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## Sympathetic stimulation of adult cardiomyocytes requires association of AKAP5 with a sub-population of L-type calcium channels

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### Abstract

**Rationale**—Sympathetic stimulation of the heart increases the force of contraction and rate of ventricular relaxation by triggering PKA-dependent phosphorylation of proteins that regulate intracellular calcium. We hypothesized that scaffolding of cAMP signaling complexes by AKAP5 is required for efficient sympathetic stimulation of calcium transients.

**Objective**—We examined the function of AKAP5 in the  $\beta$ -adrenergic signaling cascade.

**Methods and Results**—We used calcium imaging and electrophysiology to examine the sympathetic response of cardiomyocytes isolated from wild type and AKAP5 mutant animals. The  $\beta$ -adrenergic regulation of calcium transients and the phosphorylation of substrates involved in calcium handling were disrupted in AKAP5 knockout cardiomyocytes. The scaffolding protein, AKAP5 (also called AKAP150/79), targets adenylyl cyclase, PKA, and calcineurin to a caveolin 3-associated complex in ventricular myocytes that also binds a unique sub-population of  $Ca_v1.2$  L-type calcium channels. Only the caveolin 3-associated  $Ca_v1.2$  channels are phosphorylated by PKA in response to sympathetic stimulation in wild type heart. However, in the AKAP5 knockout heart the organization of this signaling complex is disrupted, adenylyl cyclase 5/6 no longer associates with caveolin 3 in the T-tubules, and non-caveolin 3 associated calcium channels become phosphorylated after  $\beta$ -adrenergic stimulation although this does not lead to an enhanced calcium transient. The signaling domain created by AKAP5 is also essential for the PKA-dependent phosphorylation of ryanodine receptors and phospholamban.

**Conclusions**—These findings identify an AKAP5-organized signaling module that is associated with caveolin 3 and is essential for sympathetic stimulation of the calcium transient in adult heart cells.

### Keywords

AKAP;  $Ca^{2+}$  channels; adenylyl cyclase; cAMP; PKA

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**DISCLOSURES:** none

## INTRODUCTION

The function of the heart is to pump blood to the pulmonary and systemic circulation. To do this, atrial and ventricular myocytes contract in response to an action potential in a process known as excitation-contraction (EC) coupling. During EC coupling, brief openings of sarcolemmal voltage-gated L-type  $\text{Ca}^{2+}$  channels (LTCC) in the transverse tubules and surface sarcolemma create local elevations in  $[\text{Ca}^{2+}]_i$  that activate closely apposed ryanodine receptors (RyR) in the sarcoplasmic reticulum (SR) by the mechanism of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) 1. This causes release of  $\text{Ca}^{2+}$  from the SR thereby inducing a global, cell-wide increase in  $[\text{Ca}^{2+}]_i$  that activates contractile proteins. LTCCs are rapidly inactivated by a  $\text{Ca}^{2+}$ -dependent mechanism and SR  $\text{Ca}^{2+}$  release is also terminated, which allows the SR  $\text{Ca}^{2+}$ -ATPase (SERCA) to recover the released  $\text{Ca}^{2+}$  prior to the next heart beat. A major negative regulator of SERCA is phospholamban (PLN), which binds to and decreases the affinity of SERCA for  $\text{Ca}^{2+}$ . Phosphorylation of PLN by Protein Kinase A (PKA) causes dissociation of PLN from SERCA, increasing the rate of  $\text{Ca}^{2+}$  reuptake into the SR 2. Unlike the action potential, the amplitude of the global  $[\text{Ca}^{2+}]_i$  transient, and hence the force of contraction during EC coupling, are not all-or-none. Instead, the force of cardiac contraction is graded by the amplitude of the global  $[\text{Ca}^{2+}]_i$  transient. This represents the mechanistic basis of a vital function of the heart: to match its pumping force to physiological needs.

Sympathetic stimulation of the heart through  $\beta$ -adrenergic receptors ( $\beta$ -AR) increases the force of contraction (inotropy) and accelerates the rate of relaxation (lusitropy) as part of the “fight or flight” response 3. Chronic activation of this pathway has been linked to an increased risk of heart failure and arrhythmias. In this pathway, activation of  $\beta$ -ARs results in the production of cAMP and the activation of PKA. Both  $\beta_1$ -AR and  $\beta_2$ -AR are expressed in heart and both are capable of  $\text{G}_s$ -mediated activation of adenylyl cyclase. However, activation of the  $\beta_2$ -AR appears to produce only a transient and highly localized burst of cAMP which may be limited by subsequent coupling to  $\text{G}_{\beta i}$  4. The duration and spatial dispersion of cAMP is limited by PDE4D isoforms which have been reported to be recruited directly to  $\beta$ -ARs 5. The specificity of cAMP signaling is also thought to be modulated by the binding of PKA to A-kinase anchoring proteins (AKAPs), which target the kinase to specific intracellular locations and provide spatial and temporal control over cAMP signaling events. The importance of AKAPs has been demonstrated through use of peptides (e.g. Ht31) that compete for PKA binding and thereby disrupt interaction with all AKAPs 6. However, over 11 AKAPs have been identified in cardiomyocytes and elucidating the specific role of each AKAP remains a long-term objective 7, 8. Several AKAPs have been identified that interact with  $\text{Ca}^{2+}$  regulating proteins in adult cardiomyocytes: for example, AKAP7 $\alpha$  (AKAP15/18 $\alpha$ ) binds LTCCs 9, AKAP6 (mAKAP) binds RyR<sub>2</sub> 10, AKAP12 (gravin) associates with  $\beta$ -AR 11, and AKAP7 $\delta$  (AKAP18 $\delta$ ) binds to SERCA-PLN 12. AKAP5 can interact with multiple proteins, including the LTCC ( $\text{Ca}_v1.2$ ),  $\beta$ -AR, adenylyl cyclase, PKA, Protein Kinase C (PKC), and calcineurin (protein phosphatase 2B, PP2B) 13–15. In smooth muscle, AKAP5 interacts with PKC and LTCCs and is required for angiotensin II-induced hypertension 16. In neurons, AKAP5 localizes PP2B to the LTCC and this interaction is required for nuclear signaling 17. Although AKAP5 is expressed in heart cells, its function has not been demonstrated. To gain insight into the potential role of AKAP5 in cardiac signaling, we examined adult cardiomyocytes from mice with mutations in AKAP5 and discovered that AKAP5 is essential for sympathetic stimulation of the  $\text{Ca}^{2+}$  transient.

## METHODS

The *Akap5*<sup>-/-</sup> allele was generated by gene targeting using 129S1/SvImJ ES cells and standard techniques as previously described<sup>13</sup>. Mice were age-matched littermates of 3 to 6 months of age maintained on a C57Bl/6 background after >10 backcrosses to C57Bl/6. Animals were handled in accordance with the guidelines of the University of Washington Institutional Animal Care and Use Committee. Adult cardiomyocytes were isolated and incubated as previously described<sup>18</sup>. Electrophysiology was performed using whole cell patch-clamp as described in online Supplemental Methods. Whole cell [Ca<sup>2+</sup>]<sub>i</sub> transients were measured using Fluo-4 loaded cardiomyocytes subjected to field stimulation at 1 Hz. Immunocytochemistry was performed using standard techniques as described in Supplementary Methods. Immunoprecipitation and western blotting were done using heart extracts homogenized in lysis buffer containing (in mmol/L): 10 Na<sub>2</sub>HPO<sub>4</sub>, 150 NaCl, 5 EGTA, 5 EDTA, 5 NaF, with 1% TritonX-100 and 0.5% Na Deoxycholate. The cAMP measurements were performed on isolated cardiomyocytes in laminin coated wells and the cAMP determined with direct cAMP EIA (Assay Design, Ann Arbor, MI, USA). Significant differences were determined by two-way ANOVA and post hoc Student's unpaired t test, as appropriate. Pearson's correlation coefficients<sup>19</sup> were calculated using Imaris software (Bitplane AG, Zurich, Switzerland) on nine to 16 images per point.

## RESULTS

### Ca<sup>2+</sup> transients in AKAP5 KO cardiomyocytes do not respond to β-AR stimulation

Immunocytochemistry revealed AKAP5 expression in adult ventricular cardiomyocytes predominantly localized near the T-tubules (Figure 1A) in wild type (WT) with no staining in AKAP5 knockout (KO) cardiomyocytes (Figure 1B). In order to measure calcium transients, adult cardiomyocytes were loaded with the calcium indicator dye, Fluo-4, and paced at 1 Hz in a perfusion chamber where the cells could be recorded by confocal microscopy. Treatment with 100 nmol/L isoproterenol (Iso) to activate the β-AR pathway increased the amplitude and reduced the decay time ( $\tau_{1/2}$ ) of the [Ca<sup>2+</sup>]<sub>i</sub> transient in WT cells (Figure 1C). In contrast, β-AR activation did not increase the amplitude or the decay rate of the [Ca<sup>2+</sup>]<sub>i</sub> transient in AKAP5 KO cells (Figure 1D).

Because β-ARs signal through cAMP/PKA to phosphorylate Ca<sub>v</sub>1.2, RyR<sub>2</sub>, and PLN 20, the lack of response in AKAP5 KO cardiomyocytes suggests that the cAMP/PKA pathway is disrupted. To determine where in the β-AR-signaling pathway AKAP5 is required, direct activation of AC was examined using forskolin (Fsk) to bypass the β-AR. Stimulation of AC with low concentrations of Fsk caused an increase in the amplitude and rate of decay of the [Ca<sup>2+</sup>]<sub>i</sub> transient in WT (Figure 2A) but not in KO cardiomyocytes (Figure 2C). To test whether cAMP was still being generated in KO cardiomyocytes we measured total cellular cAMP after stimulation with Iso and found equivalent increases in WT (Figure 2B) and KO cells (Figure 2D). We next tested the ability of higher concentrations of agonist or cAMP to overcome the deficits observed in AKAP5 KO myocytes. Stimulation of the β-AR with higher concentrations of Iso (100 μmol/L) began to induce increased [Ca<sup>2+</sup>]<sub>i</sub> transients in KO cells but these higher doses were also toxic to the WT cells (Online Figure I). High doses of Fsk (10 μmol/L) could overcome the deficit in KO myocytes and no toxicity was observed in either WT or KO cells. High dose forskolin produced a 1.5 fold increase in intracellular cAMP compared with Iso as shown in Online Figure I. In contrast, when a cell permeable and phosphodiesterase (PDE)-resistant cAMP analog (8-CPT-cAMP) was used to elicit an increased [Ca<sup>2+</sup>]<sub>i</sub> transient, both KO and WT showed the same dose-response relationship and there was no difference in the onset of increased calcium transients after drug administration as shown in Online Figure I. These data demonstrate that AKAP5 is required for a sensitive response to either β-AR stimulation or cyclase activation and suggest

that AKAP5 is helping to localize cAMP generation near PKA targets that modulate the  $[Ca^{2+}]_i$  transient. Exogenous cAMP analogs overcome the requirement for localized cAMP generation as expected.

AKAP5 was first described as a PKA binding protein, but has now been reported to scaffold many additional signaling molecules, including  $Ca_v1.2$ , PP2B, PKC,  $\beta$ -ARs, and AC 6, 15. To directly test the requirement for PKA binding, we utilized another AKAP5 mutant mouse line, AKAP5 D36 21, that expresses a truncated AKAP5 that lacks the last 36 amino acids which contain the PKA binding site (Figure 2E) and one potential site of interaction with  $Ca_v1.2$ . This D36 mutant protein has been shown to express normally in the brain and continues to bind PP2B although PKA binding is completely eliminated<sup>22</sup>. In heart the D36 mutant protein continues to form a complex with both  $Ca_v1.2$  and AC6 as does wild type AKAP5 (Online Figure IV B,C). Stimulation with Iso elicited a similar  $[Ca^{2+}]_i$  transient response in WT and D36 cardiomyocytes demonstrating that AKAP5-anchored PKA is not essential for  $\beta$ -AR stimulation of  $[Ca^{2+}]_i$  transients.

### $Ca_v1.2$ responses to $\beta$ -AR signaling

AKAP5 is localized near the T-tubules (Figure 1A) along with  $Ca_v1.2$  LTCCs, which are juxtaposed within 15 nm of the  $RyR_2$  in the T-tubule/SR junctions of ventricular myocytes. Increased LTCC activity is a hallmark of  $\beta$ -AR activation in these cells. Thus, to determine the mechanisms by which loss of AKAP5 caused the elimination of  $\beta$ -AR-dependent modulation of EC coupling, we simultaneously recorded  $[Ca^{2+}]_i$  transients and LTCC currents in WT and KO cells. Surprisingly,  $\beta$ -AR stimulation with Iso (100 nmol/l) enhanced LTCC current in both WT and KO cells to a similar extent (Figure 3A). However, this increase in  $Ca^{2+}$  current did not trigger an increase in  $[Ca^{2+}]_i$  transients in the KO cells (Online Figure II) consistent with the results in Figure 1. When we analyze calcium transients in individual KO cells before and after Iso and calculate the ratio for each cell we can detect a 9% increase in peak calcium as depicted in Online Figure III A,B. The increase in calcium current in the KO appears to be offset in part by an Iso-stimulated increase in the activity of the  $Na^+/Ca^{2+}$  exchanger (NCX) as shown in Online Figure III and this might explain why the KO cells do not become overloaded with  $Ca^{2+}$  in response to Iso.

When a high concentration of forskolin (10  $\mu$ mol/l) was used both WT and KO cells responded with current increases that were 1.5 fold higher than that seen with Iso (Figure 3B,C). Interestingly this treatment with high forskolin overcomes the defect in KO cells and produces an increased calcium transient of similar magnitude to WT. High forskolin also elevated cAMP about 1.5 fold greater than Iso treatment (Online Figure I).

### $Ca^{2+}$ sparks and phosphorylation of $RyR_2$ and PLN

We investigated the effect of  $\beta$ -AR stimulation on calcium release from the SR directly by measuring  $Ca^{2+}$  sparks.  $Ca^{2+}$  sparks are spontaneous calcium release events caused by the opening of a cluster of  $RyR_2$  in the absence of LTCC opening<sup>23</sup>. Iso treatment increased  $Ca^{2+}$  spark rate in WT but not in KO myocytes (Figure 4A, B, left panel). Blocking  $Ca_v1.2$  with nifedipine had no significant effect on spark rate in either WT or KO indicating that the sparks were independent of spontaneous  $Ca_v1.2$  openings. The amplitude of the  $Ca^{2+}$  sparks was also increased in WT but not KO cells after  $\beta$ -AR stimulation (Online Figure III D,E).

$\beta$ -AR stimulation increases spark frequency in isolated cardiomyocytes but the relative contribution of  $RyR_2$  phosphorylation and SR  $Ca^{2+}$  loading remains controversial<sup>24, 25</sup>. Previous work has shown that an increase in the rate and amplitude of the  $Ca^{2+}$  sparks during  $\beta$ -AR activation is linked to increased SR  $Ca^{2+}$  load<sup>18, 26</sup>. SR  $Ca^{2+}$  load was measured in WT and KO cells by caffeine-induced release before and after Iso stimulation.

The SR  $\text{Ca}^{2+}$  load was similar under control conditions but  $\beta$ -AR activation increased SR  $\text{Ca}^{2+}$  load in WT but not KO cells (Figure 4A, B, middle panel). The failure of the KO cells to load more  $\text{Ca}^{2+}$  into the SR after  $\beta$ -AR stimulation could be due to a change in the regulation of SERCA in the KO cells. Perfusion of cells with high (5 mmol/L) external  $\text{Ca}^{2+}$  was used to increase SR load independently of the  $\beta$ -AR pathway and this resulted in increased  $\text{Ca}^{2+}$  spark rates in both WT (Figure 4A, right panel) and KO (Figure 4B, right panel) cells. We conclude that the defect in  $\beta$ -AR stimulated sparks is likely to be due to a loss of PKA-dependent regulation of calcium loading in the SR although RyR<sub>2</sub> regulation may also contribute.

We next examined the PKA dependent phosphorylation of RyR<sub>2</sub> and PLN under basal and  $\beta$ -AR-stimulated conditions. PKA phosphorylates Ser-2808 on the RyR<sub>2</sub> 10 and Ser-16 on PLN 27 in response to  $\beta$ -AR stimulation. In WT cells, there was a significant increase in the phosphorylation of Ser-2808 after 100 nmol/L Iso, 100 nmol/L Fsk or 20  $\mu\text{M}$  8-CPT-cAMP treatment as shown by western blot (Figure 4C, top panel) or immunocytochemistry (Figure 4C, bottom panel). However, in KO cells, Iso and Fsk treatment did not induce phosphorylation of Ser-2808 while 8-CPT-cAMP did (Figure 4D). PKA-dependent phosphorylation of PLN on Ser-16 attenuates PLN-dependent inhibition of the SERCA 2. Phosphorylation of PLN occurred in parallel with that observed for RyR<sub>2</sub>. Robust phosphorylation was observed after treatment with Iso, Fsk, or 8-CPT-cAMP in WT cells (Figure 4E) but the response to Iso and Fsk was lost in KO cells (Figure 4F). These observations raised an intriguing question: how could the phosphorylation and activation of RyR<sub>2</sub> and PLN depend on AKAP5 when the activation of  $\text{Ca}_v1.2$  did not?

### AKAP5-dependent complexes in cardiomyocytes

In order to address this question we first characterized the biochemical interactions between signaling molecules that are mediated by AKAP5.  $\text{Ca}_v1.2$  is located in the sarcolemma and enriched in the T-tubules, while RyR<sub>2</sub> and PLN are in the SR membrane 1. AKAP5 KO myocytes lack  $\beta$ -AR-dependent phosphorylation of RyR<sub>2</sub> and PLN suggesting that AKAP5 is required for targeting cAMP signaling to the SR. We employed immunoprecipitation experiments with adult heart lysate to show that AKAP5 is in a complex that includes AC6, RII $\alpha$ , PP2B, and  $\text{Ca}_v1.2$  (Figure 5A). Since AC6 and  $\text{Ca}_v1.2$  are present in the sarcolemma we interpret this result to indicate that AKAP5 is physically associated with the sarcolemma and T-tubule proteins but must be in close proximity with RyR and PLN in the SR. Both AC6 and AC5 are expressed in cardiac myocytes but the commercial antibody used to detect adenylyl cyclase in our immunoprecipitations was shown recently to react specifically with AC6 and not AC5 28; we have used an AC5-specific antibody to repeat these immunoprecipitations and find that both AC5 and AC6 associate with the AKAP5 complex (Online Figure IV A).

The caveolin family of proteins form oligomers that can create 50 to 100 nm invaginations of the plasma membrane called caveolae that are enriched for cholesterol and sphingolipids 29. The major caveolin expressed in cardiomyocytes is CAV3 and its overall role in cardiac physiology has been studied previously by examining the cardiac phenotypes of CAV3 KO mice. The loss of CAV3 is associated with cardiomyopathy and cardiomyocyte hypertrophy as well as altered signaling leading to an increase in cAMP and an activation of the p42/44 MAPK cascade 30, 31. Localization of  $\text{Ca}_v1.2$  to a caveolin 3 (CAV3) organized macromolecular signaling complex has been reported to be essential for regulation of the channels by  $\beta_2$ -ARs 32, 33. We found that AKAP5 also associates with CAV3 in immunoprecipitation experiments (Figure 5A).  $\beta_1$  and  $\beta_2$ -AR were both found associated with AKAP5 and CAV3 by immunoprecipitation and their association with CAV3 did not depend on AKAP5 since  $\beta$ -ARs remained in the complex in the AKAP5 KO (Figure 5B). It was previously reported that only the  $\beta_2$ -AR is associated with CAV3 32 and consistent with

this finding we see a greater fraction of the total cellular  $\beta_2$ -AR in the CAV3 immunoprecipitation compared with  $\beta_1$ -AR; however, a significant fraction of  $\beta_1$ -AR was also found in the complex under our immunoprecipitation conditions (Figure 5B). The immunoprecipitation data suggest that the complex is linked by CAV3 and includes AKAP5, AC5/6,  $\text{Ca}_v1.2$ , PP2B,  $\beta$ AR, and PKA. Our immunoprecipitation data from AKAP5 KO heart shows that of the proteins examined only AC5/6 and PP2B appear to depend on AKAP5 for their association with CAV3. Previously it was reported that  $\text{Ca}_v1.2$  interacts directly and with high affinity with PP2B 34 but at least under the conditions of our assay, AKAP5 is a major contributor to this association.  $\text{Ca}_v1.2$ ,  $\beta$ -ARs, and PKA remain at similar levels in the complex with CAV3 in the absence of AKAP5. This does not exclude the possibility of additional protein-protein interactions between AKAP5 and either  $\text{Ca}_v1.2$  or  $\beta$ -ARs as reported previously<sup>13, 35</sup> but they do not seem to be essential for complex formation.

### Functionally distinct populations of $\text{Ca}_v1.2$ in cardiomyocytes

The association of multiple signaling molecules bridged by CAV3 suggested the possibility that the  $\text{Ca}_v1.2$  channels in the CAV3 complex with AKAP5 might constitute a unique population that responds preferentially to  $\beta$ -AR stimulation compared to  $\text{Ca}_v1.2$  in other regions of the plasma membrane. In order to test this hypothesis we had to develop a more quantitative analysis of the  $\text{Ca}_v1.2$  channels that associated with CAV3 and AKAP5 compared with those that did not. We used a CAV3 antibody to immunoprecipitate the complex because this antibody gave us quantitative precipitation of the CAV3 protein. Ventricular myocytes were exposed to either 100 nmol/l Iso or 10  $\mu\text{mol/l}$  forskolin for 90 sec and extracts were prepared and immuno-precipitated with antibody against CAV3. We then determined the amount of total  $\text{Ca}_v1.2$  and P- $\text{Ca}_v1.2$  in the input homogenate, the CAV3 associated fraction, and the supernatant from the immunoprecipitation.

The pore forming  $\alpha 1$  subunit of the LTCC,  $\text{Ca}_v1.2$ , is phosphorylated on Ser-1928 by PKA<sup>36</sup>. Phosphorylation at this site serves as an indicator that PKA has been activated in close proximity to the channel although recent evidence demonstrates that Ser-1928 is not essential for PKA stimulation of calcium current<sup>37, 38</sup> suggesting that other sites on  $\text{Ca}_v1.2$  or the  $\beta$ -subunits of the channel might be critical. Our results demonstrated that approximately 50% of the total  $\text{Ca}_v1.2$  is associated with the CAV3 complex but surprisingly, only the CAV3-associated  $\text{Ca}_v1.2$  was phosphorylated on Ser-1928 in response to Iso in WT mice (Figure 6A).  $\text{Ca}_v1.2$  is partially cleaved in vivo leaving Ser-1928 in the distal C-terminal fragment but this fragment remains bound to the channel as a regulatory subunit<sup>39</sup>. The upper P- $\text{Ca}_v1.2$  band at  $\sim 240$  kDa represents the uncleaved  $\text{Ca}_v1.2$  and the lower doublet at 55–60 kDa is the phosphorylated C-terminal tail released after in vivo processing. The C-terminal tail migrated as several bands which may be due to differences in cleavage and processing or possibly additional phosphorylation sites that alter migration. When high forskolin was used we observed phosphorylation of Ser-1928 on  $\text{Ca}_v1.2$  in both the IP and supernatant (S) fractions and an overall 2-fold increase in phosphorylation of Cav1.2 compared with Iso. In contrast, analysis of the KO revealed that Iso was targeting the non-CAV3 associated  $\text{Ca}_v1.2$  for phosphorylation with much less phosphorylation of the CAV3-associated channels. High forskolin leads to phosphorylation of both the CAV3 associated and non-CAV3 associated fractions in both WT and KO cells. A quantitative analysis of these results averaged over 3 experiments is shown in Figure 6B.

This surprising result distinguishes two populations of  $\text{Ca}_v1.2$  channels in WT myocytes: a CAV3 and AKAP5 associated population that is highly sensitive to Iso and forskolin and a non-CAV3 associated population of channels that are only phosphorylated when cAMP levels are much higher after 10  $\mu\text{mol/l}$  forskolin treatment or after treatment with cell-permeable cAMP analogs. In order to determine whether this preferential phosphorylation of

CAV3-associated  $Ca_v1.2$  took place under in vivo conditions we treated WT and KO mice with either isoproterenol or the  $\beta$ -AR antagonist, propranolol for 5 min and then immediately isolated the ventricular portion of the heart for immunoprecipitation experiments similar to those shown in Figure 6. The results shown in Online Figure V confirm that the same differential phosphorylation of channels is occurring in vivo.

We have used specific  $\beta$ -AR antagonists together with Iso to examine whether  $Ca_v1.2$  phosphorylation is dependent on  $\beta_1$  or  $\beta_2$ -AR using cultured myocytes. In WT myocytes,  $Ca_v1.2$  phosphorylation was inhibited by 44% using the  $\beta_1$ -AR selective antagonist, and by 20% with the  $\beta_2$ -AR specific antagonist. Both antagonists together blocked 95% of the Iso dependent increase in  $Ca_v1.2$  phosphorylation suggesting that there may be some compensation when only one receptor is blocked (Online Figure VI). This is in good agreement with our previous results using immunocytochemistry to quantitate  $Ca_v1.2$  phosphorylation in rat ventricular myocytes 36. In the AKAP5 KO myocytes,  $Ca_v1.2$  phosphorylation was predominantly  $\beta_1$ -AR specific in AKAP5 KO myocytes (Online Figure VI). The Iso dependent increase in LTCC current was completely lost in mice with a null mutation in the *Adrb1* ( $\beta_1$ -AR) gene as was the Iso-dependent stimulation of the calcium transient (Online Figure VI). We conclude that AKAP5 is required to facilitate phosphorylation of a sub-population of  $Ca_v1.2$  channels that are associated with CAV3 and that this channel population is primarily responding through stimulation of the  $\beta_1$ -AR. The role of  $\beta_2$ -ARs in the regulation of calcium channel physiology remains unclear.

### Subcellular localization of AKAP5 and CAV3 binding partners

We next examined whether we could visualize the co-localization of AC6 with CAV3 by immunocytochemistry and whether co-localization would be disrupted in the AKAP5 KO as predicted from the immunoprecipitation experiments in Figure 5. In WT cells AC6 staining was localized to T-tubules with CAV3; however, in KO cells, AC6 showed a significantly reduced co-localization with CAV3 (Figure 7). We utilized the Pearson product-moment correlation coefficient as a quantitative measure of the co-localization of CAV3 and AC6 in cardiomyocytes from WT and KO heart and found a significant decrease in the association of AC6 with CAV3 (Figure 7B) as predicted from the immunoprecipitation experiments. The antibody specific for AC5 did not give a signal by immunocytochemistry but the immunoprecipitation data indicates that, like AC6, it is also released from the CAV3-associated signaling complex in the AKAP5 KO (Online Figure IV A). The association of PP2B with CAV3 also depends on AKAP5 based on the immunoprecipitation experiments but we could distinguish no changes in the localization of PP2B comparing WT and KO cardiomyocytes (Online Figure VII). This result might be expected if only a small fraction of the total PP2B in the cardiomyocyte is associated with AKAP5.

## DISCUSSION

Our results show that AKAP5 plays an essential role in the sympathetic regulation of both the amplitude and rate of decay of calcium transients in cardiac cells. Co-immunoprecipitation experiments reveal a signaling complex containing AKAP5, AC5/6, PKA, PP2B,  $Ca_v1.2$ , and  $\beta$ -ARs that are tightly associated with CAV3 and we postulate that this complex generates a micro-domain of cAMP that is juxtaposed to the  $RyR_2$  containing junctional regions of the SR as depicted in the WT panel of Figure 8. When AKAP5 is missing in the KO, AC5 and AC6 are released from the CAV3 complex as judged by both co-immunoprecipitation and immunocytochemistry and the response of the calcium transient to  $\beta$ -AR stimulation is lost. More than 90% of the AKAP5 in cardiomyocytes is complexed with CAV3 based on our immunoprecipitation experiments; this leads us to suggest that the

CAV3 complex is also responsible for generating the micro-domain of cAMP that is required to stimulate PKA-dependent phosphorylation of both the RyR<sub>2</sub> and PLN.

We were surprised to find that AKAP5 KO cardiomyocytes had lost the ability to modulate calcium transients in response to Iso but still retained Iso-dependent increases in whole cell LTCC current (Figure 3). One explanation for this paradox is that the Ca<sub>v</sub>1.2 channels that are conducting increased Ca<sup>2+</sup> current in KO cells are not the same population of channels that we identified in the CAV3/AKAP5 complex. In support of this possibility, we identified two populations of Ca<sub>v</sub>1.2 channels in the cardiomyocyte that can be distinguished biochemically and functionally. The Ca<sub>v</sub>1.2 population associated with CAV3 and AKAP5 is phosphorylated by PKA in WT cells in response to β-AR stimulation and this event appears to be coupled to the β-AR-induced Ca<sup>2+</sup> loading of the SR and an increased calcium transient. A second Ca<sub>v</sub>1.2 population is depicted in the WT panel of Figure 8 that is not in the CAV3 complex and does not respond to β-AR stimulation in WT cells. However, in the KO myocytes this second non-CAV3 associated population is phosphorylated by β-AR stimulation. We believe this is because AC6 has been released from AKAP5 in the KO and that it now migrates closer to the non-CAV3 associated Ca<sub>v</sub>1.2 channels as depicted in the KO panel of Figure 8. Co-localization experiments using immunocytochemistry confirmed this change in AC6 association with CAV3 as shown in Figure 7. It appears to be the non-CAV3 associated Ca<sub>v</sub>1.2 channels that are responsible for the increased LTCC current when KO myocytes are treated with Iso. It is not clear whether these channels are in junctional complexes with the SR but since there is no concomitant phosphorylation of PLN to cause an increased calcium loading of the SR in the KO after Iso we hypothesize that the increased LTCC current alone is not sufficient to produce an enhanced calcium transient that is comparable to WT cells.

These findings compel modification of the generally accepted model for β-AR-mediated modulation of cardiac contraction. Our suggestion that the CAV3-associated Ca<sub>v</sub>1.2 channels are functionally linked to β-AR regulation of SR calcium release conflicts with the observation that caveolae are typically visualized at the surface membrane of cardiac cells and not within the T-tubule network. However, mounting evidence exists for a substantial fraction of CAV3 that is not associated with the structurally distinct caveolae in adult cardiomyocytes and it has been suggested that CAV3 may form functional scaffolding domains that are independent of caveolae 40. The presence of CAV3 within the T-tubule structures where it co-localizes with RyR<sub>2</sub> and a fraction of LTCCs 41 supports this idea and is consistent with our results. Co-immunoprecipitation experiments have also shown an interaction between CAV3 and both the RyR<sub>2</sub> 42 and junctophilin-2, a membrane spanning protein that contributes to the junctional membrane complex between the T-tubule membrane and the SR in heart 43. In our immunoprecipitations we did not see any interactions between RyR<sub>2</sub> and either CAV3 or AKAP5 but if this complex is dependent on junctophilin or other intermediary proteins it may be very sensitive to detergent and homogenization conditions.

Our studies provide new insights into the compartmentalization of β-AR signaling complexes and their linkage to functionally distinct calcium channel populations in the heart. Many questions are raised by our results. Are the Ca<sub>v</sub>1.2 channels associated with CAV3 in the T-tubules unique biochemically? A recent report suggests that the localization of Ca<sub>v</sub>1.2 to CAV3 depends on association with specific channel β-subunits 44. Are the Ca<sub>v</sub>1.2 channels that are not linked to CAV3 regulated by other hormonal signals that stimulate cAMP in the heart such as glucagon, GLP-1, adenosine, or PGE1? Cardiovascular stress results in chronic over-stimulation of the β-AR pathway, cardiac hypertrophy, and ultimately cardiac failure. Do changes or disruption of the AKAP5-signaling complex contribute to the pathophysiology of heart failure? Analysis of the *in vivo* physiological



phenotypes of AKAP5 KO mice are complicated by the expression and functional role of AKAP5 in vascular smooth muscle where it regulates PKC dependent effects on vascular tone and hence, blood pressure<sup>16</sup>. AKAP5 is also expressed in neurons where it regulates a multitude of events underlying synaptic plasticity and behavior<sup>13, 22, 45</sup>. Direct or indirect effects of the AKAP5 KO on the autonomic nervous system have not been examined. Analysis of the in vivo physiological phenotypes associated uniquely with the loss of AKAP5 function in the cardiomyocyte will require a cell type specific KO.

## NOVELTY AND SIGNIFICANCE

### What Is Known?

- $\beta$ -adrenergic stimulation of heart cells produces a highly compartmentalized increase in cAMP and PKA activity that culminates in increased contractility.
- Scaffolding proteins like A Kinase Anchoring Proteins (AKAPs) are implicated in controlling this process and contribute to specificity and sub-cellular localization of responses.
- PKA-dependent phosphorylation of both the L-type  $\text{Ca}^{2+}$  channel (LTCC) and phospholamban (PLN) are believed to regulate the amplitude and kinetics of the calcium transient evoked by an action potential during excitation-contraction coupling.

### What New Information Does This Article Contribute?

- AKAP5 (also known as AKAP150 in rodents and AKAP79 in humans) is essential for ventricular myocytes to increase the amplitude and decay rate of the cytosolic  $\text{Ca}^{2+}$  transient in response to  $\beta$ -adrenergic stimulation, yet the whole-cell LTCC current remains intact in AKAP5 knockout myocytes.
- The role of AKAP5 is to recruit adenylyl cyclase to a membrane-associated complex of signaling molecules which directs the PKA phosphorylation of PLN, RyR, and a sub-population of LTCCs.
- The increase in LTCC current in AKAP5 knockout myocytes is due to phosphorylation of a different subpopulation of LTCCs than in wild-type myocytes.
- Ventricular myocytes compartmentalize LTCCs such that only about 50% are responsive to physiological levels of  $\beta$ -adrenergic stimulation.

This study was designed to determine the role of AKAP5 in ventricular myocytes and resulted in the unexpected finding that AKAP5 is essential for the compartmentalized production of cAMP in the mouse heart. Our studies relied on targeted mutation of the AKAP5 gene in mice. Other AKAPs have been studied in the heart, including AKAP7 (AKAP15/18) which is thought to interact with LTCCs and PLN, AKAP9 (Yotiao) which interacts with and regulates a cardiac  $\text{K}^+$  channel, and AKAP6 (mAKAP) which interacts with the RyR. which interacts with the coordinate the  $\beta$ -adrenergic response pathway prior to this study. We also discovered that only a sub-population (about 50%) of the ventricular LTCCs are associated with AKAP5 and only this sub-population is efficiently phosphorylated by PKA when cells are treated with  $\beta$ -adrenergic receptor agonists. Acute  $\beta$ -adrenergic stimulation of the heart is beneficial and increases cardiac output; however, chronic over-stimulation is associated with heart failure and pathological changes in cardiac tissue. Our results implicate AKAP5 in normal cardiac signalling, suggesting a new target to investigate in the context of signaling changes that occur during heart failure.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Non-standard Abbreviations and Acronyms

<b>LTCC</b>	L-type Ca <sup>2+</sup> channel
<b>RyR</b>	ryanodine receptors
<b>SR</b>	sarcoplasmic reticulum
<b>CICR</b>	Ca <sup>2+</sup> -induced Ca <sup>2+</sup> release
<b>SERCA</b>	sarcoplasmic reticulum Ca <sup>2+</sup> -ATPase
<b>PLN</b>	phospholamban
<b>β-AR</b>	β-adrenergic receptors
<b>AKAP</b>	A-kinase anchoring protein
<b>PKC</b>	protein kinase C
<b>PP2B</b>	calcineurin, protein phosphatase 2B
<b>WT</b>	wild type
<b>KO</b>	knockout
<b>Iso</b>	isoproterenol
<b>AC</b>	adenylyl cyclase
<b>Fsk</b>	forskolin
<b>PDE</b>	phosphodiesterase
<b>CAV3</b>	caveolin 3
<b>EC</b>	excitation-contraction
<b>NCX</b>	Na <sup>+</sup> /Ca <sup>2+</sup> exchanger

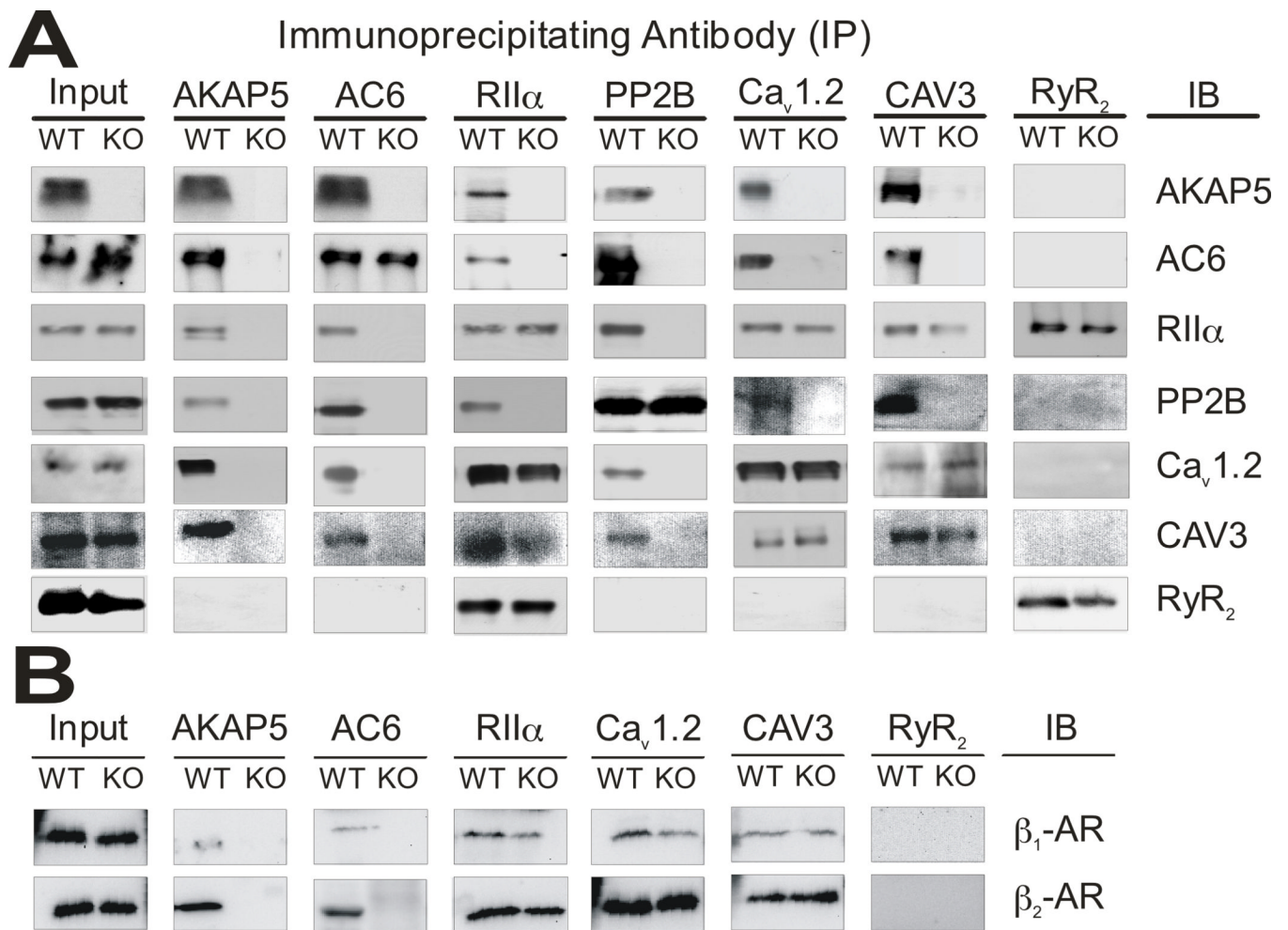
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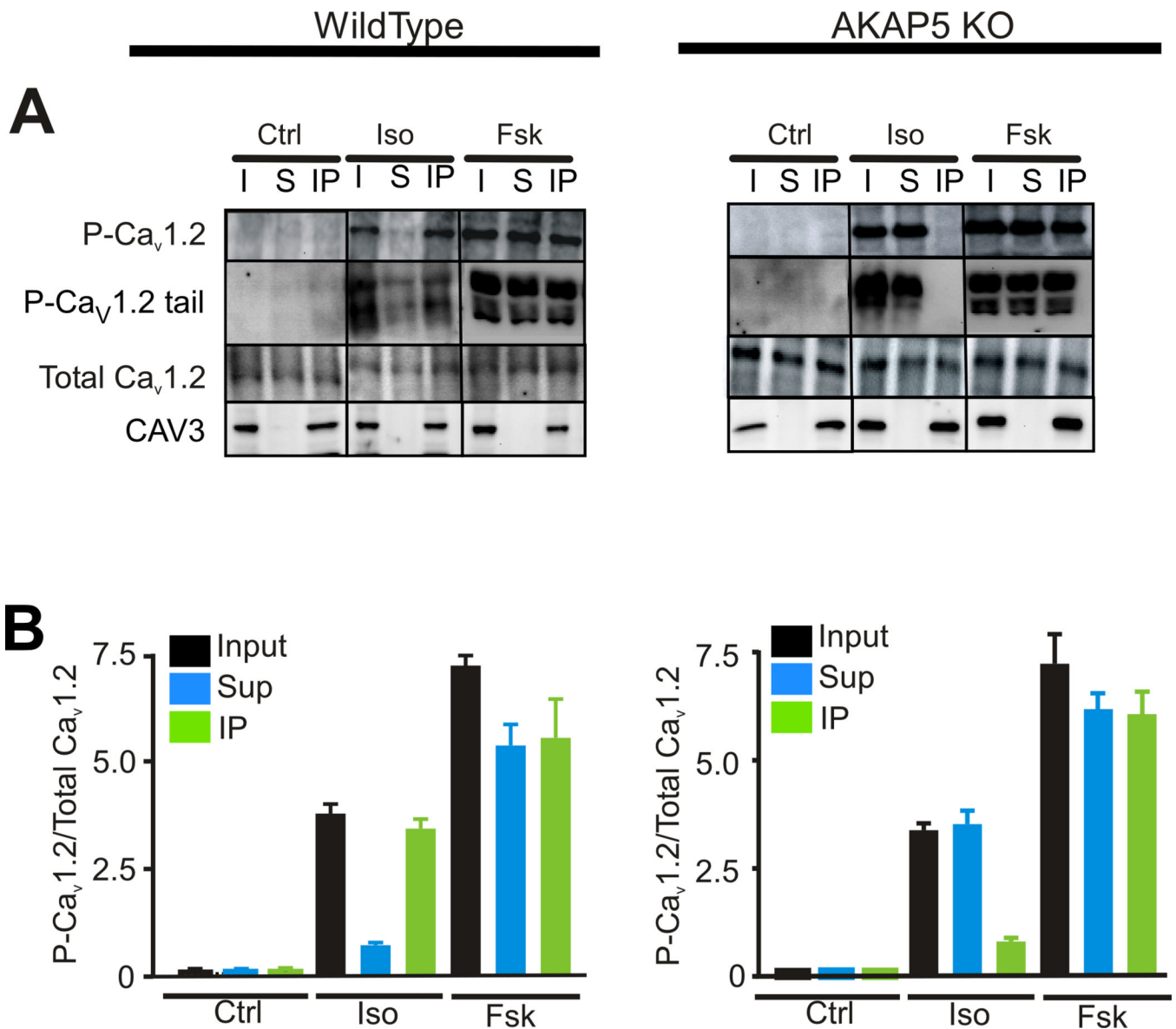
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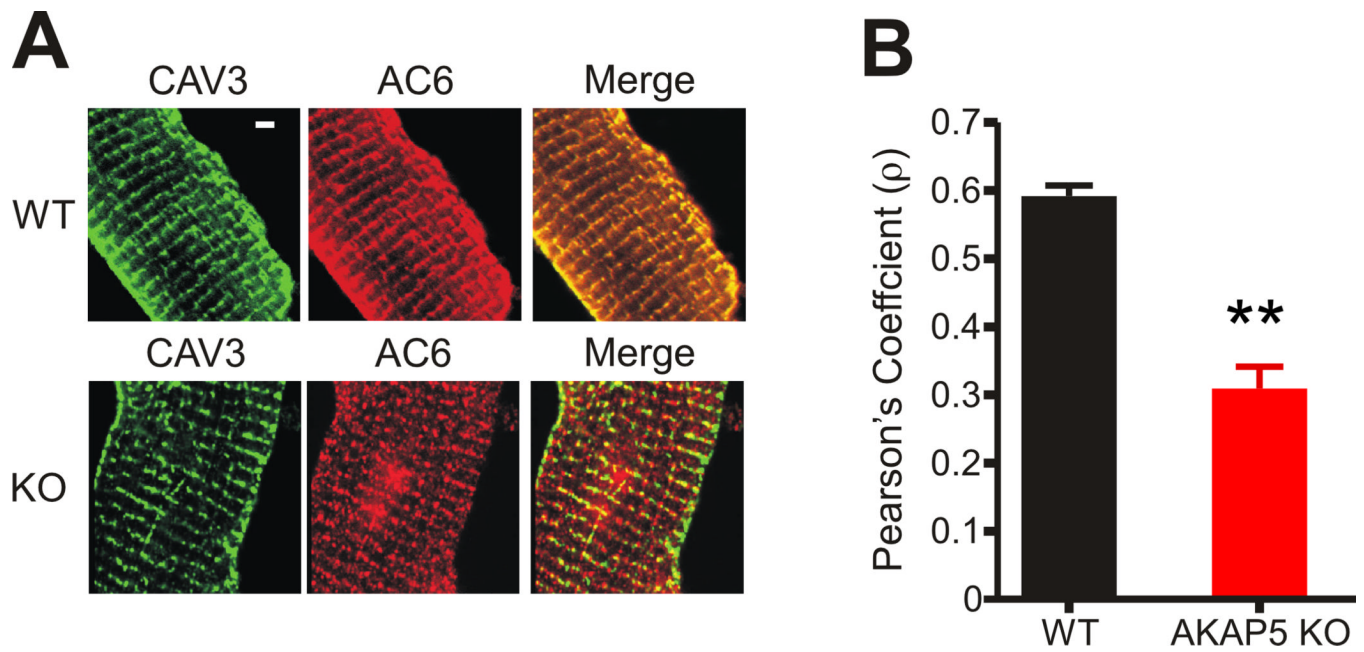
**Figure 1. AKAP5 is required for  $\beta$ -adrenergic stimulation of  $[Ca^{2+}]_i$  transients**

**A,B** Staining of isolated mouse adult cardiomyocytes for  $\alpha$ -actinin and AKAP5 in WT and KO. Scale bar = 15  $\mu$ m. **C–D**, Isoproterenol, Iso, (100 nmol/L) stimulation of whole cell  $[Ca^{2+}]_i$  transients in cardiomyocytes isolated from WT and KO animals compared with no treatment (Ctrl). Cells were field stimulated at 1 Hz as described in Methods. Left panels are a representative  $[Ca^{2+}]_i$  trace, middle panels are bar graphs of the amplitude of the  $[Ca^{2+}]_i$  transient and right panels are the half life of  $[Ca^{2+}]_i$  transient decay. Calcium transients were measured with Fluo-4 and converted to calcium concentrations as described in Methods (mean  $\pm$  s.e.m., n=9 animals, 3–4 cells analyzed per animal; \*\*P<0.001).



**Figure 2. Forskolin, cAMP, and the role of PKA binding to AKAP5**

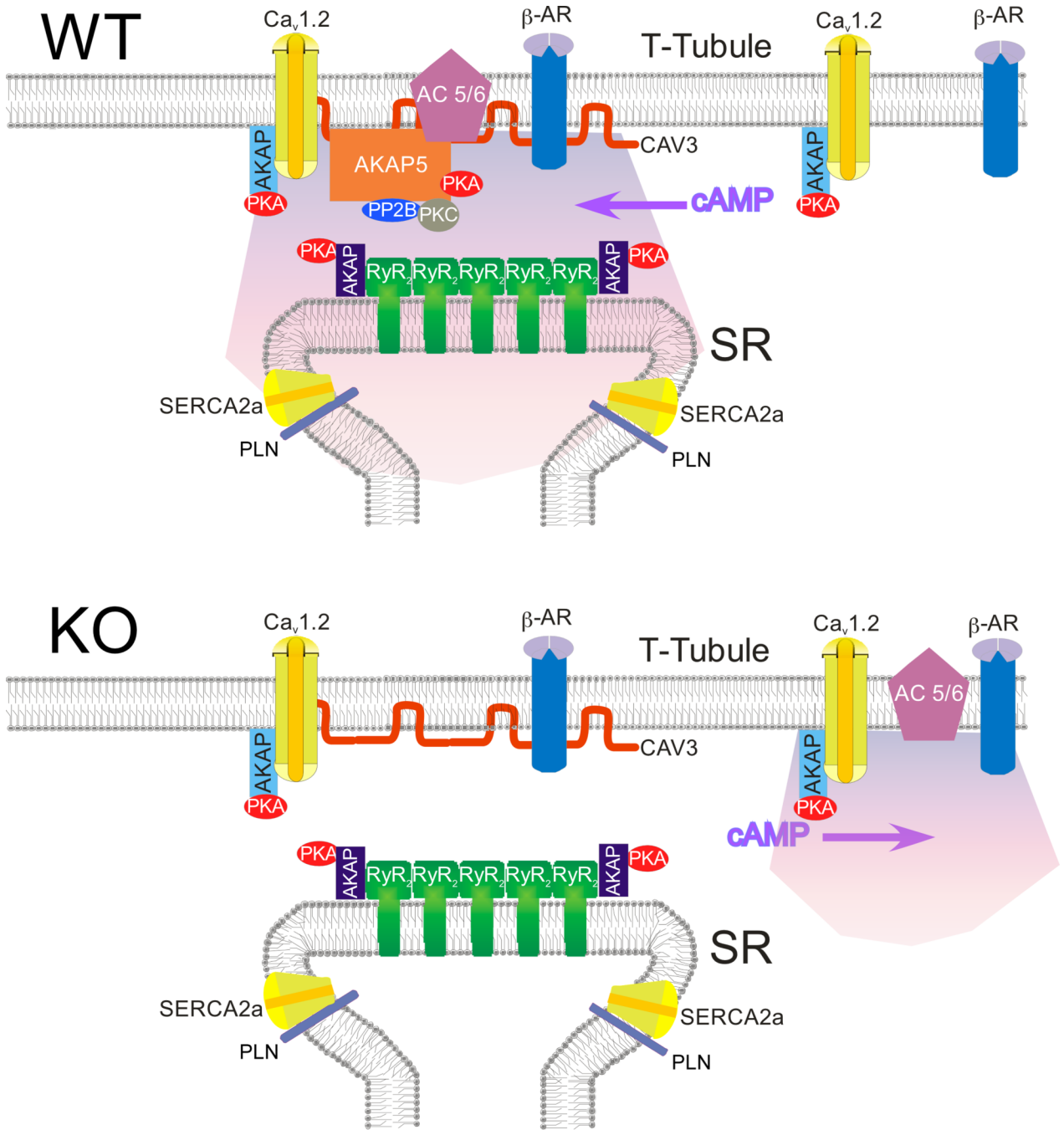
**A, C** Forskolin, Fsk, (100 nmol/L) stimulation of  $[Ca^{2+}]_i$  transients in isolated cardiac myocytes from WT and KO animals (mean  $\pm$  s.e.m., n=9 animals, 3–4 cells analyzed per animal; \*\*P<0.001). **B, D**, Stimulation of cAMP production in isolated ventricular myocytes from WT and KO animals after a 90 sec treatment with 100 nmol/L Iso. **E**, AKAP5D36 lacks the last 36 amino acids and the binding site for PKA. Iso (100 nmol/L) stimulation of whole cell  $[Ca^{2+}]_i$  transients in both WT and D36 cardiac myocytes (mean  $\pm$  s.e.m., n=4 animals; \*\*P<0.001).



**Figure 3.  $Ca_v1.2$  function and AKAP5**

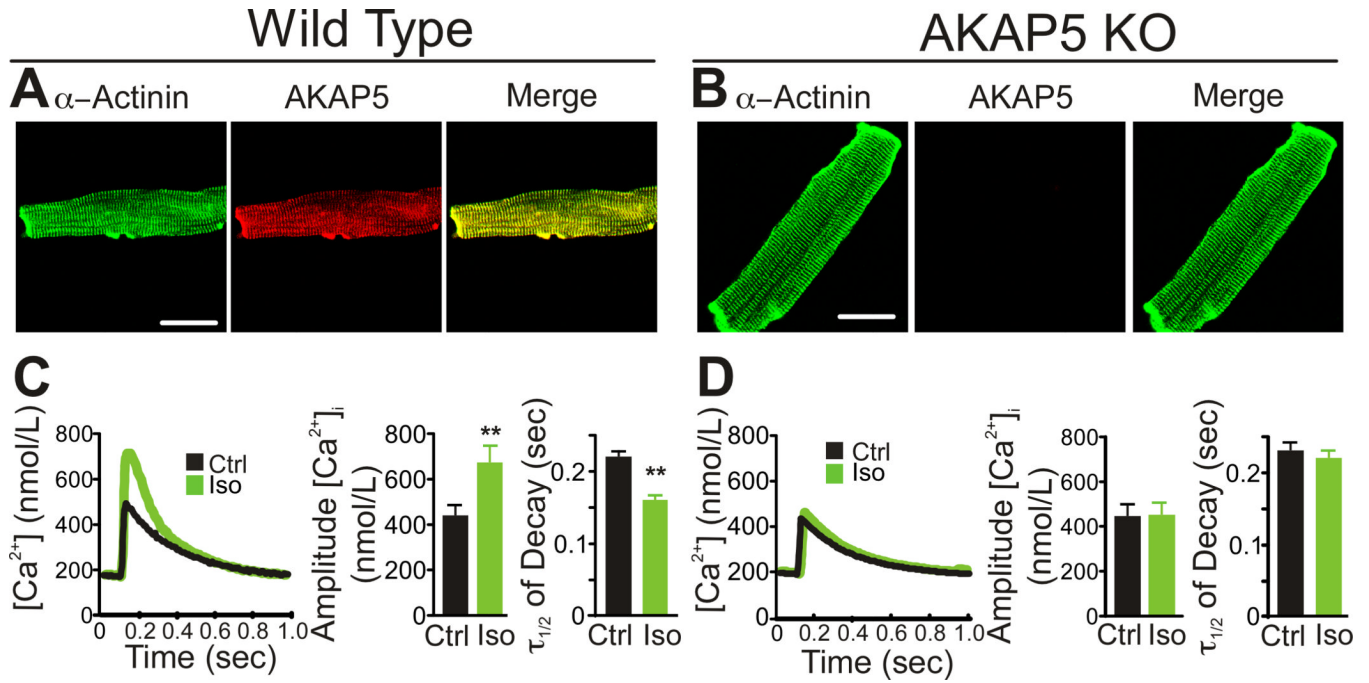
**A**, Whole cell patch clamp recording of  $I_{Ca}$  as a function of voltage in WT (left panel) and KO (right panel) cardiomyocytes before and after stimulation by isoproterenol (100 nmol/L) (n=12 cells from 3 animals). **B**, Whole cell patch clamp recording of  $I_{Ca}$  as a function of voltage in WT (left panel) and KO (right panel) cardiomyocytes before and after stimulation with forskolin (Fsk, 10  $\mu$ mol/L) (n=10 cells from 3 animals). **C**, The fold change of the stimulated  $I_{Ca}$  measured at 10 mV for isoproterenol (Iso, 100 nmol/L) or forskolin (Fsk, 10  $\mu$ mol/L) (mean  $\pm$  s.e.m., n=3 animals; \*\*P<0.001)





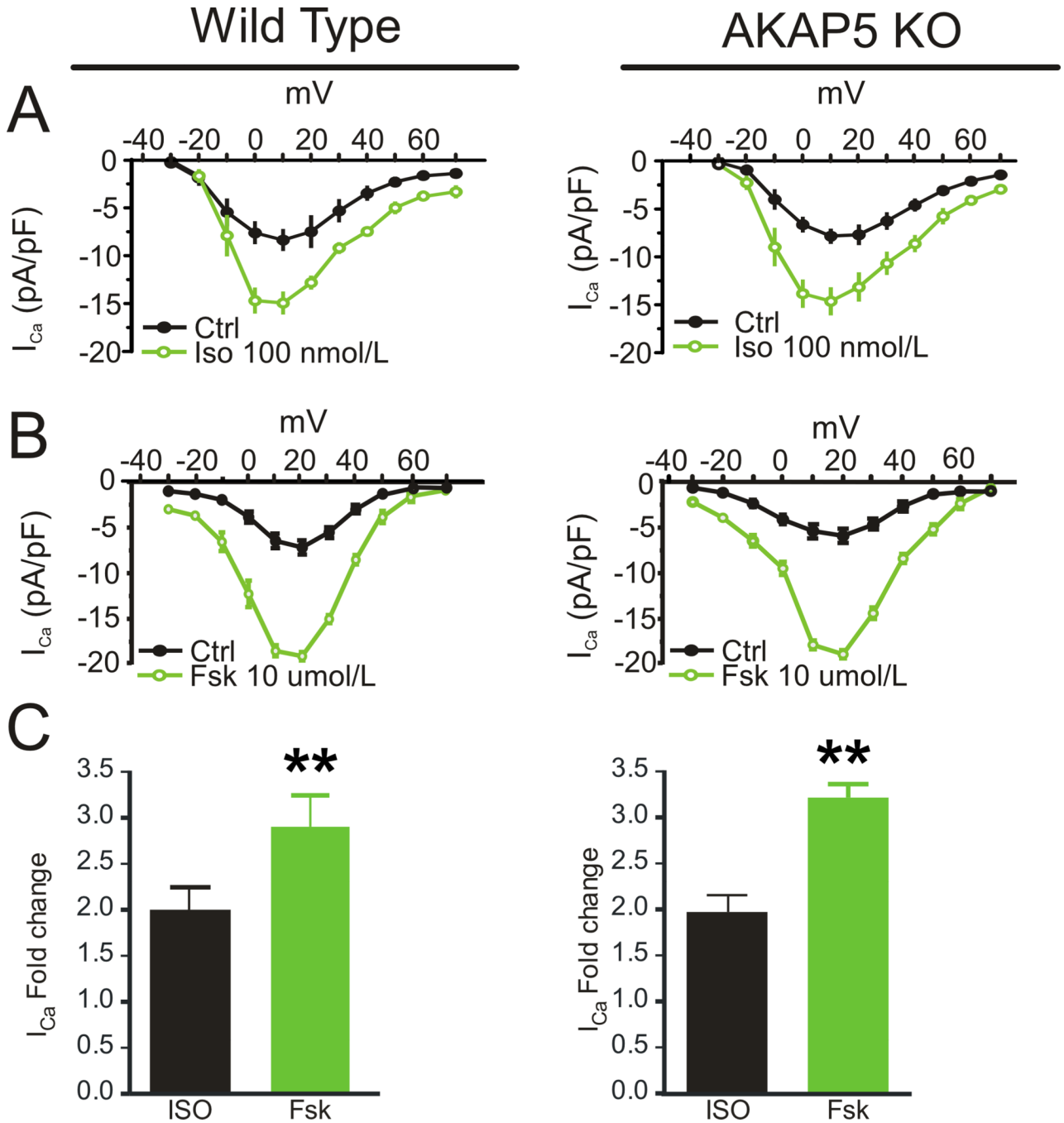
**Figure 4. β-adrenergic stimulation of calcium sparks and phosphorylation of RyR<sub>2</sub> and PLN**  
**A, B**, Left panel: Iso (100 nmol/L) stimulation of [Ca<sup>2+</sup>]<sub>i</sub> sparks in WT and KO. Nifedipine (Nif, 25 nmol/L) was used to block L-type Ca<sup>2+</sup> channels. Middle panel: SR [Ca<sup>2+</sup>]<sub>i</sub> load as measured by caffeine-induced [Ca<sup>2+</sup>]<sub>i</sub> release under control and Iso (100 nmol/L) stimulation in WT and KO cells. Right panel: Cells perfused with different concentrations of external Ca<sup>2+</sup> to measure increases in [Ca<sup>2+</sup>]<sub>i</sub> spark rate in WT and KO cells (4 animals, 8 cells/animal). **C, D**, Top panels: Western blots from isolated Wt and KO cardiomyocytes showing phosphorylated RyR<sub>2</sub> at Ser2808 and total RyR<sub>2</sub> after 3 min treatment with Iso (100 nmol/L), Fsk (100 nmol/L), or 8-CPT-cAMP (20 μM). Bottom panels:

Immunocytochemistry of isolated cardiomyocytes to detect phosphorylated RyR<sub>2</sub> at Ser2808 and total RyR<sub>2</sub> after a 3 min treatment with Iso (100 nmol/L), Fsk (100 nmol/L), or 8-CPT-cAMP (20 μM). **E, F**, Western blots from isolated WT and KO cardiomyocytes to detect phosphorylated PLN at Ser16 and total PLN after a 3 min treatment with Iso (100 nmol/L), Fsk (100 nmol/L), or 8-CPT-cAMP (20 μM). Westerns and images are representative of 3 separate experiments. (\*\*P<0.001)



#### Figure 5. AKAP5 dependent signaling complexes

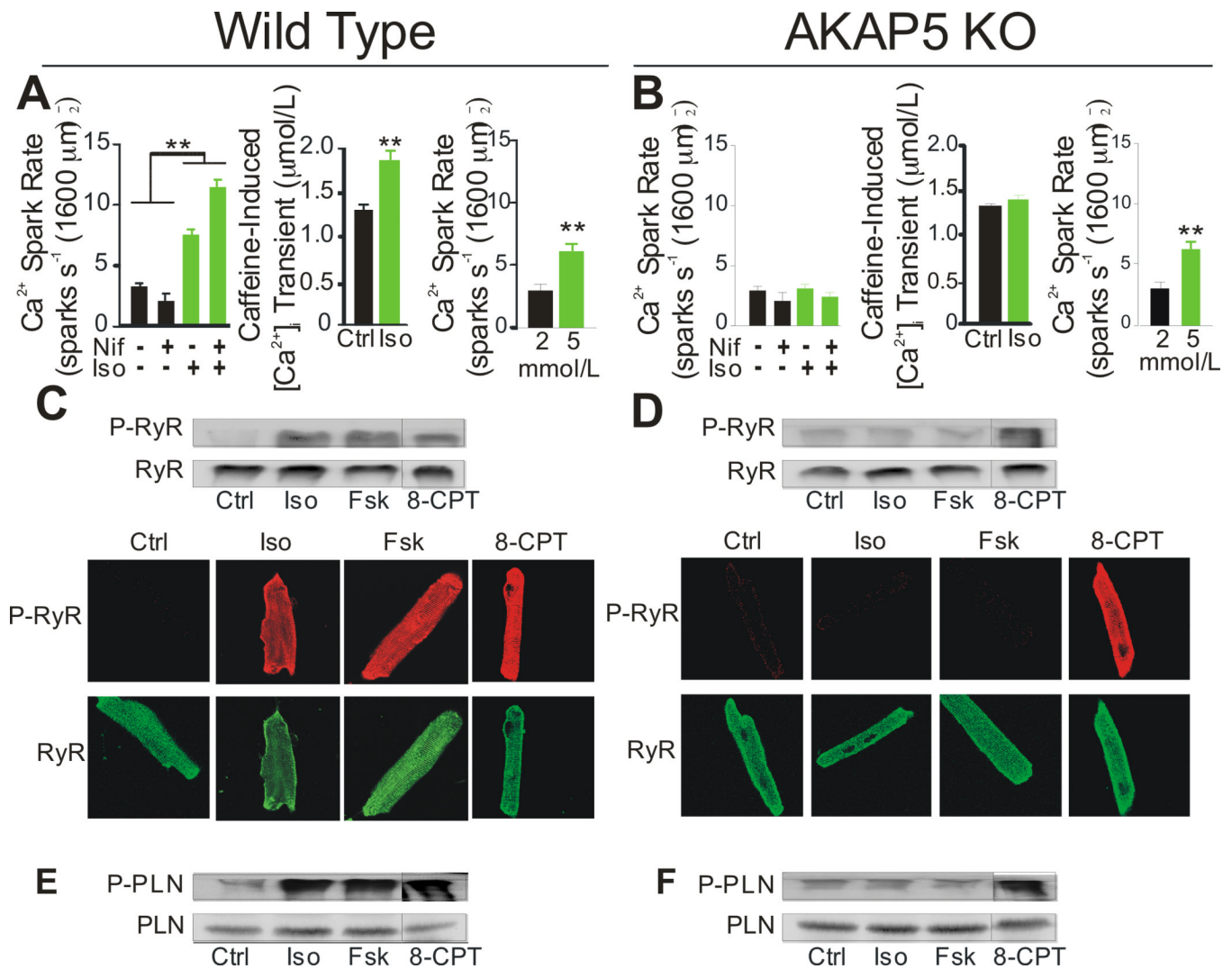
Immunoprecipitations were done from heart extracts of WT and KO animals. Tissue was homogenized in buffer containing 1% TX-100 and 0.5% sodium deoxycholate and antibody-bound proteins were recovered using protein G magnetic beads as described in Supplemental Methods. **A**, Immunoprecipitations with antibodies against AKAP5, AC6, RII $\alpha$ , PP2B, Ca<sub>v</sub>1.2, CAV3, and RyR<sub>2</sub> were run on western blots and probed with the antibodies listed under immunoblot (IB). **B**, Immunoprecipitations with antibodies against AKAP5, AC6, RII $\alpha$ , Ca<sub>v</sub>1.2, CAV3, and RyR<sub>2</sub> were probed with antibodies for  $\beta$ 1 and  $\beta$ 2-AR.



**Figure 6. Phosphorylation of  $Ca_v1.2$  in cardiomyocytes after isoproterenol or high forskolin**

**A**, Isolated WT and KO cardiomyocytes were treated with isoproterenol (Iso, 100 nmol/L), or forskolin (Fsk, 10 umol/L) for 90 sec. Extracts were made and immunoprecipitated with an antibody against CAV3 as in Figure 5. An equal fraction of input (In), supernatant from the CAV3 immunoprecipitation (Sup) and the immunoprecipitated fraction (IP) were loaded for western blotting and probed with antibodies against Cav1.2 and P- $Ca_v1.2$  (Ser1928) and CAV3. Intact P- $Ca_v1.2$  (~240 kDa) and the P- $Ca_v1.2$  tail (~60 kDa) are shown. Total  $Ca_v1.2$  at ~240 kDa and CAV3 at 18 kDa are shown. **B**, The P- $Ca_v1.2$ / $Ca_v1.2$  ratio based

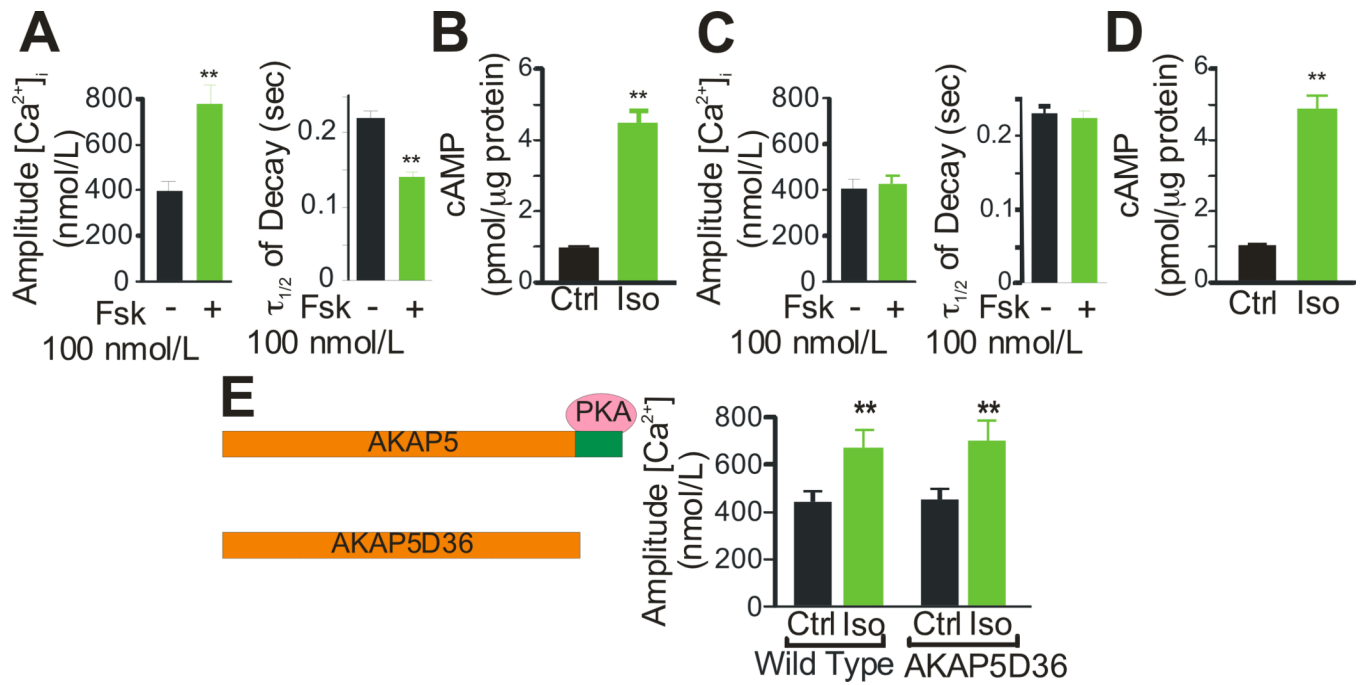
on densitometry and averaged from 3 separate experiments is shown for WT cells (left panel) and KO cells (right panel) (mean  $\pm$  s.e.m, N=3).



**Figure 7. Immuno-localization of CAV3 and AC6 in WT and KO cardiomyocytes**

**A**, Immunocytochemistry of CAV3 (left panel) and AC6 (center panel) with the merged image (right panel) in WT and KO ventricular cardiomyocytes. Bar = 5 μm.

**B**, Pearson's product-moment coefficient as a measure of co-localization of CAV3 and AC6. WT, n=30. KO, N=29 cells (mean ± s.e.m, \*\* = P<0.001).



**Figure 8. Model of the hypothetical signaling complex at the T-Tubule/SR junction that responds to sympathetic stimulation**