaction was tested in the presence of Ht31 (ablates cTnT-PKA-R binding) and Ht31P (no effect), as reported in Fig. 7*A*.

Native PKA-R and cTnT Form Stable Complexes in the Rat Heart—To determine whether natively expressed PKA-R and cTnT form stable complexes, we performed co-immunoprecipitation assays using rat heart homogenates. As shown in Fig. 7*B*, native cTnT-PKA-RI and -RII complexes were immunoprecipitated with an anti-cTnT antibody, but not by a control IgG. Ht31 peptide, but not HT31P, prevented cTnT-PKA interaction. These data strongly suggest that native cTnT and PKA-R form stable complexes in the heart.

PKA-R Have Differential Affinities for Detergent-extracted Cardiac Myofibrils and Papillary Muscle Strips—To elucidate whether PKA-R tether specifically to the cardiac myofilaments we used isolated myofibrils and papillary muscle strips that were treated with Triton X-100 to remove all membranes and membrane-associated proteins. Interestingly, immunoblot analysis revealed PKA-RII, but not PKA-RI, associated with myofilaments from both preparations. Fig. 8 indicates a robust reactivity of myofilaments with anti-PKA-catalytic and anti-PKA-RII antibodies, but not anti-PKA-RI. This is not entirely unexpected because dual AKAPs tend to bind PKA-RII with higher affinity than PKA-RI (30). It is likely that myofilament-bound PKA-RI have been removed by detergent treatment. Similar disruption of PKA-RI anchoring was reported in a recent study (11).

PKA Type II Is Associated with Cardiac Thin Filaments—To investigate whether endogenous cardiac PKA is associated with thin filaments (*i.e.* cTnT) we have used a gelsolin assay. Gelsolin is a Ca^{2+}-dependent actin filament-severing molecule (31). It selectively removes cardiac thin filaments leaving behind relatively intact thick, intermediate and titin filaments, anchored at the Z lines (32–34). Fig. 9 shows data from a representative experiment. Incubation of myofibrils with gelsolin leads to selective removal of actin filaments (thin filaments) as indicated by extraction of actin, Tm, and troponins from the pellet fraction and their enrichment in the supernatant fraction (Fig. 9*A*). Immunoblot analyses of gelsolin-treated myofilaments indicate a ~75% decrease in endogenous PKA-cat and PKA-RII protein concentrations. PKA extraction is proportional with the reduction of cTnT (Fig. 9, *B* and *C*).

PKA Type II Is Bound to Cardiac Troponin in the Myofibrillar Milieu—To show that PKA holoenzyme (RII + cat) is part of the same complex with cTnT, in the myofibrillar milieu, we used a cTn exchange method to replace endogenous cTn and therefore displace any PKA bound to cTnT. The schematic of the troponin exchange maneuver is shown in Fig. 10*A*. Immunoblot analysis of control (*Cont*) and exchanged (*Exch*) papillary muscle fibers show that ~72% of the endogenous cTn (*Endo*) was replaced by recombinant cTn (*Rec*) (Fig. 10, *B* and *C*). Notice that recombinant cTnT migrates with a different mobility (*i.e.* slower) on SDS-PAGE due to the presence of a Myc tag (used to differentiate between endogenous and recombinant cTnT). The cTn exchange procedure led to a considerable decrease in PKA bound to the myofilaments, indi-

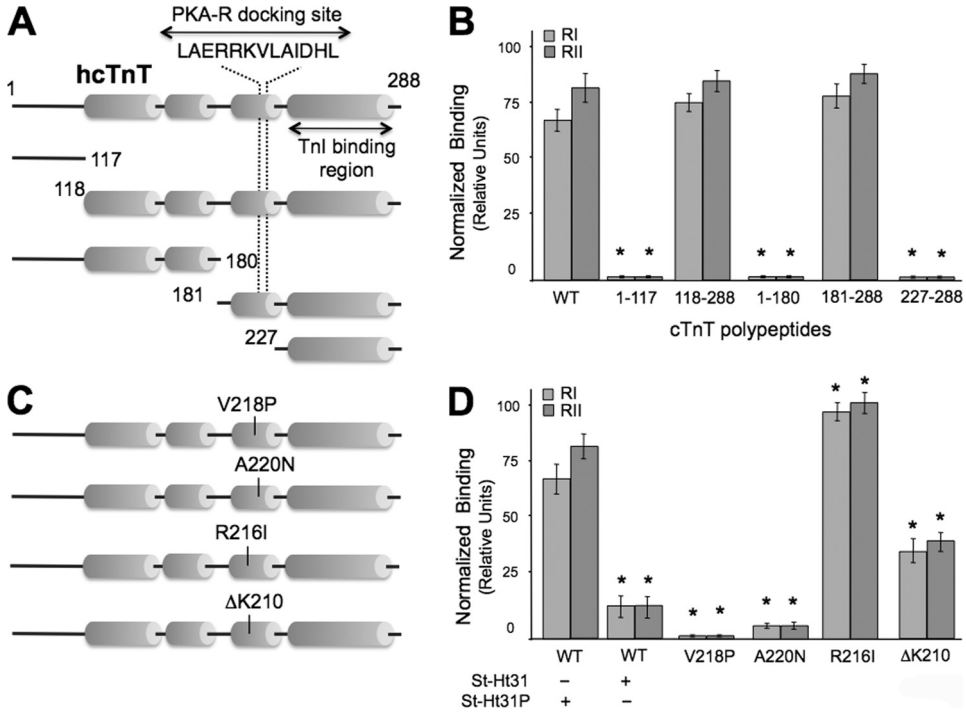

FIGURE 5. **Residues 212–224 of cTnT are essential for PKA-R binding.** *A*, schematic representation of cTnT truncations tested with two-hybrid assays. PKA-R docking site and the cTnI binding region are also shown. *B*, interaction between cTnT-wt-(1–288) or cTnT truncations with PKA-RI and PKA-RII was determined by the β-gal assay. Data normalized to cTnI-cTnT-wt binding (arbitrary set to 100). Only cTnT fragments that contained the proposed PKA binding site interacted positively with PKA-RI and PKA-RII. *C*, schematic representation of cTnT mutants tested in the Y2H system: V218P and A220N (loss-of-function), R216I (gain-of-function), and K210del (cardiomyopathy-causing deletion). *D*, interaction between cTnT-wt or -mutants with PKA-RI and PKA-RII assessed by β-gal assay. Membrane-permeable St-Ht31 (50 μM) disrupts cTnT-PKA interaction. cTnI was used as a positive control and laminin C as a negative control. Each screen was repeated at least 5 times. *, $p < 0.05$.

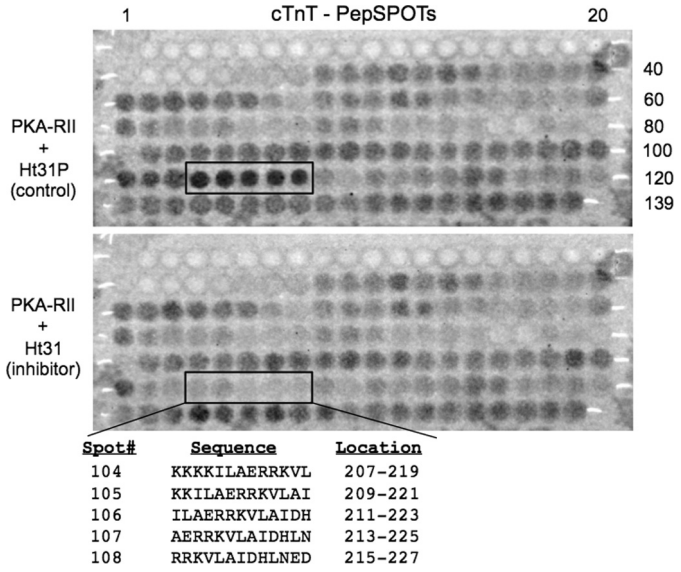

FIGURE 6. **Fine mapping of cTnT residues interacting with PKA-R.** The 288-amino acid residues of human cTnT were displayed on a SPOTS blot membrane as a series of 139 overlapping 13-mer peptides frameshifted by two residues. The cTnT SPOTS blot was tested for interaction with PKA-RII in a solid phase assay (in the presence of 20 μM Ht31 or Ht31P). PKA-RII has the highest affinity for the peptides spanning amino acids 207–227, as predicted from multiple sequence alignment and Y2H data. The other spots represent nonspecific interactions. Data shown are from a single experiment and are representative of 3 separate experiments.

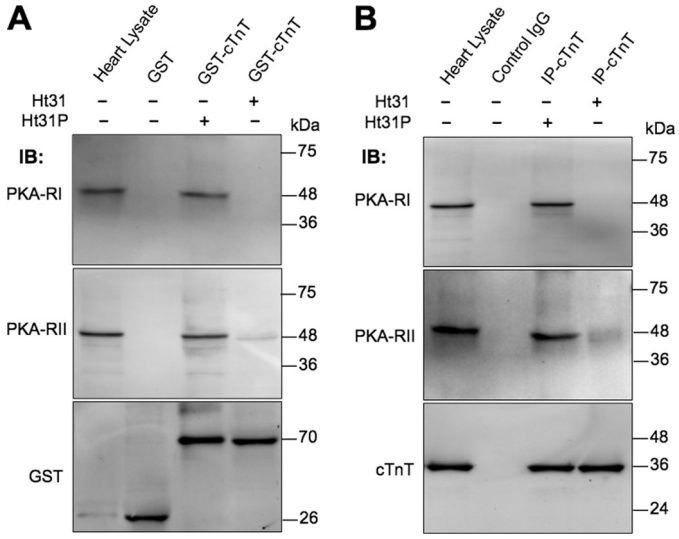

FIGURE 7. **Endogenously expressed PKA-R interacts directly and specifically with cTnT.** *A*, rat heart homogenates were used to test for endogenous cardiac PKA-R binding to cTnT. GST-TnT interaction with PKA-RI and RII was detected by immunoblotting (*IB*) with specified anti-PKA antibodies. GST alone shows no interaction with PKA-R. Ht31 and Ht31P peptides were used to test the specificity of interaction. Anti-GST antibody was used as control. *B*, rat heart homogenates were used to immunoprecipitate native cTnT-PKA-R complexes. Immunoblots of the IP complexes show endogenous association of cTnT-PKA-R in stable complexes. Data shown are from a single experiment and are representative of 3 separate experiments. Molecular weight markers are shown on the *left*.

cating that ~51% of PKA-cat and ~55% of PKA-RII were removed together with endogenous cTn. PKA activity was determined in the presence and absence of PKI (PKA inhibitor peptide) before and after the exchange (Fig. 10*D*). Our data indicate a significant blunting (by ~50%) of myofilament bound PKA activity after the cTn exchange procedure.

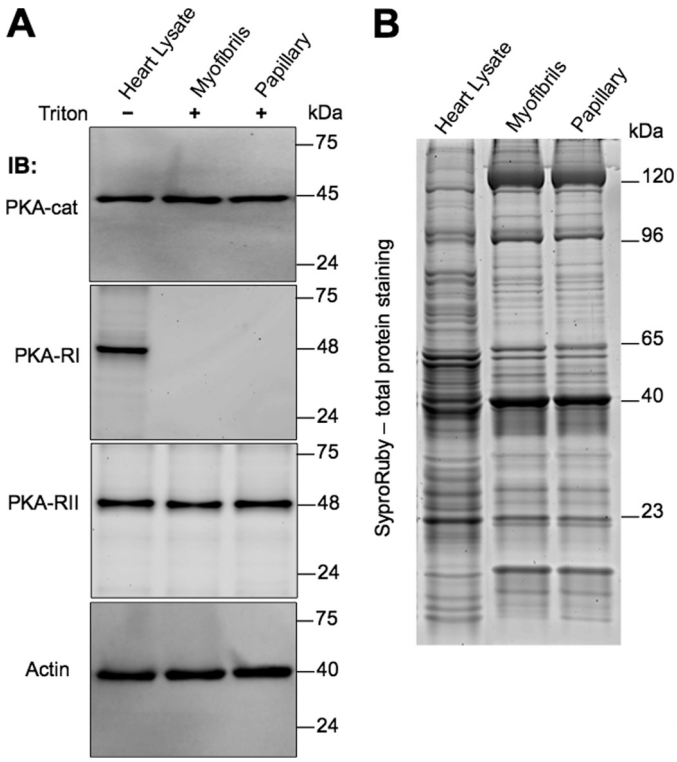

FIGURE 8. **PKA-R have differential affinities to detergent-extracted cardiac myofibrils and papillary muscle strips.** *A*, Triton X-100-extracted rat cardiac myofibrils or left ventricular papillary muscle fibers indicate that PKA type II holoenzyme (*RII* and *cat*) is associated with cardiac myofilaments. PKA-RI appears to have been removed by the detergent treatment. Similar data were obtained in 3 separate experiments. *B*, representative gel used to resolve detergent-treated myofibrils and papillary fibers prior to transfer to PVDF membranes. SyproRuby-total protein staining indicates similar loading of myofibrils and papillary muscle strips. Molecular mass markers are shown on the *left*. *IB,* immunoblot.

DISCUSSION

This study provides novel evidence that cTnT is a dual-specificity AKAP, and that native cTnT forms stable complexes in the rat heart with PKA-RI and -RII. The disruptor peptide Ht31, but not Ht31P (control), abolished cTnT/PKA-R association. Using gelsolin and cTn exchange assays we show that PKA-RII associates with thin filaments and endogenous cTnT, in isolated rat myofibrils. In addition, our study indicates that PKA-R docks to an amphipathic domain on cTnT and mutation of specific residues abolished cTnT/PKA-R interaction.

PKA regulates many aspects of cardiac contractility. In healthy myocardium, stimulation of the β-AR signaling pathway enhances the heart rate, force of contraction, and relaxation (35). At the cardiomyocyte level these events are mediated by PKA, which is anchored by various AKAPs in the vicinity of key Ca^{2+} handling and contractile proteins, and regulates their activity through phosphorylation. For example, AKAP18α, a small membrane-associated protein, has been shown to target PKA to the L-type Ca^{2+} channel (36). PKA-dependent phosphorylation of the channel increases its open probability. Similarly, muscle-specific AKAP (mAKAP), a 255-kDa protein, has been shown to facilitate anchoring and PKA-dependent phosphorylation of RyR_2 at the SR (15). Phosphorylation of RyR_2 releases the inhibitory action of FKBP12.6 resulting in an increase in open channel probability. Last, AKAP18δ has been identified as the anchoring protein responsible for PKA-dependent regulation of SERCA2, through phosphorylation of its inhibitory protein phospholamban (37). Phospholamban phosphorylation relieves its inhibitory action on SERCA2 thereby boosting Ca^{2+} re-uptake into the SR during diastole.

PKA-dependent phosphorylation of cardiac sarcomeric proteins is more studied and better understood than all the other kinases targeting the sarcomere. PKA-dependent phosphorylation of cTnI and MyBP-C seem to dominate the control of myofilament function during β-AR stimulation (9). Phosphorylation of cTnI at its cardiac-specific sites Ser^{23}-Ser^{24} depresses myofilament Ca^{2+} sensitivity and controls the kinetics of Ca^{2+} binding to cTnC (38). Like cTnI, MyBP-C has a N-terminal phosphorylation motif that is unique to the cardiac isoform. Phosphorylation of MyBP-C affects the radial movement of cross-bridges from the thick filament (39, 40). PKA phosphorylation of both cTnI and MyBP-C is essential in shortening the heart beat cycle time required to adjust to augmented frequency in exercise (41, 42). PKA also phosphorylates titin, the giant third filament protein, and controls its mechanical properties (reduces passive tension) (43).

Despite the importance of PKA-mediated phosphorylation in the control of myofilament function, little is known regarding its targeting to the sarcomere (44). Docking of PKA near substrates is attained by interaction of regulatory domains with AKAPs. Both, PKA-RI and PKA-RII are ubiquitously expressed in cardiac myocytes. Studies over the past decade have illuminated our understanding of their non-redundant biological role, usually through sequestering each PKA type to distinct intracellular signaling compartments. Recent studies employing various imaging techniques show overlapping staining patterns at the cardiac sarcomere for both PKA-RI and PKA-RII (11, 45). The striated staining is largely abolished in the presence of peptide disruptors. These data are in agreement with our present findings that PKA-R interact with cTnT and are anchored at the myofilaments.

However, what could be the functional advantage of having both PKA-types anchored to cardiac sarcomeres? Probably the two isoforms could impart a more dynamic modulation of myofilament function in response to varying cAMP levels, and the combined regulation could provide a more refined physiological response. It is well known that the two PKA types possess distinct activation and docking properties. For example, PKA type I is more readily activated by cAMP (activation constant, K_{act} of 50–100 nM) than PKA type II (K_{act} of 200–400 nM) (46). In addition, PKA-RI appear to have a weaker binding affinity to the AKAP(s) compared with PKA-RII, therefore it could be easier released from its binding site in a process related to the mechanical state of the sarcomere, as shown for other proteins (47, 48).

The interaction between cTnT and PKA-regulatory subunits is not unanticipated. Evidence from an Y2H screen of a human skeletal muscle cDNA library proposed that skeletal TnT interacts with the leucine zipper domain of the regulatory subunit of cGMP-dependent protein kinase I (or PKG)

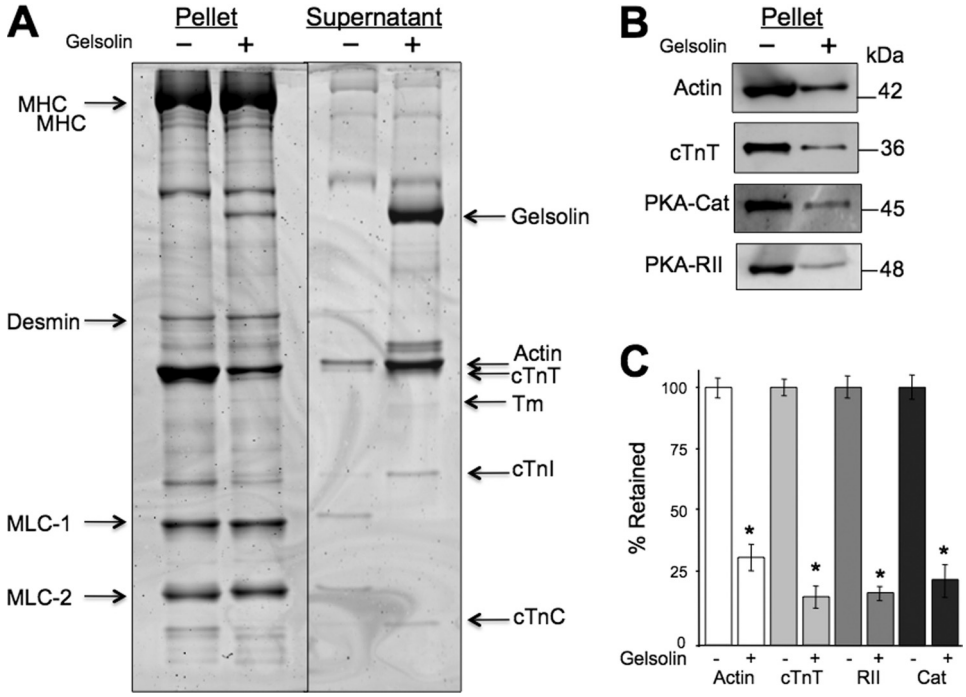

FIGURE 9. **Gelsolin-dependent removal of cardiac thin filaments significantly reduces sarcomeric PKA abundance.** *A*, rat cardiac myofibrillar proteins (pellets and supernatants) resolved by SDS-PAGE following gelsolin treatment. Myosin heavy chain (*MHC*), myosin light chains 1 and 2 (*MLC*), and desmin, indicate intact thick and intermediate filaments, whereas thin filament proteins (actin, troponins, and tropomyosin) are selectively extracted by gelsolin treatment. *B*, immunoblot analysis of control (−) and gelsolin-extracted (+) pellets. PKA-catalytic subunit and -RII are extracted together with actin and cTnT indicating the association of the holoenzyme with cardiac thin filaments. *C*, analysis of optical densities of thin filament proteins retained after gelsolin extraction *versus* control. Similar data were obtained in 3 separate experiments. *, $p < 0.05$.

(49). PKG and PKA are part of the same family of kinases. A major difference is their respective regulatory subunits require distinct binding motifs.

All members of the diverse AKAP family share a conserved PKA-anchoring domain, consisting of an amphipathic helix of 10–16 residues, which interacts with a hydrophobic groove in the docking/dimerization domain of the regulatory subunits (25, 26). Crystal structure of the core domain of the cardiac troponin complex shows that cTnT residues 202–226 form an amphipathic helix, are solvent exposed, and apparently are not required for intra-troponin interactions (50). This region shows homology to amphipathic helices found in other AKAPs that serve as docking domains for PKA-R. It was shown that the amino acid composition of the amphipathic helix dictates the binding preference for PKA-RI, -RII, or both (51).

A recent study by Jarnaess *et al.* (52) demonstrates that some dual specificity AKAPs contain an additional PKA-RI binding region (termed RISR) that is generally located upstream of the amphipathic helix. The RISR motif was suggested to be an enhancer for PKA-RI binding to the amphipathic helix and that AKAPs that contain both regions have a higher affinity for PKA-RI compared with those that only have the amphipathic helix. Our study provides strong evidence that cardiac troponin T is a dual AKAP. To determine whether cTnT also possesses a possible RISR domain we took a closer look at its sequence. *In silico* sequence analysis identified a region in cTnT with high homology to the RISR minimal binding motif located just upstream of the amphipathic helix (see Fig. 11) spanning a critical phosphorylation region centered on Thr^{203} (20, 21). Whether the putative RISR region is important in enhancing PKA-RI binding to cTnT, and whether Thr^{203} phosphorylation alters the complex formation, remains to be seen.

Recent data indicate that β-AR signaling abnormalities in heart failure include a profound decrease in PKA activation, likely due to defects in AKAP-based molecular complexes leading to impaired compartmentation of cAMP/PKA signaling (2, 28, 53). As a result, studies of human and experimental heart failure models show a significant decrease in the phosphorylation levels of PKA substrates cTnI, MyBP-C, and titin, which may account for a large part of the contractile dysfunction of the failing myocardium (9, 54). Interestingly, targeted disruption of AKAP-PKA complexes by adenovirus-mediated gene transfer of the Ht31 inhibitory peptide or TAT-conjugated AKAD (A kinase anchoring disruptor) elicited detrimental effects on chronotropy, inotropy, and lusitropy via a redistribution of PKA away from AKAPs and its substrates, and a blunting of PKA-dependent phosphorylation of key substrates (45, 55, 56). PKA-dependent phosphorylation of myofilament proteins is necessary for optimal contractile function, is associated with cardioprotection, and confers resistance to calpain-mediated proteolysis of cTnI and MyBP-C (38, 57, 58). Collectively, these studies strongly suggest that altered PKA anchoring at the myofilaments has a profound impact on cardiac function.

More work is needed to understand not only what PKA does in the heart to modulate contractile function, but also when and where it happens. Of particular importance also is to understand whether cTnT-PKA complexes are altered

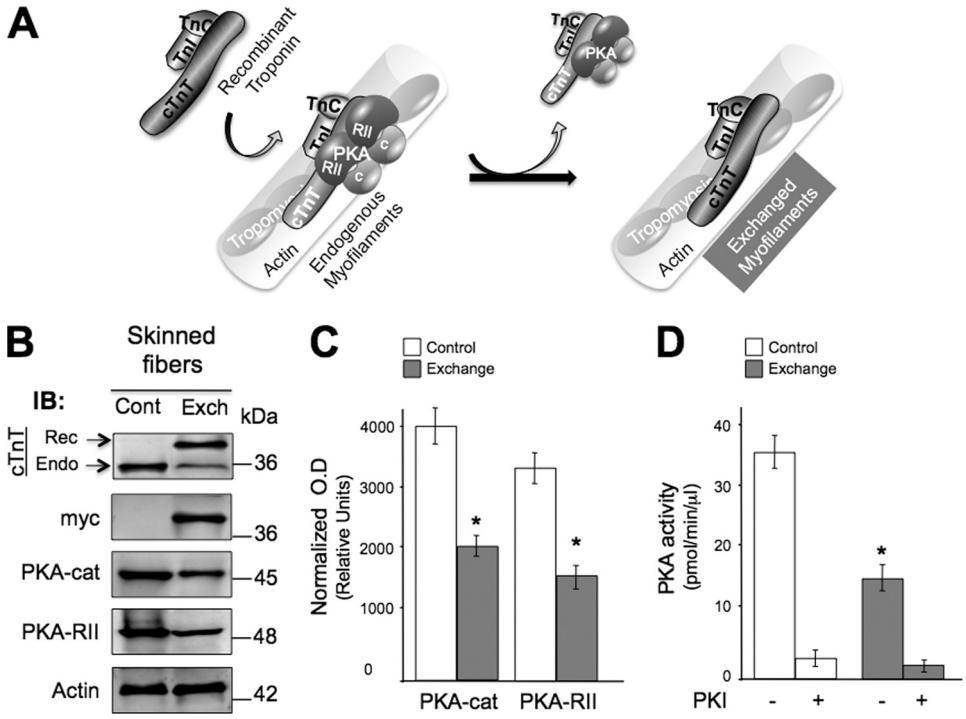

FIGURE 10. **Exchanging endogenous cTn complexes with recombinant cTn displaces the myofilament bound PKA holoenzyme type II.** *A*, schematic representation of the cTn exchange protocol. Recombinant cTn complex (*gray*) added to the detergent-extracted mouse papillary muscle fibers replaces the endogenous cTn on the myofilaments. This action dislodges any PKA holoenzyme (2R, *red,* and 2Cat, *purple* subunits) bound to endogenous cTnT. *B*, immunoblot analysis of detergent-extracted fibers (skinned) incubated in exchange buffer with and without (*Cont*) the recombinant cTn (*Exch*). *Top panel* shows the incorporation of recombinant Myc-cTnT (*Rec*) and the displacement of the endogenous TnT (*End*). *Middle panels* indicate the levels of PKA-cat and PKA-RII decreased in the exchanged fibers. Actin immunoblotting was used as control. *C*, optical density (*O.D.*) of PKA immunoblots (*IB*) normalized to actin. *D*, kinase activity assay shows a significant decrease of PKA activity following cTn exchange. PKA inhibitor (15 μM PKI) verifies that the activity is PKA-specific and not from another kinase. Data shown are from a single experiment and is representative of 3 separate experiments. *, $p < 0.05$.

FIGURE 11. **Identification of a putative PKA-RI specified region (*RISR*) in cTnT.** Sequence alignment of cTnT residues 191–230 and the RISR minimal binding region described in Ref. 52. Conserved residues are shown in *white*. The amphipathic helix location is also shown.

during heart disease progression, and whether cTnT phosphorylation has a regulatory role in complex formation (implicating a cross-talk between different kinases at the sarcomere).

Taken together our data indicate that cTnT is a dual AKAP that tethers PKA-R at the myofilaments. We predict that these macromolecular complexes are localized at the at the I-Z-I region away from myosin-actin cross-bridges. Generally, AKAP-PKA clusters are signaling modules composed of many other proteins such as phosphodiesterases and phosphatases, and even other kinases. Although numerous studies discuss the important role of phosphorylation events in regulating cardiac myofilament function, essentially nothing is known about the mechanisms that promote anchoring of these signaling molecules in the proximity of their substrates. This information is vital to a complete understanding of cardiac muscle regulation. In addition, direct and specific manipulation of sarcomeric proteins and signaling complexes, circumventing receptor-ligand pathways, could pave the way for myofilament-targeted therapeutic approaches in muscle diseases (59).

REFERENCES

1. Steinberg, S. F., and Brunton, L. L. (2001) *Annu. Rev. Pharmacol. Toxicol.* **41,** 751–773
2. El-Armouche, A., and Eschenhagen, T. (2009) *Heart Fail. Rev.* **14,** 225–241
3. Taylor, S. S., Kim, C., Vigil, D., Haste, N. M., Yang, J., Wu, J., and Anand, G. S. (2005) *Biochim. Biophys. Acta* **1754,** 25–37
4. Taskén, K., and Aandahl, E. M. (2004) *Physiol. Rev.* **84,** 137–167
5. McConnachie, G., Langeberg, L. K., and Scott, J. D. (2006) *Trends Mol. Med.* **12,** 317–323
6. Kobayashi, T., and Solaro, R. J. (2005) *Annu. Rev. Physiol.* **67,** 39–67
7. Solaro, R. J. (2010) *J. Biomed. Biotechnol.* **2010,** 105648
8. Bers, D. M. (2002) *Nature* **415,** 198–205
9. Solaro, R. J. (2008) *J. Biol. Chem.* **283,** 26829–26833
10. Fischmeister, R., Castro, L. R., Abi-Gerges, A., Rochais, F., Jurevicius, J., Leroy, J., and Vandecasteele, G. (2006) *Circ. Res.* **99,** 816–828
11. Di Benedetto, G., Zoccarato, A., Lissandron, V., Terrin, A., Li, X., Houslay, M. D., Baillie, G. S., and Zaccolo, M. (2008) *Circ. Res.* **103,** 836–844
12. Michel, J. J., and Scott, J. D. (2002) *Annu. Rev. Pharmacol. Toxicol.* **42,** 235–257
13. Zaccolo, M. (2009) *Br. J. Pharmacol.* **158,** 50–60
14. Houslay, M. D., Baillie, G. S., and Maurice, D. H. (2007) *Circ. Res.* **100,** 950–966
15. Dodge-Kafka, K. L., Bauman, A., and Kapiloff, M. S. (2008) *Handb. Exp. Pharmacol.* **186,** 3–14
16. David, H., Meyer, R., Marx, I., Guski, H., and Wenzelides, K. (1979) *J. Mol. Cell Cardiol.* **11,** 631–638
17. Wu, S. C., and Solaro, R. J. (2007) *J. Biol. Chem.* **282,** 30691–30698
18. Cànaves, J. M., Leon, D. A., and Taylor, S. S. (2000) *Biochemistry* **39,** 15022–15031

19. Sfichi-Duke, L., Garcia-Cazarin, M. L., Sumandea, C. A., Sievert, G. A., Balke, C. W., Zhan, D. Y., Morimoto, S., and Sumandea, M. P. (2010) *J. Mol. Cell Cardiol.* **48,** 934–942
20. Sumandea, M. P., Vahebi, S., Sumandea, C. A., Garcia-Cazarin, M. L., Staidle, J., and Homsher, E. (2009) *Biochemistry* **48,** 7722–7731
21. Sumandea, M. P., Pyle, W. G., Kobayashi, T., de Tombe, P. P., and Solaro, R. J. (2003) *J. Biol. Chem.* **278,** 35135–35144
22. Fields, S., and Song, O. (1989) *Nature* **340,** 245–246
23. Estojak, J., Brent, R., and Golemis, E. A. (1995) *Mol. Cell. Biol.* **15,** 5820–5829
24. Carr, D. W., Stofko-Hahn, R. E., Fraser, I. D., Bishop, S. M., Acott, T. S., Brennan, R. G., and Scott, J. D. (1991) *J. Biol. Chem.* **266,** 14188–14192
25. Kinderman, F. S., Kim, C., von Daake, S., Ma, Y., Pham, B. Q., Spraggon, G., Xuong, N. H., Jennings, P. A., and Taylor, S. S. (2006) *Mol. Cell* **24,** 397–408
26. Gold, M. G., Lygren, B., Dokurno, P., Hoshi, N., McConnachie, G., Taskén, K., Carlson, C. R., Scott, J. D., and Barford, D. (2006) *Mol. Cell* **24,** 383–395
27. Hinkle, A., Goranson, A., Butters, C. A., and Tobacman, L. S. (1999) *J. Biol. Chem.* **274,** 7157–7164
28. Diviani, D. (2008) *Curr. Opin. Pharmacol.* **8,** 166–173
29. Mauban, J. R., O'Donnell, M., Warrier, S., Manni, S., and Bond, M. (2009) *Physiology* **24,** 78–87
30. Pidoux, G., and Taskén, K. (2010) *J. Mol. Endocrinol.* **44,** 271–284
31. Sun, H. Q., Yamamoto, M., Mejillano, M., and Yin, H. L. (1999) *J. Biol. Chem.* **274,** 33179–33182
32. Kawai, M., and Ishiwata, S. (2006) *J. Muscle Res. Cell Motil.* **27,** 455–468
33. Trombitás, K., and Granzier, H. (1997) *Am. J. Physiol.* **273,** C662–C670
34. Trombitás, K., Greaser, M. L., and Pollack, G. H. (1997) *J. Muscle Res. Cell Motil.* **18,** 345–351
35. Ruehr, M. L., Russell, M. A., and Bond, M. (2004) *J. Mol. Cell Cardiol.* **37,** 653–665
36. Hulme, J. T., Lin, T. W., Westenbroek, R. E., Scheuer, T., and Catterall, W. A. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 13093–13098
37. Lygren, B., Carlson, C. R., Santamaria, K., Lissandron, V., McSorley, T., Litzenberg, J., Lorenz, D., Wiesner, B., Rosenthal, W., Zaccolo, M., Taskén, K., and Klussmann, E. (2007) *EMBO Rep.* **8,** 1061–1067
38. Layland, J., Solaro, R. J., and Shah, A. M. (2005) *Cardiovasc. Res.* **66,** 12–21
39. Colson, B. A., Bekyarova, T., Fitzsimons, D. P., Irving, T. C., and Moss, R. L. (2007) *J. Mol. Biol.* **367,** 36–41
40. Stelzer, J. E., Fitzsimons, D. P., and Moss, R. L. (2006) *Biophys. J.* **90,** 4119–4127
41. Kentish, J. C., McCloskey, D. T., Layland, J., Palmer, S., Leiden, J. M., Martin, A. F., and Solaro, R. J. (2001) *Circ. Res.* **88,** 1059–1065
42. Stelzer, J. E., Patel, J. R., Walker, J. W., and Moss, R. L. (2007) *Circ. Res.* **101,** 503–511
43. Yamasaki, R., Wu, Y., McNabb, M., Greaser, M., Labeit, S., and Granzier, H. (2002) *Circ. Res.* **90,** 1181–1188
44. Russell, M. A., Lund, L. M., Haber, R., McKeegan, K., Cianciola, N., and Bond, M. (2006) *Arch. Biochem. Biophys.* **456,** 204–215
45. Patel, H. H., Hamuro, L. L., Chun, B. J., Kawaraguchi, Y., Quick, A., Rebolledo, B., Pennypacker, J., Thurston, J., Rodriguez-Pinto, N., Self, C., Olson, G., Insel, P. A., Giles, W. R., Taylor, S. S., and Roth, D. M. (2010) *J. Biol. Chem.* **285,** 27632–27640
46. Dostmann, W. R., and Taylor, S. S. (1991) *Biochemistry* **30,** 8710–8716
47. Hoshijima, M. (2006) *Am. J. Physiol. Heart Circ. Physiol.* **290,** H1313–1325
48. Frank, D., Kuhn, C., Katus, H. A., and Frey, N. (2006) *J. Mol. Med.* **84,** 446–468
49. Yuasa, K., Michibata, H., Omori, K., and Yanaka, N. (1999) *J. Biol. Chem.* **274,** 37429–37434
50. Takeda, S., Yamashita, A., Maeda, K., and Maéda, Y. (2003) *Nature* **424,** 35–41
51. Feliciello, A., Gottesman, M. E., and Avvedimento, E. V. (2001) *J. Mol. Biol.* **308,** 99–114
52. Jarnaess, E., Ruppelt, A., Stokka, A. J., Lygren, B., Scott, J. D., and Taskén, K. (2008) *J. Biol. Chem.* **283,** 33708–33718
53. Zakhary, D. R., Fink, M. A., Ruehr, M. L., and Bond, M. (2000) *J. Biol. Chem.* **275,** 41389–41395
54. Hamdani, N., Kooij, V., van Dijk, S., Merkus, D., Paulus, W. J., Remedios, C. D., Duncker, D. J., Stienen, G. J., and van der Velden, J. (2008) *Cardiovasc. Res.* **77,** 649–658
55. Fink, M. A., Zakhary, D. R., Mackey, J. A., Desnoyer, R. W., Apperson-Hansen, C., Damron, D. S., and Bond, M. (2001) *Circ. Res.* **88,** 291–297
56. McConnell, B. K., Popovic, Z., Mal, N., Lee, K., Bautista, J., Forudi, F., Schwartzman, R., Jin, J. P., Penn, M., and Bond, M. (2009) *J. Biol. Chem.* **284,** 1583–1592
57. Nagayama, T., Takimoto, E., Sadayappan, S., Mudd, J. O., Seidman, J. G., Robbins, J., and Kass, D. A. (2007) *Circulation* **116,** 2399–2408
58. Sadayappan, S., Osinska, H., Klevitsky, R., Lorenz, J. N., Sargent, M., Molkentin, J. D., Seidman, C. E., Seidman, J. G., and Robbins, J. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103,** 16918–16923
59. Solaro, R. J. (2009) *IDrugs* **12,** 243–251