Sympathetic Stimulation of Adult Cardiomyocytes Requires Association of AKAP5 With a Subpopulation of L-Type Calcium Channels

C. Blake Nichols, Charles F. Rossow, Manuel F. Navedo, Ruth E. Westenbroek, William A. Catterall, Luis F. Santana, G. Stanley McKnight

Rationale: Sympathetic stimulation of the heart increases the force of contraction and rate of ventricular relaxation by triggering protein kinase (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 cardiac transients.

Objective: We examined the function of AKAP5 in the β-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 β-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 subpopulation of Ca$_{1.2}$ L-type calcium channels. Only the caveolin 3-associated Ca$_{1.2}$ 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 noncaveolin 3-associated calcium channels become phosphorylated after β-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. (Circ Res. 2010;107:747-756.)

Key Words: AKAP • Ca$^{2+}$ channels • adenylyl cyclase • cAMP • PKA

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 Ca$^{2+}$ channels (LTCCs) in the transverse-tubules (T-tubules) and surface sarcolemma create local elevations in [Ca$^{2+}$], that activate closely apposed ryanodine receptors (RyRs) in the sarcoplasmic reticulum (SR) by the mechanism of Ca$^{2+}$-induced Ca$^{2+}$ release (CICR). This causes release of Ca$^{2+}$ from the SR, thereby inducing a global, cell-wide increase in [Ca$^{2+}$], that activates contractile proteins. LTCCs are rapidly inactivated by a Ca$^{2+}$-dependent mechanism and SR Ca$^{2+}$ release is also terminated, which allows the SR Ca$^{2+}$-ATPase (SERCA) to recover the released Ca$^{2+}$ before the next heart beat. A major negative regulator of SERCA is phospholamban (PLN), which binds to and decreases the affinity of SERCA for Ca$^{2+}$. Phosphorylation of PLN by Protein kinase (PKA) causes dissociation of PLN from SERCA, increasing the rate of Ca$^{2+}$ reuptake into the SR. Unlike the action potential, the amplitude of the global [Ca$^{2+}$], 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 [Ca$^{2+}$], 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 β-adrenergic receptors (β-ARs) increases the force of contraction (inotropy) and accelerates the rate of relaxation (lusitropy) as part...
of the “fight or flight” response. Chronic activation of this pathway has been linked to an increased risk of heart failure and arrhythmias. In this pathway, activation of β-ARs results in the production of cAMP and the activation of PKA. Both β1-AR and β2-AR are expressed in heart and both are capable of Goα-mediated activation of adenyl cyclase. However, activation of the β2-AR appears to produce only a transient and highly localized burst of cAMP which may be limited by subsequent coupling to Goi. The duration and spatial dispersion of cAMP is limited by PDE4D isoforms which have been reported to be recruited directly to β-ARs. 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 (eg, Ht31) that compete for PKA binding and thereby disrupt interaction with all AKAPs. However, over 11 AKAPs have been identified in cardiomyocytes and elucidating the specific role of each AKAP remains a long-term objective. Several AKAPs have been identified that interact with Ca2+ regulating proteins in adult cardiomyocytes: for example, AKAP7α (AKAP15/18α) binds LTCCs, AKAP6 (mAKAP) binds RyR2, AKAP12 (gravin) associates with β-AR, and AKAP7δ (AKAP18δ) binds to SERCA-PLN. AKAP5 can interact with multiple proteins, including the LTCC (Ca1.2), β-AR, adenyl cyclase, PKA, PKC, and calcineurin (protein phosphatase 2B, PP2B). In smooth muscle, AKAP5 interacts with PKC and LTCCs and is required for angiotensin II-induced hypertension. In neurons, AKAP5 localizes PP2B to the LTCC and this interaction is required for nuclear signaling. 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 Ca2+ transient.

Methods
The Akap5+/− allele was generated by gene targeting using 129S1/SvImJ ES cells and standard techniques as previously described. 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. Electrophysiology was performed using whole cell patch-clamp as described in the expanded Methods section (see the Online Data Supplement at http://circres.ahajournals.org). Whole cell [Ca2+]i transients were measured using Fluoro-4-loaded cardiomyocytes subjected to field stimulation at 1 Hz. Immunocytochemistry was performed using standard techniques as described in the Online Data Supplement. Immunoprecipitation and Western blotting were done using heart extracts homogenized in lysis buffer containing (in mmol/L): 10 Na2HPO4, 150 NaCl, 5 EGTA, 5 EDTA, 5 NaF, with 1% Triton X-100, and 0.5% Na deoxycholate. The cAMP measurements were performed on isolated cardiomyocytes in laminin-coated wells and the cAMP determined using forskolin (Fsk) to bypass the β-AR signaling pathway increased the amplitude and reduced the decay time (τ1/2) of the [Ca2+]i transient in WT cells (Figure 1C). In contrast, β-AR activation did not increase the amplitude or the decay rate of the [Ca2+]i transient in AKAP5 KO cells (Figure 1D). Because β-ARs signal through cAMP/PKA to phosphorylate Ca1.2, RyR2, and PLN, 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 reduced the decay time of the [Ca2+]i transient (Figure 2B) 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

### Non-standard Abbreviations and Acronyms

<table>
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<tr>
<td>AC</td>
<td>adenyl cyclase</td>
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<tr>
<td>AKAP</td>
<td>A-kinase anchoring protein</td>
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<td>β-AR</td>
<td>β-adrenergic receptor</td>
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<td>CAV3</td>
<td>caveolin 3</td>
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<td>CICR</td>
<td>Ca2+-induced Ca2+ release</td>
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<td>EC</td>
<td>excitation–contraction</td>
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<td>Fsk</td>
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<td>isoproterenol</td>
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<td>KO</td>
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<td>LTCC</td>
<td>L-type Ca2+ channel</td>
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<td>NCX</td>
<td>Na+/Ca2+ exchanger</td>
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<td>PLN</td>
<td>phospholamban</td>
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<td>PP2B</td>
<td>calcineurin, protein phosphatase 2B</td>
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<td>RyR</td>
<td>ryanodine receptor</td>
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<tr>
<td>SERCA</td>
<td>sarcoplasmic reticulum Ca2+-ATPase</td>
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<td>SR</td>
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### Results

**Ca2+ 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). 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 (τ1/2) of the [Ca2+]i transient in WT cells (Figure 1C). In contrast, β-AR activation did not increase the amplitude or the decay rate of the [Ca2+]i transient in AKAP5 KO cells (Figure 1D).
with higher concentrations of Iso (100 μmol/L) began to induce increased \([\text{Ca}^{2+}]_i\) 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 Fsk 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 \([\text{Ca}^{2+}]_i\) 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 \([\text{Ca}^{2+}]_i\) 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,1,2, PP2B, PKC, β-ARs, and AC.6,15 To directly test the requirement for PKA binding, we used 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

**Figure 1. AKAP5 is required for β-adrenergic stimulation of \([\text{Ca}^{2+}]_i\) transients. A and B, Staining of isolated mouse adult cardiomyocytes for α-actinin and AKAP5 in WT and KO. Scale bar, 15 μm. C and D, Iso (100 nmol/L) stimulation of whole cell \([\text{Ca}^{2+}]_i\) 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, Representative \([\text{Ca}^{2+}]_i\) traces. Middle, Bar graphs of the amplitude of the \([\text{Ca}^{2+}]_i\) transient. Right, Half-lives of \([\text{Ca}^{2+}]_i\) transient decay. Calcium transients were measured with Fluo-4 and converted to calcium concentrations as described in Methods (means±SEM; n=9 animals; 3 to 4 cells analyzed per animal; **P<0.001).**

**Figure 2. Fsk, cAMP, and the role of PKA binding to AKAP5. A and C, Fsk (100 nmol/L) stimulation of \([\text{Ca}^{2+}]_i\) transients in isolated cardiac myocytes from WT and KO animals (means±SEM; n=9 animals; 3 to 4 cells analyzed per animal; **P<0.001). B and D, Stimulation of cAMP production in isolated ventricular myocytes from WT and KO animals after a 90-second 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 \([\text{Ca}^{2+}]_i\) transients in both WT and D36 cardiac myocytes (means±SEM; n=4 animals; **P<0.001).**
potential site of interaction with Cav1.2.17 This D36 mutant protein has been shown to express normally in the brain and continues to bind PP2B although PKA binding is completely eliminated.22 In heart the D36 mutant protein continues to form a complex with both Ca1.2 and AC6 as does native AKAP5 (Online Figure IV, B and 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 β-AR stimulation of [Ca$^{2+}$]i transients.

Ca$^{2+}$ Sparks and Phosphorylation of RyR$_2$ and PLN

We investigated the effect of β-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.23 Iso treatment increased Ca$^{2+}$ spark rate in WT but not in KO myocytes (Figure 4A and 4B, left). Blocking Ca1.2 with nifedipine had no significant effect on spark rate in either WT or KO indicating that the sparks were independent of spontaneous Ca1.2 openings. The amplitude of the Ca$^{2+}$ sparks was also increased in WT but not KO cells after β-AR stimulation (Online Figure III, D and E).

β-AR stimulation increases spark frequency in isolated cardiomyocytes, but the relative contribution of RyR$_2$ phosphorylation and SR Ca$^{2+}$ loading remains controversial.24,25 Previous work has shown that an increase in the rate and amplitude of the Ca$^{2+}$ sparks during β-AR activation is linked to increased SR Ca$^{2+}$ load.18,26 SR Ca$^{2+}$ load was measured in WT and KO cells by caffeine-induced release before and after Iso stimulation. The SR Ca$^{2+}$ load was similar under control conditions but β-AR activation increased SR Ca$^{2+}$ load in WT but not KO cells (Figure 4A and 4B, middle). The failure of the KO cells to load more Ca$^{2+}$ into the SR after β-AR stimulation could be attributable to a change in the regulation of SERCA in the KO cells. Perfusion of cells with high (5 mmol/L) external Ca$^{2+}$ was used to increase SR load independently of the β-AR pathway and this resulted in increased Ca$^{2+}$ spark rates in both WT (Figure 4A, right) and KO (Figure 4B, right) cells. We conclude that the defect in β-AR-stimulated sparks is likely to be caused by a loss of PKA-dependent regulation of calcium loading in the SR although RyR$_2$ regulation may also contribute.

We next examined the PKA dependent phosphorylation of RyR$_2$ and PLN under basal and β-AR-stimulated conditions. PKA phosphorylates Ser2808 and Ser16 on PLN,27 in response to β-AR stimulation. In WT cells, there was a significant increase in the phosphorylation of Ser2808 after 100 nmol/L Iso, 100 nmol/L Fsk or 20 μmol/L 8-CPT-cAMP treatment as shown by Western blot (Figure 4C, top) or immunocytochemistry (Figure 4C, bottom). However, in KO cells, Iso and Fsk treatment did not induce phosphorylation of Ser2808, whereas 8-CPT-cAMP did (Figure 4D). PKA-dependent phosphorylation of PLN on Ser16 attenuates PLN-dependent inhibition of the SERCA.28 Phosphorylation of PLN occurred in parallel with that observed for RyR$_2$. 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 RyR2 and PLN depend on AKAP5 when the activation of Cav1.2 did not?

AKAP5-Dependent Complexes in Cardiomyocytes

To address this question we first characterized the biochemical interactions between signaling molecules that are mediated by AKAP5. Cav1.2 is located in the sarcolemma and enriched in the T-tubules, whereas RyR2 and PLN are in the SR membrane.1 AKAP5 KO myocytes lack β-AR-dependent phosphorylation of RyR2 and PLN suggesting that AKAP5 is required for targeting cAMP signaling to the SR. We used immunoprecipitation experiments with adult heart lysate to show that AKAP5 is in a complex that includes AC6, RIIα, PP2B, and Cav1.2 (Figure 5A). Because AC6 and Ca1.2 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 AC528; we have used an AC5-specific antibody to repeat these immunoprecipitations.
immunoprecipitation compared with protein interactions between AKAP5 and either Cav1.2 or CAV3 in the absence of AKAP5. This does not exclude the possibility of additional protein interactions directly and with high affinity with PP2B34 but at least under our immunoprecipitation conditions (Figure 5B). It was previously reported that only the beta-ARs were found associated with AKAP5 and CAV3 by immunoprecipitation and their association with CAV3 did not depend on AKAP5 because beta-ARs remained in the complex in the AKAP5 KO (Figure 5B). It was previously reported that only the beta-AR is associated with CAV3 and consistent with this finding we see a greater fraction of the total cellular beta-AR in the CAV3 immunoprecipitation compared with beta-AR; however, a significant fraction of the beta-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, Ca1.2, 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 Ca1.2 interacts directly and with high affinity with PP2B34 but at least under the conditions of our assay, AKAP5 is a major contributor to this association. Ca1.2, 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 Ca1.2 or beta-ARs as reported previously but they do not seem to be essential for complex formation.

Functionally Distinct Populations of Ca1.2 in Cardiomyocytes

The association of multiple signaling molecules bridged by CAV3 suggested the possibility that the Ca1.2 channels in the CAV3 complex with AKAP5 might constitute a unique population that responds preferentially to beta-AR stimulation compared to Ca1.2 in other regions of the plasma membrane. To test this hypothesis we had to develop a more quantitative analysis of the Ca1.2 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 umol/L Fsk for 90 seconds and extracts were prepared and immuno-precipitated with antibody against CAV3. We then determined the amount of total Ca1.2 and phosphorylated (P)-Ca1.2 in the input homogenate, the CAV3 associated fraction, and the supernatant from the immunoprecipitation.

The pore forming a1 subunit of the LTCC, Ca1.2, is phosphorylated on Ser1928 by PKA.36 Phosphorylation at this site serves as an indicator that PKA has been activated in close proximity to the channel although recent evidence demonstrates that Ser1928 is not essential for PKA stimulation of calcium current suggesting that other sites on Ca1.2 or the beta-subunits of the channel might be critical. Our results demonstrated that approximately 50% of the total Ca1.2 is associated with the CAV3 complex but surprisingly, only the CAV3-associated Ca1.2 was phosphorylated on Ser1928 in response to Iso in WT mice (Figure 6A). Ca1.2 is partially cleaved in vivo leaving Ser1928 in the distal C-terminal fragment but this fragment remains bound to the channel as a regulatory subunit.39 The upper P-Ca1.2 band at ~240 kDa represents the uncleaved Ca1.2 and the lower doublet at 55 to 60 kDa is the phosphorylated C-terminal tail released after in vivo processing. The C-terminal tail migrated as several bands, which may be attributable to differences in cleavage and processing or possibly additional phosphorylation sites that alter migration. When high Fsk was used we observed phosphorylation of Ser1928 on Ca1.2 in both the immunoprecipitate and supernatant 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 Ca1.2 for phosphorylation with much less phosphorylation of the CAV3-associated channels. High Fsk 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 Cav1.2 channels in WT myocytes: a CAV3 and AKAP5 associated population that is highly sensitive to Iso and Fsk and a non-CAV3 associated population of channels that are only phosphorylated when cAMP levels are much higher after 10 μmol/L Fsk treatment or after treatment with cell-permeable cAMP analogs.

To determine whether this preferential phosphorylation of CAV3-associated Cav1.2 took place under in vivo conditions we treated WT and KO mice with either Iso or the β-AR antagonist, propranolol, for 5 minutes 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 β-AR antagonists together with Iso to examine whether Ca,1.2 phosphorylation is dependent on β1 or β2-AR using cultured myocytes. In WT myocytes, Ca,1.2 phosphorylation was inhibited by 44% using the β1-AR selective antagonist, and by 20% with the β2-AR specific antagonist. Both antagonists together blocked 95% of the Iso dependent increase in Ca,1.2 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,1.2 phosphorylation in rat ventricular myocytes.

In the AKAP5 KO myocytes, Ca,1.2 phosphorylation was predominantly β1-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 (β1-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 subpopulation of Ca,1.2 channels that are associated with CAV3 and that this channel population is primarily responding through stimulation of the β1-AR. The role of β2-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 colocalization of AC6 with CAV3 by immunocytochemistry and

![Figure 7. Immunolocalization of CAV3 and AC6 in WT and KO cardiomyocytes. A, Immunocytochemistry of CAV3 (left) and AC6 (middle) with the merged image (right) in WT and KO ventricular cardiomyocytes. Bar, 5 μm. B, Pearson’s product–moment coefficient as a measure of colocalization of CAV3 and AC6. WT, n=30 cells; KO, N=29 cells. Means±SEM. **P<0.001.

![Figure 8. Model of the hypothetical signaling complex at the T-tubule/SR junction that responds to sympathetic stimulation.](https://example.com/figure8)
whether colocalization 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 colocalization with CAV3 (Figure 7). We used the Pearson product-moment correlation coefficient as a quantitative measure of the colocalization 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. Coimmunoprecipitation experiments reveal a signaling complex containing AKAP5, AC5/6, PKA, PP2B, Ca,1.2, and β-ARs that are tightly associated with CAV3 and we postulate that this complex generates a microdomain of cAMP that is juxta-tight associated with CAV3 and we postulate that this complex is also responsible for generating the microdomain of cAMP that is required to stimulate PKA-dependent phosphorylation of both the RyR2 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,1.2 channels that are conducting increased Ca2+ 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,1.2 channels in the cardiomyocyte that can be distinguished biochemically and functionally. The Ca,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 Ca2+ loading of the SR and an increased calcium transient. A second Ca,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,1.2 channels as depicted in the KO panel of Figure 8. Colocalization 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,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 because 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,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 colocalizes with RyR2 and a fraction of LTCCs supports this idea and is consistent with our results. Coimmunoprecipitation experiments have also shown an interaction between CAV3 and both the RyR2 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 RyR2 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,1.2 channels associated with CAV3 in the T-tubules unique biochemically? A recent report suggests that the localization of Ca,1.2 to CAV3 depends on association with specific channel β-subunits.44 Are the Ca,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 overstimulation 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.18 AKAP5 is also expressed in neurons, where it regulates a multitude of events underl-
ing synaptic plasticity and behavior.13,22,45 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.

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Disclosures

None.

References


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**Novelty and Significance**

**What Is Known?**

- Beta-{\textsubscript{1}}-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 subcellular localization of responses.
- Protein kinase (PK)A-dependent phosphorylation of both the L-type 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 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 adenyl cyclase to a membrane-associated complex of signaling molecules which directs the PKA phosphorylation of PLN, ryanodine receptor (RyR), and a subpopulation of LTCCs.
- The increase in LTCC current in AKAP5 knockout myocytes is caused by phosphorylation of a different subpopulation of LTCCs than in wild-type myocytes.

- Ventricular myocytes compartmentalize LTCCs such that only ~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 K^{+} channel; and AKAP6 (mAKAP), which interacts with the RyR. No specific AKAP had been shown to coordinate the beta-adrenergic response pathway before this study. We also discovered that only a subpopulation (~50%) of the ventricular LTCCs are associated with AKAP5 and only this subpopulation 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 overstimulation is associated with heart failure and pathological changes in cardiac tissue. Our results implicate AKAP5 in normal cardiac signaling, suggesting a new target to investigate in the context of signaling changes that occur during heart failure.
Sympathetic Stimulation of Adult Cardiomyocytes Requires Association of AKAP5 With a Subpopulation of L-Type Calcium Channels
C. Blake Nichols, Charles F. Rossow, Manuel F. Navedo, Ruth E. Westenbroek, William A. Catterall, Luis F. Santana and G. Stanley McKnight

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METHODS

Cardiomyocyte isolation
The Akap5<sup>−/−</sup> allele was generated by gene targeting using standard techniques as previously described. Mice were age matched littersmates of 3 to 6 months of age maintained on a C57Bl/6 background. Animals were handled in accordance with the guidelines of the University of Washington Institutional Animal Care and Use Committee. Hearts were obtained from mice, 3 – 6 months of age, euthanized by an intraperitoneal injection of pentobarbitone (100 mg kg<sup>−1</sup>). Ventricular myocytes were isolated following the procedure of Powell, Noma, Shioya & Kozlowski for guinea-pig and modified as previously reported. The cells were kept at room temperature (22-25 °C) in normal Tyrode (NT) solution composed of (mmol/L): 140 NaCl, 5 KCl, 10 Hepes, 10 glucose, 2 CaCl<sub>2</sub>, and 1 MgCl<sub>2</sub>; pH 7.4, until use (0.5-6 h after isolation).

Electrophysiology
Membrane Ca<sup>2+</sup> currents were measured using the whole-cell configuration of the patch-clamp technique with an Axopatch-200A amplifier (Axon Instruments). Patch pipettes were pulled with a Flaming Brown-type puller (Sutter Instrument Co., Novato, CA, USA) to a nominal resistance of 1-3 MΩ and filled with an internal solution of the following composition (in mmol/L): 110 potassium aspartate, 30 KCl, 10 Hepes, 5 Mg<sup>2+</sup>-ATP and 10 NaCl (pH 7.3). The 15 mV tip potential produced by this solution was corrected offline. During experiments cells were continuously superfused with normal Tyrode solution. Once a successful whole-cell patch-clamp was established, the external solution was changed to one containing (in mmol/L): 140 NaCl, 5 CsCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 Glucose, 10 Heps, and 0.01 TTX. Cells were held at -80 mV. To ensure that steady-state loading of the SR Ca<sup>2+</sup> was attained, four depolarizing pulses (1 Hz) of 50 ms duration to 0 mV were applied before the cell was depolarized to a given test potential using the following protocol: Five hundred milliseconds after the fourth conditioning pulse, cells were depolarized to -40 mV slowly (1 mV every 12-5 ms) and held at this potential for 50 ms (to inactivate Na<sup>+</sup> current) before rapid (step) depolarization to a test potential. All electrophysiological signals were analyzed using pCLAMP 6.01 software (Axon Instruments).

Field stimulation for calcium imaging
Cardiomyocytes were placed in a perfusion chamber and incubated with normal Tyrode at 22 – 25 °C. Field stimulation was performed with two platinum wires (0.5 cm separation) placed at the bottom of the perfusion chamber. An IonOptix Myopacer (IonOptix Corp, Milton, MA, USA) stimulator was used to deliver square voltage pulses (4 ms duration) with amplitude of 35 volts at a frequency of 1 Hz.

Measurements of [Ca<sup>2+</sup>]<sub>i</sub>
We used the fluorescent Ca<sup>2+</sup> indicator Fluo-4 to measure changes in [Ca<sup>2+</sup>]<sub>i</sub>. Cells were loaded with the membrane-permeable acetoxymethyl-ester form of Fluo-4 (Fluo-4 AM, Invitrogen, Carlsbad, CA, USA) as previously described for measurement of [Ca<sup>2+</sup>]<sub>i</sub> that did not involved patch-clamping (i.e. Ca<sup>2+</sup> sparks, SR Ca<sup>2+</sup> load in paced and un-stimulated cells). For experiments that involved the simultaneous measurement of electrophysiological signals and [Ca<sup>2+</sup>]<sub>i</sub>, cells were loaded with the penta-potassium salt of Fluo-4 (50 µM) through the patch pipette. Confocal imaging of whole-cell [Ca<sup>2+</sup>], was performed using a Bio-Rad Radiance 2000 confocal system (Cambridge, MA, USA) coupled to a Nikon TE300 inverted microscope equipped with a Nikon 60X oil immersion lens (NA = 1.4). This system was operated by Lasersharp 2000 (v. 4.0) software. Images were analyzed with custom software written in IDL.
language (Research Systems, Boulder, CO, USA). Background-subtracted fluorescence signals were normalized by dividing the fluorescence (F) intensity at each time point by the resting fluorescence (F₀). Calibration of fluorescence signals was performed using the 'pseudo-ratio' equation ⁵:

\[
[\text{Ca}^{2+}]_i = \frac{K_d (F/F_0)}{(K_d/[\text{Ca}^{2+}]_{\text{rest}} + 1 - (F/F_0))},
\]

where F is the fluorescence intensity, F₀ is the resting fluorescence, K_d is the dissociation constant of Fluo4 (1100 nmol/L) and [Ca^{2+}]_{\text{rest}} is the resting Ca^{2+} concentration (150 nmol/L). The rate of decay of [Ca^{2+}]_i transients was obtained by fitting the decaying phase of calibrated fluorescence signals with a standard single exponential function. \(F_{\text{max}}\) was determined at the end of each experiment by exposing cells to solution NT (see above) to which the Ca^{2+} ionophore ionomycin (10 µM), 2,3-butanediol monoxime (BDM, 20 mmol/L; to prevent contraction), and 20 mmol/L external Ca^{2+} had been added.

Measurement of SR Ca^{2+} content and Ca^{2+} Sparks

Caffeine induced [Ca^{2+}]_i transients were induced as previously described ⁶. The amplitude of the [Ca^{2+}]_i transient evoked by the application of a Ca^{2+}- and Na^{+}-free (substituted with N-methyl-d-glucamine) solution containing 20 mmol/L caffeine (10 s; via a picospritzer) was used as an indicator of SR Ca^{2+} content. To ensure steady-state SR Ca^{2+} load, cells were subjected to a minimum of 10 preconditioning pulses (1 Hz) before caffeine was applied. Isoproterenol treatment was applied for 90 sec prior to measurement of SR Ca^{2+} content and Ca^{2+} sparks.

Ca^{2+} sparks were measured by loading cells with the potassium salt of Fluo-4-AM (50 µM, Invitrogen, Carlsbad, CA, USA). Fluorescence signals were then collected from unpaced cells using a Nikon swept field confocal microscope. Background-subtracted fluorescence signals were normalized by dividing the fluorescence (F) intensity at each time point by the resting fluorescence (F₀). Images were scanned using an argon ion laser beam for illumination at 488 nm (LiveScan Swept Field Confocal Microscope, Nikon, Melville, NY, USA). Emitted fluorescence was detected from 505 - 630 nm. XY confocal images were acquired every 45 to 55 ms. Automated analysis of images for Ca^{2+} sparks was performed using custom routines, written in Interactive Data Language (IDL version 6.2) as reported previously ⁷, ⁸.

NCX function was assessed as the rate constant of [Ca^{2+}]_i decline (\(k_{\text{Ca}}\)) in the presence of caffeine⁹. To ensure steady-state SR Ca^{2+} load, cells were subjected to a minimum of 10 preconditioning pulses (1 Hz) before caffeine was applied. The time point used to measure NCX function with caffeine treatment was 90 sec after isoproterenol application.

Immunoprecipitation and Western blot analysis

Lysate buffer contained (in mmol/L): 10 Na₂HPO₄, 150 NaCl, 5 EGTA, 5 EDTA, 5 NaF, with 1% TritonX-100 and 0.5% Na Deoxycholate. Before use protease and phosphatase inhibitor cocktail (P8340 and P2850, respectively, Sigma-Aldrich, St. Louis, MO, USA) was added. For isolate cardiomyocytes, 200 µL of lysate was added to each well then scraped and a hand held polytron PT-1200E (Kinematica, Bohemia, NY, USA ) used to homogenize cells. For the in vivo experiments, 5 mg kg⁻¹ of isoproterenol stabilized with 10 mg kg⁻¹ ascorbic acid or 5 mg kg⁻¹ propranolol was dosed intraperitoneal followed within 30 seconds with an intraperitoneal injection of pentobarbitone (100 mg kg⁻¹) to euthanize the mouse. The hearts were removed and washed once in ice cold PBS, pH 7.4. Hearts were homogenized at 5% (wt/vol) in lysate buffer with a polytron PT-10-35 (Kinematica, Bohemia, NY, USA) for 10 seconds. The homogenate was then spun at 10,000 x g for 10 min at 4 °C and the pellet discarded. The protein concentration of the supernatant was determined using the BCA assay (Thera Scientific, Rockford, IL, USA). For immunoprecipitation, one mg of protein was diluted into a 500 µL volume of IP buffer (in mmol/L): 10 Na₂HPO₄, 150 NaCl, 5 EGTA, 5 EDTA, 5 NaF, 0.1%
tritonX-100 with fresh protease and phosphatase inhibitors. Two to four µg of capture antibody was added to the lysate and mixed at 4 – 6°C overnight. The next day 100 µL of magnetic protein G beads were added to the lysate and washed per manufactures instruction (Millipore, Billerica, MA, USA). SDS-PAGE and Western blots used standard techniques\textsuperscript{10}. The antibodies used for immunoprecipitation were: AKAP 150 (N-19): sc-6446, Adenyl Cyclase V/VI (C-17) sc-590, and PKA IIα reg (C-20) sc-908 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); Ryanodine Receptor, 559279 (Calbiochem, Merck KGaA, Darmstadt, Germany); Caveolin 3, 910420, Caveolin 1, 611338 (BD Transduction Laboratories, San Jose, CA, USA); Calcineurin (α-Subunit), C1956 (Sigma-Aldrich, St. Louis, MO, USA). Antibodies used for western blotting were as follows: AKAP150 C-20, sc-6445, Adenyl Cyclase V/VI (C-17) sc-590, and PKA IIα reg (C-20) sc-908 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); Ryanodine Receptor, 559279 (Calbiochem, Merck KGaA, Darmstadt, Germany); Caveolin 3, 910420, Caveolin 1, 611338 (BD Transduction Laboratories, San Jose, CA, USA); Calcineurin (α-Subunit), C1956 (Sigma-Aldrich, St. Louis, MO, USA); β1-Adrenergic Receptor, ab3546-100 and β1-Adrenergic Receptor, ab13989-50 (Abcam, Cambridge, MA, USA); CNC1 rabbit polyclonal for the α1c subunit of Ca\textsubscript{v}1.2 and the phospho-Ser1928 Ca\textsubscript{v}1.2 were generated as described previously\textsuperscript{11}. The antibodies specific for phospho-Ser2808 on the RyR\textsubscript{2} were either a gift from A.R.Marks (Columbia University) or purchased (A010-30, Badrilla, United Kingdom). The antibody specific for AC5 was a gift from S.F.Vatner, New Jersey Medical School\textsuperscript{13}.

**Immunocytochemistry**

Standard immunocytochemistry techniques were described previously\textsuperscript{4}. Cells were incubated in M199 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with pen/strep (Invitrogen, Carlsbad, CA, USA) at 37 °C for 2 – 4 hr prior to treatment with drugs for the times indicated in Figure legends. Media was removed and the cells fixed with an ice cold solution of 2% paraformaldehyde in methanol for 10 min. Fixed cells were washed three times for 5 min each in phosphate buffered saline (PBS, pH 7.6). Blocking buffer, consisting of 20 % normal goat serum (Jackson ImmunoResearch Laboratories, Inc.) in an antibody dilution buffer (1 % IgG-free, protease-free BSA and 0.1 % Triton X-100 in PBS) was used for 30 min at room temperature to enhance cell permeability and to decrease non-specific binding of the antibodies. Cells were then washed three times in PBS. Myocytes were incubated overnight at 4 °C with the primary antibodies in antibody dilution buffer (1:500). The following day, cells were incubated for 2 h at room temperature with Alexa Fluor 488-conjugated (1:500) and Alexa Fluor 568-conjugated [1:500] secondary antibodies diluted in antibody dilution buffer. Following another wash with PBS, cells were mounted on slides using ProLong Antifade medium (Molecular Probes, Invitrogen, Carlsbad, CA, USA).

Slides were imaged using the Zeiss LSM 510 META at the Keck Microscopy Facility, University of Washington, using the Argon ion laser with lines at 488 nm, and Helium-neon laser with a 543 nm. Images were collected using a 63X Oil Immersion (PLAN APO N.A. 1.40). The fluorescence emitted by Alexa-488 and Alexa-568 was separated by the appropriate set of filters. Primary antibodies used were the same as above for western blots and we also used α-actinin A7811 (Sigma-Aldrich, St. Louis, MO, USA) as a marker for Z-lines.

**Cyclic AMP Assay**

All experiments were performed at 37°C. The cardiomyocytes were plated in 6 well plates coated in mouse laminin, 10 µg per well, (23017-015, Invitrogen, Carlsbad, CA, USA). Cells were incubated in M199 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with pen/strep (Invitrogen, Carlsbad, CA, USA). One heart was divided between four wells. After 6 hours media was removed, and then replaced with media and either 100 nmol/L isoproterenol or vehicle
spited in at 1000X. After 90 sec the media was removed and to lyse the cells and stabilize the cAMP, 0.1 mol/L HCl in 100% ethanol was add to the cells. The cAMP concentration was measured using cAMP detection EAI as pre instruction (Assay Design, Ann Arbor, MI, USA). The protein concentration was measured for each well using 1% SDS to solubilize the protein and measured by BCA assay (Therma Scientific, Rockford, IL, USA), and used to normalize cAMP concentration between samples.

**Statistical Analysis**

Significant differences were determined by two-way ANOVA and post hoc Student’s unpaired t test, as appropriate. Pearson’s correlation coefficients were calculated using Imaris software (Bitplane AG, Zurich, Switzerland).

**REFERENCES**


Online Figure 1. Response of WT and KO cardiomyocytes to stimulation of the cAMP/PKA pathway. 

A, Stimulation of cAMP production in isolated ventricular myocytes from WT and KO animals after a 90 sec treatment with 100 nmol/L isoproterenol (Iso) or 10 umol/L forskolin (Fsk). B, Forskolin (Fsk, 10 umol/L) stimulation of [Ca^{2+}]_t transients in isolated cardiac myocytes from WT and KO animals. Calcium transients were measured with Fluo-4 and converted to calcium concentrations as described in methods. (mean ± s.e.m., n=9 animals, 3-4 cells analyzed per animal; **P<0.001). C, Time course of calcium transient response in field stimulated cardiomyocytes treated with various concentrations of exogenous cAMP analog, 8-CPT-cAMP, measured with Fluo-4 and converted to calcium concentrations as described in Methods (mean, N=3). D, Isoproterenol dose response of [Ca^{2+}]_t transients in isolated cardiac myocytes from WT and KO animals. Calcium transients were measured with Fluo-4 and converted to calcium concentrations as described in methods. # = WT myocytes responded to 1000 umol/L isoproterenol by rounding up preventing measurement. @ = Six of 12 WT cells treated with 100 umol/L isoproterenol responded with spontaneous transients that decreased the field stimulation [Ca^{2+}] transients amplitude. (mean ± s.e.m., n=3 animals, 3-4 cells analyzed per animal)
Online Figure II. AKAP5 KO whole cell [Ca\(^{2+}\)] \(_i\) transients fail to respond to isoproterenol even though the \(I_{\text{Ca}}\) increases. Simultaneous recording of the whole cell [Ca\(^{2+}\)] \(_i\) transients from the same cells shown in Figure 3A for LTCC current measurements. Transients were measured at +10 mV using Fluo-4.
Online Figure III. Ca^{2+} dynamics in the cardiomyocyte. A, The peak amplitude of the [Ca^{2+}]_{i} transient is shown for a representative WT and KO myocyte. During isoproterenol stimulation there is a robust increase in the peak amplitude of the [Ca^{2+}]_{i} transient in WT as expected and a very small increase in KO myocytes. B, The [Ca^{2+}] was normalized for each cell by comparing the peak amplitude of the [Ca^{2+}]_{i} transient after stimulation with Iso to that before stimulation. A small 1.09 fold difference is seen in KO myocytes after isoproterenol (Iso, 100 nmol/L) stimulation whereas the WT cells demonstrate a 1.51 fold change. (mean ± s.e.m, P<0.001, N=9). C, Average rate constant $k_{Ca}$ for [Ca^{2+}]_{i} decay during caffeine exposure used as an indirect indicator of NCX function. Ten pulses were used to fill the SR of the myocyte before caffeine (10 mmol/L) treatment. (mean ± s.e.m, 4 animals, 8 cells/animal, **P<0.001 vs. WT and KO). Details are described in Methods. D-E, Isoproterenol (Iso, 100 nmol/L) stimulation of [Ca^{2+}]_{i} spark amplitude in WT (D) and KO (E) cardiomyocytes. Nifedipine (Nif, 25 nmol/L) was used to block L-type Ca^{2+} channels (mean ± s.e.m, N=3).
Online Figure IV. Both AC5 and AC6 interact with AKAP5 and the D36 mutant still interacts with both AC6 and Ca\textsubscript{v}1.2. 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 Online Methods. **A,** Immunoprecipitations with antibodies against AKAP5, AC6, and AC5 were run on western blots and probed with the antibodies listed under immunoblot (IB). **B,** Immunoprecipitation with antibodies for Ca\textsubscript{v}1.2 were run on western blots and probed with the antibodies listed under immunoblot (IB). **C,** Immunoprecipitation with antibodies against AC6, were run on western blots and probed with the antibodies listed under immunoblot (IB).
Online Figure V. In vivo phosphorylation of Ca,1.2 in response to isoproterenol or propranolol. A, WT and KO mice were administered Iso (5mg/kg i.p.) or propranolol (5mg/kg i.p.). After 5 min, hearts were isolated and extracts were immunoprecipitated with an antibody against CAV3. An equal fraction of the input (In), the supernatant from the CAV3 immunoprecipitation (Sup) and the immunoprecipitated fraction (IP) was loaded for western blotting and probed with antibodies against Ca,1.2, CAV3, and P-Ca,1.2 (Ser1928). Both the intact P-Ca,1.2 at ~240 kDa and the C-tail at ~54 kDa are shown. B, The P-Ca,1.2/Ca,1.2 ratio based on densitometry and averaged from 4 separate experiments is shown in the right panel (mean ± s.e.m., *P<0.05).
**Online Figure VI.** Phosphorylation of Cav1.2 by β1 or β2 Adrenergic Receptor stimulation in WT myocytes and effects of Iso on LTCC current and calcium transient in β1-AR KO myocytes. 

**A.** Isolated cardiomyocytes were treated with isoproterenol (Iso, 100 nmol/L), isoproterenol plus β2-AR selective blocker ICI118551 (1 umol/L), isoproterenol plus β1-AR specific blocker CGP20712A (1 umol/L), or isoproterenol plus both ICI and CGP inhibitors (mean ± s.e.m, N=3). Extracts were subjected to western blot with antibodies against P-Cav1.2 and Cav1.2 as described in Online Figure V and the ratio of phospho to total Cav1.2 was measured by densitometry and normalized to the WT control value. The percent inhibition by antagonist is shown above the bar. 

**B.** ICa records from a representative β1 KO (Adb1 KO) ventricular myocyte before and after the application of 100 nM Iso. ICa was evoked by a step depolarization to 0 mV for 300 ms from the holding potential of -40 mV. Bar plot shows the mean ± SEM of the amplitude of ICa at 0 mV before and after Iso (n = 7, p > 0.05). 

**C.** Action potential-evoked [Ca2+]i transients in a representative β1-KO myocyte before and after Iso. Bar plot of the mean ± SEM of the amplitude of an action potential-evoked [Ca2+]i transient before and after Iso.
Online Figure VII. Localization and quantification of PP2B in WT and AKAP5 KO cardiomyocytes. 

A. Immunocytochemistry of PP2B (left panel), Ca,1.2 (center panel), and merged image (left panel) in WT and KO ventricular cardiomyocytes. Bar = 5 μm. 

B. PP2B protein expression in WT and KO heart compared to α-actin on western blots. 

C. Level of PP2B expression normalized to α-actin as a loading control. (mean ± s.e.m, N=3).