Adenylyl Cyclase Subtype-Specific Compartmentalization: Differential Regulation of L-type Ca^{2+} Current in Ventricular Myocytes

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Abstract

Rationale—Adenylyl cyclase (AC) represents one of the principal molecules in the β-adrenergic receptor (βAR) signaling pathway, responsible for the conversion of ATP to the second messenger, cAMP. AC type 5 (ACV) and 6 (ACVI) are the two main isoforms in the heart. While highly homologous in sequence, these two proteins nevertheless play different roles during the development of heart failure. Caveolin-3 is a scaffolding protein, integrating many intracellular signaling molecules in specialized areas called caveolae. In cardiomyocytes, caveolin is predominantly located along invaginations of the cell membrane known as t-tubules.

Objective—We take advantage of AC_V and AC_{VI} knockout mouse models to test the hypothesis that there is distinct compartmentalization of these two isoforms in ventricular myocytes.

Methods and Results—We demonstrate that AC$_V$ and AC$_{VI}$ isoforms exhibit distinct subcellular localization. AC$_{VI}$ isoform is localized in the plasma membrane outside of the t-tubular region, and is responsible for β$_1$AR signaling-mediated enhancement of the L-type Ca^{2+} current ($I_{Ca,L}$) in ventricular myocytes. In contrast, AC$_V$ isoform is localized mainly in the t-tubular region where its influence on $I_{Ca,L}$ is restricted by phosphodiesterase (PDE). We further demonstrate that the interaction between caveolin-3 with AC$_V$ and PDE is responsible for the compartmentalization of AC$_V$ signaling.

Conclusions—Our results provide new insights into the compartmentalization of the two AC isoforms in the regulation of $I_{Ca,L}$ in ventricular myocytes. Since caveolae are found in most mammalian cells, the mechanism of βAR and AC compartmentalization may also be important for βAR signaling in other cell types.

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DISCLOSURES
None.

INTRODUCTION

Adenylyl cyclase (AC)[1,2] represents one of the principal effector molecules in the β-adrenergic receptor (βAR) signaling pathway, responsible for the conversion of ATP to the second messenger, cAMP. cAMP activates protein kinase A (PKA) which phosphorylates a number of key Ca^{2+}-cycling proteins in cardiac myocytes, including phospholamban, ryanodine receptors, troponin I, and L-type Ca^{2+} channel $(Ca_v 1.2)$. Phosphorylation of these target proteins increases cytoplasmic Ca^{2+}, resulting in an increased contractile response of cardiomyocytes. Unlike other components of the βAR signaling pathway, over-expression of AC did not promote cardiac hypertrophy, making it an attractive therapeutic target for heart failure.[3]

The mammalian AC gene family contains at least ten members[4] with a number of AC isoforms detected in the heart at the transcript level, however, only two isoforms (AC_V and AC_{VI}) are found in abundance.[5,6] These two isoforms share approximately 65% protein sequence identity and both belong to a group of Ca^{2+}-inhibitable AC, which can be inhibited by physiological concentrations of Ca^{2+}.[5,7] A fundamental difference between the two isoforms is that AC_V appears to cause detrimental effects in the aging model of cardiomyopathy, whereas over-expression of the AC_{VI} isoform confers beneficial effects in models of cardiac hypertrophy.[8,9]

Subcellular compartmentalization of the β-AR signaling proteins allows a variety of biological functions to be regulated at the same time by only a few β-AR subtypes.[10,11] Signaling proteins that are located close to each other can form a working unit, also termed signalosome. Different subcellular localizations have been shown to occur through a number of scaffolding proteins. Previous studies by Ostrom and Insel have provided insights into the roles of caveolin-rich domain in subcellular localization of AC and β-ARs in cardiac myocytes.[12,13] Additionally, it has been documented that the interactions between different compartments of the βAR signaling are limited by phosphodiesterase (PDE)-dependent cAMP degradation.[14-16]

Caveolin is an essential molecule for the formation of caveolae, or membrane invaginations, which are found in most cell types.[17] Caveolae increase the cell surface area, which is important for vesicular trafficking, signal transduction, and macromolecular transport. In the heart, the most abundant isoform of caveolin is caveolin-3, which is associated with long membrane invaginations known as t-tubules in addition to the surface membrane.[18]

Compartmentalization contributes to the complexity of subcellular organization and alterations in a compartment's signaling pathway could lead or contribute to cardiac pathology. For example, an increase in $β_2AR$ reactivity or redistribution of $β_2AR$ from t-tubule could contribute to cardiac toxicity during the progression of cardiomyopathy.[19] Even though the concepts of compartmentalization are well documented, there is still limited understanding of how different compartments are organized and some of the key effectors in these specialized regions. Specifically, it is not known whether there is similar subcellular compartmentalization of the key effector molecules, AC_V and AC_{VI}, in the βAR signaling pathways. Moreover, it has not been established whether different βAR subtypes, namely $β_1AR$ and $β_2AR$, mediate their downstream effects *via* different isoforms of ACs in the heart. Indeed, new insights into the distinct localization and function of the two main AC

isoforms will help to shed light on these critical effector molecules during the development and progression of cardiac hypertrophy and failure, possibly provide new therapeutic rationales for these common cardiac conditions.

In order to accomplish these goals, we took advantage of AC_V, AC_{VI}, and AC_V/AC_{VI} knockout models to test the central hypothesis that there is subcellular compartmentalization of the two main AC isoforms in ventricular myocytes. $I_{Ca,L}$ was recorded as a robust readout of the βAR signaling. The effects of β_1AR *vs.* β_2AR signaling *via* the two AC isoforms were tested. Moreover, we provide new mechanistic insights into the pivotal roles of caveolin-3 as a scaffolding protein which links the specific isoform of AC and PDE leading to the specialized subcellular organization, forming a separate functional compartment. Finally, we provide evidence that caveolin-3 represents a critical molecule for the compartmentalization of the βAR signaling cascade in cardiac myocytes.

METHODS

Detailed materials and methods are provided in the Online Supplement.

Animals

All animal care and use procedures were approved by the University of California, Davis Institutional Animal Care and Use Committee. Experiments were performed in accordance with National Institutes of Health and institutional guidelines. Genetically targeted mouse models including AC_V knockout (AC_V KO)[20] and AC_{VI} knockout (AC_{VI} KO)[7] were used for the studies. The double knockout (AC_V/AC_{VI} KO) mice were generated by crossing the homozygous single KO mice.

Patch-clamp recordings

Single left ventricular myocytes were isolated from 10-12 week old animals. All experiments were performed using the conventional whole-cell patch-clamp technique at room temperature. In some experiments, a detubulation technique consisting of an osmotic shock to internalize the t-tubules was used.[21]

Cell culture and transfection

Human Embryonic Kidney (HEK) 293 cells were transfected with AC_V-HA, AC_{VI}-AU1, and Caveolin3 constructs using Lipofectamine, LTX and PLUS reagents (Invitrogen).

Western blot analyses

Western blot analyses were performed as previously described.[22] Primary antibodies used in the study include anti-β-tubulin (Abcam ab6046, 1:4,000 dilution) and anti-Ca_v1.2 (Sigma C1603, 1:200 dilution) antibodies.

Computer modeling

Rosetta-Membrane *de novo* method[23] was used for structure prediction of caveolin. We first generated 10,000 caveolin models of the transmembrane regions of caveolin followed by model clustering as described previously.[23] The center model of the largest cluster was then used for modeling of N- and Ctermini of caveolin using Rosetta loop modeling method.[24]

The AC_V and mutated AC_V molecule were modeled using the PHYRE database.[25] Only the peptide segment before the first transmembrane segment was included in the modeling. After both AC_V models were created, the peptides were truncated to 20 amino acids to mimic our experiments (amino acids 84- 104 of the mouse AC_V sequence).

Molecular graphics and analyses were performed using the UCSF Chimera package.[26] The Dock 6.4 was used for the docking of the peptides.[27,28] The docking of the peptides used a rigid algorithm (the individual parts of the peptide were kept in the same orientation). The caveolin-3 peptide was treated as a receptor and the AC_V wild type and mutant peptide was treated as a ligand. The model was run six times with a range of grid from 0.1 to 10 Å to confirm the results.

RESULTS

AC_{VI} isoform is critical for the observed β-AR enhancement of $I_{Ca,L}$ in mouse ventricular myocytes

We first evaluated the distinct role of AC_V and AC_{VI} in βAR stimulation of $I_{Ca,L}$ using whole-cell patch-clamp technique (Figure 1). Ventricular myocytes were isolated from four different groups of animals including WT, AC_V KO, AC_{VI} KO, and the double knockout (AC_V/AC_{VI} KO). Application of isoproterenol (ISO) at a concentration of 1 μM significantly increased $I_{Ca,L}$ in WT ventricular myocytes (Figure 1**a**). Similar results were observed in the AC_V KO group (Figure 1**a**, middle panels). Remarkably, ISO had no effects on $I_{Ca,L}$ in myocytes isolated from AC_{VI} KO (Figure 1a, lower panels) or AC_V/AC_{VI} KO myocytes (Online Figure I). In addition, the basal current was significantly decreased (-1.6±0.4 pA/pF at 0 mV) in AC_{VI} KO myocytes (Figure 1**a**, lower panels). There was no statistical difference in $I_{Ca,L}$ between AC_{VI} KO and the double KO myocytes. Summary data for the current density elicited at 0 mV are shown in Figure 1**b**.

$I_{Ca,L}$ was recorded 10 minutes after patch rupture for the current to stabilize. Online Figure IIa shows the time course of the current at baseline and up to 30 minutes after ISO demonstrating a relatively stable current in our recording conditions. Indeed, in all our patch-clamp experiments, we have ensured that current recordings were stable with good access before the application of ISO or blockers.

Taken together, these results suggest that the observed βAR enhancement of $I_{Ca,L}$ occur mainly *via* the AC_{VI} isoform. There are three main possibilities as to why AC_V does not contribute to the observed $I_{Ca,L}$ enhancement. First, AC_V isoform may become degraded in the absence of AC_{VI}.[7] Second, AC_V isoform may not couple directly to βAR. Third, it is possible that the stimulatory effect of the AC_V isoform may be compartmentalized and masked by the action of PDE. Indeed, suppression of β2AR stimulation by PDE was previously documented.[15] To directly test the latter possibility, we investigated the role of AC_V and AC_{VI} separately in the β1AR and β2AR signaling pathways.

β2AR-mediated enhancement of $I_{Ca,L}$ signals mainly via AC_V isoform

Application of a selective β1AR blocker, CGP-20712A, completely abolished the effect of ISO in WT cardiomyocytes (Figure 2**a**, upper panel). However, the β2AR-dependent enhancement of $I_{Ca,L}$ can be revealed by the application of selective PDE3 and PDE4 blockers, rolipram and cilostamide (Figure 2**b** & **d**). Similar to WT ventricular myocytes, application of CGP-20712A also abolished the effect of ISO in the AC_V KO (Figure 2**b**, middle panel). In contrast, additional application of PDE blockers failed to reveal the β2AR-dependent increase of $I_{Ca,L}$ (Figure 2**b** & **d**). Administration of CGP-20712A in AC_{VI} KO cardiomyocytes did not change the outcome of ISO stimulation, as there was no increase in $I_{Ca,L}$ (Figure 2**a**, lower panel). More importantly, the addition of PDE blockers in AC_{VI} KO mice revealed the β2AR-dependent enhancement of $I_{Ca,L}$ (Figure 2**b** & **d**) that was not statistically different from the WT group. Taken together, these results suggest that β2AR regulation of $I_{Ca,L}$ may signal mainly *via* the AC_V isoform and is under the strong influence of PDE (Schematic representation in Figure 2**c**).

For experiments using PDE blockers, cells were first treated with the blockers for 30 minutes before the addition of ISO. Online Figure IIb shows the time course of the current at baseline, after PDE blockers and then ISO in the presence of the blockers.

The significant decrease in the basal $I_{Ca,L}$ in AC_{VI} KO and double KO myocytes was not associated with significant changes in the expression at the protein or transcript levels (Online Figure III). Moreover, application of PDE3 and PDE4 blockers alone did not alter $I_{Ca,L}$ (Online Figure IIb) suggesting minimal basal phosphorylation of $I_{Ca,L}$. Future experiments are required to further explore the mechanisms underlying the decrease in $I_{Ca,L}$ in AC_{VI} KO mice. Nonetheless, the data so far support distinct functional roles of the two isoforms of AC in ventricular myocytes.

Two distinct populations of β₁ARs in ventricular myocytes

To directly examine the downstream signaling pathway of β₁ARs, we tested the effect of ISO in the presence of ICI-118,551 (a specific β₂AR blocker). In this condition, ISO increased $I_{Ca,L}$ in the WT myocytes by 145.8±18.8% at 0 mV (Figure 3**a**, upper panel). Application of PDE blockers in this condition further potentiated the effect of ISO by 392±63% (Figure 3**b** & **d**). A similar enhancement of $I_{Ca,L}$ in AC_V KO myocytes was observed by the application of ISO in the presence of ICI-118,551 (108±16.3% increase in the current). However, in contrast to the WT myocytes, PDE blockers failed to further potentiate the effects of ISO in AC_V KO myocytes (Figure 3**b** & **d**).

As predicted from the results in Figure 1a (lower panels), there was no enhancement of $I_{Ca,L}$ by ISO in the presence of ICI-118,551 in AC_{VI} KO myocytes. However, the application of PDE blockers revealed a marked increase of $I_{Ca,L}$ by 814.2±46% (Figure 3**b** & **d**). These results suggest that there may exist two populations of β₁ARs; one that is associated with AC_{VI} and another that is associated with AC_V and is masked by PDE under basal conditions (see diagram in Figure 3**c**). However, PDE inhibition is known to have effects that are independent of ßAR stimulation which may confound the interpretations. Therefore, additional experiments were performed to further test our hypothesis (Figure 4-6).

AC_V and AC_{VI} are the two key isoforms of AC in ventricular myocytes

In order to test the key isoforms of AC in ventricular myocytes, the effects of ISO on $I_{Ca,L}$ was tested in AC_V/AC_{VI} KO myocytes in the absence and presence of PDE blockers. There were no observable effects of ISO in these conditions (Online Figure I**a-c**), suggesting that AC_V and AC_{VI} represent the two main isoforms of AC in ventricular myocytes.

Parallel assessment of cAMP levels in ventricular myocytes isolated from AC_V/AC_V KO and WT mice was performed as previously described.[29] The same protocol for patch-clamp recordings was used. Specifically, cells were first incubated with PDE inhibitors for 30 minutes prior to the addition of ß-adrenergic agonist plus PDE inhibitors (Online Figure I**d**). Results demonstrate a lack of cAMP stimulation in AC_V/AC_V KO consistent with our patch-clamp data.

AC_V isoform is compartmentalized within the t-tubules under the strong influence of PDE while AC_{VI} is localized outside of the t-tubules

Our findings thus far support the signaling of β₂AR mainly *via* the AC_V isoform. Since previous studies have provided evidence for the localization of β₂AR within the t-tubules,[19] we tested the hypothesis that the AC_V is similarly compartmentalized within the t-tubules. After the detubulation procedure, there were no changes in the effects of ISO on $I_{Ca,L}$ in the WT, AC_V KO and AC_{VI} KO myocytes (Online Figure IV**a-b** compared to Figure 1**a**). However, there was a dramatic decrease in the cell capacitance and the amplitude of $I_{Ca,L}$

(Online Figure IV**c**, and **e**). In order to verify successful detubulation, DI-8-ANEPPS was used to demonstrate the loss of the striation pattern (Online Figure IV**d**).

More importantly, after detubulation, the application of PDE blockers failed to reveal the β_2AR enhancement of $I_{Ca,L}$ current in WT and AC_{VI} KO cardiomyocytes (Figure 4**a**, right column compared to Figure 2**b**). In addition, detubulation led to the absence of β_1AR enhancement of $I_{Ca,L}$ in AC_{VI} KO cardiomyocytes in the presence of PDE blockers (Figure 4**b**, lower panels compared to Figure 3**b**). In contrast, in WT and AC_V KO cardiomyocytes, PDE blockers could potentiate the effect of β_1AR stimulation after detubulation (Figure 4**b**, upper and middle panels). Taken together, the data support the notion that β_2ARs signal mainly *via* the AC_V isoform. AC_V isoform is compartmentalized within the t-tubules and is under the strong influence of PDE. Moreover, AC_{VI} isoform appears to be localized outside of the t-tubules and is under weaker influence of PDE.

Finally, from the detubulation experiments, the fraction of L-type Ca^{2+} channels which are localized in the t-tubule *versus* the sarcolemmal membrane are estimated to be ~ 86% (Online Figure IV**e**). In addition, we estimated the ratio of Ca^{2+} channels in the t-tubules which are regulated by β_1 *vs*. β_2ARs to be 2.4:1 (Online Figure IV**e**).

Caveolin-3 may anchor and localize AC_V within t-tubules

In order to investigate the mechanisms for the proposed compartmentalization of AC_V within the t-tubules, we tested the hypothesis that caveolin-3, a scaffolding protein, may interact with both AC_V and PDE, thus, localizing AC_V within t-tubules under the strong influence of PDE. Indeed, comparison of the amino acid sequence alignment between the N termini of AC_V and AC_{VI} isoforms reveals a putative caveolin-binding domain in AC_V but not AC_{VI} isoform (Figure 5**a**). The proposed caveolin-binding domain is found to be conserved in both mouse and human AC_V isoform and contains four aromatic amino acids including phenylalanine (PHE) at positions 94, 96, and 98, and tryptophan (TRP) at position 104 in mouse AC_V (Figure 5**a**, Online Figure V**a**).

To test the hypothesis that the putative caveolin-binding domain may interact with the previously documented caveolin-scaffolding domain in the N terminus of caveolin-3 (Figure 5**b**), we first modeled the caveolin-3 protein utilizing the Rosetta Stone algorithm with a modification for the transmembrane region (Online Figure V**b**). Utilizing the docking algorithm, we observed the predicted interactions between the putative caveolin-binding domain and caveolin-scaffolding domain (Online Figure V**d** & **f**). Changing each of the amino acids PHE 94, 96, 98, and TRP 104 in the putative caveolin-binding domain to alanine (Online Figure 5**e**) abolished the interactions in the docking procedure.

Inhibitory peptides derived from the putative caveolin-binding domain or caveolin-scaffolding domain unmask the stimulatory effects of ISO on $I_{Ca,L}$ in AC_{VI} KO mice

We next developed two inhibitory peptides (IPs) containing 20 amino acids derived from the N termini of AC_V and caveolin-3, which encompass the putative caveolin-binding domain (IP1) and caveolin-scaffolding domain (IP2), respectively. Two control peptides (CPs) were developed by mutating the critical aromatic amino acids (shown in bold in the outlined boxes in Figure 5**a** & **b**) to alanine.

If caveolin-3 is indeed interacting with AC_V and PDE to localize AC_V under the strong influence of PDE, we predict that intracellular application of IPs may disrupt the interaction between caveolin-3 and AC_V and unmask the stimulatory effects of ISO on $I_{Ca,L}$ in AC_{VI} KO myocytes. In fact, this is what we have observed (Figure 5**c** compared to Figure 1**a**, lower panel). In contrast, application of the two control peptides (CP1 or CP2) did not affect

the response of $I_{Ca,L}$ to ISO in AC_{VI} KO myocytes (Figure 5**c**). Nonetheless, there are other possible interpretations of the results. It is possible that the IPs may result in the relief of the tonic caveolin-3 inhibition on AC_V.

Similarly, we also predict a putative caveolin-binding domain in PDE4 isoforms which appears to be critical for the compartmentalization of AC_V isoform (Figure 6**a**). This sequence is found to be conserved in mouse and human PDE4 across all splice variants (more then 20). An inhibitory peptide was developed using 20 amino acid sequences encompassing the putative caveolin-binding domain of PDE 4b and 4d (IP3, Figure 6**a**). A control peptide was developed by mutating the aromatic amino acid residues to alanine (CP3). Intracellular application of IP3 resulted in the enhancement of ISO on $I_{Ca,L}$ in AC_{VI} KO cardiomyocytes (Figure 6**b** compared to Figure 1**a**, lower panel). In contrast, intracellular application of CP3 did not alter the response of AC_{VI} KO cardiomyocytes to ISO (Figure 6**b**).

The interactions between caveolin-3 and AC$_V$ may help to compartmentalize the β$_2$AR signaling

To further test the hypothesis that the interactions between caveolin-3 and AC_V may help to compartmentalize the β$_2$AR signaling, we tested the effects of the two inhibitory peptides (IP1 or IP2) on the β$_2$AR regulation of $I_{Ca,L}$ (Online Figure VI**a**). Currents were recorded in control and after ISO stimulation in the presence of a selective β$_1$AR blocker (CGP-20712A). Control experiments are shown to the right using control peptides (CP1 or CP2). In contrast to Figure 2**a**, there was a significant enhancement of $I_{Ca,L}$ in the presence of the inhibitory peptides (~22-30%, Online Figure VI**b** and **c**). The enhancement of $I_{Ca,L}$ was not observed in AC_V KO ventricular myocytes consistent with data shown using PDE inhibitors (Figure 2**b**, Middle Panel).

We further test the effects of PDE4 inhibitor alone on the β$_2$AR stimulation of WT ventricular myocytes (Online Figure VI**d**). PDE4 inhibitor alone was sufficient in relieving the endogenous PDE inhibition on the $I_{Ca,L}$ enhancement consistent with our data using IP3 (Figure 6).

Immunofluorescence confocal microscopic imaging of caveolin-3 after detubulation

In order to properly interpret the results of the detubulation shown in Figure 4, we further confirm that there were no significant changes in the localization of caveolin-3 after the detubulation process (see Online Figure VII and Online Movies). The data further support previous findings that caveolin-3 is localized predominantly in the t-tubules but also present on the surface membrane.[12,13]

DISCUSSION

βARs represent one of the most important signaling pathways in the control of heart rates and cardiac contractility. Interests in the βAR pathways stem from their seminal roles in both physiologic and pathologic conditions.[30-32] Indeed, the use of βAR blockers has been shown to be one of the most beneficial therapies for the treatment of heart failure, hypertension, and cardiac arrhythmias.[33]

Stimulation of L-type Ca^{2+} channels by β$_1$ and β$_2$ARs is mediated by different isoforms of AC

Functional differences between AC_V and AC_{VI} isoforms have not been extensively investigated until recently. The lack of isoform-specific antibodies and the relatively small amount of AC available on the membrane presented technical challenges which have

recently been overcome with the development of gene-targeted mouse models. Previous studies have provided evidence that AC_V disruption increases longevity and protect against stress.[34] In AC_{VI} KO animals,[7] there is a significant decrease in βAR reactivity (approximately 78% reduction). Double knockout animals were previously reported to be non viable, however, we found that double knockout animals could survive despite high mortality rates within the first 3 weeks after birth. After this period, the mice survive without gross abnormalities. This is similar to the limited survival rate of $\beta_1 AR$ KO animals.[35]

Our data support the divergent roles of the two isoforms of AC in the heart. In contrast to previous studies, our data suggest the critical roles of both isoforms. There is a complete lack of enhancement of $I_{Ca,L}$ by βAR stimulation using isoproterenol in AC_{VI} KO and AC_V/AC_{VI} double knockout myocytes. This is in contrast to the WT and AC_V KO myocytes. In order to explain this finding, we considered a possibility that the effects of βAR stimulation *via* AC_V isoform may be masked by PDE. Without PDE blockers, the stimulation of $I_{Ca,L}$ from isoproterenol was mostly mediated through $\beta_1 AR$ activation (Figures 2a & 3a). Detailed investigation involving the separation of $\beta_1 AR$ and $\beta_2 AR$ suggest that the effects of $\beta_2 AR$ stimulation *via* AC_V isoform are masked by PDE (as shown in Figure 2b). Moreover, the enhancement of $I_{Ca,L}$ *via* AC_V isoform appears to be compartmentalized with both $\beta_1 AR$ and $\beta_2 AR$, whereas the enhancement of $I_{Ca,L}$ *via* AC_{VI} is mediated mainly through $\beta_1 AR$. Using the detubulation technique, our data suggest that the enhancement effect of $I_{Ca,L}$ *via* AC_V is compartmentalized within the t-tubule while the effects of AC_{VI} are coupled to L-type Ca^{2+} channels which are localized outside of the t-tubules. Indeed, the data is in good agreement with previous findings suggesting that both $\beta_1 AR$ and $\beta_2 AR$ are located within the t-tubules, whereas only $\beta_1 AR$ is present outside the t-tubules.[19] Nonetheless, it remains a possibility that AC_V isoform is present in other cellular domains where it might not be coupled to the L-type Ca^{2+} channels directly. Likewise, AC_{VI} isoform may be localized but does not couple to L-type Ca^{2+} channel in the t-tubules. Future experiments are required to develop antibodies which are isoform-specific to directly establish the subcellular localization of the two isoforms.

Mechanisms underlying the distinct subcellular organization and compartmentalization of AC isoforms

The different isoforms of AC have similar primary structure containing an N-terminus, a six transmembrane domain, a C1 portion of the catalytic domain, a second six transmembrane domain, and a C2 portion of the catalytic domain. The most striking difference between AC_V and AC_{VI} is in the N-termini. The N-terminus of AC_V is much longer than that of AC_{VI}, leading us to hypothesize that the specific localization of AC_V within the t-tubule may be associated with the N-terminus. Since t-tubules are known to contain different lipid contents compared to the outer membrane, we investigated potential differences in the hydrophobicity of the N termini of the two isoforms. However, a hydrophobicity plot did not reveal significant differences between the N-termini of AC_V and AC_{VI} isoforms.

On the other hand, several isoforms of ACs have been shown to be suppressed by the addition of caveolin peptides.[36] Since caveolin is a scaffolding protein localized within the t-tubules in addition to the surface sarcolemma, we first tested the hypothesis that the AC_V isoform may possess a caveolin-binding domain. Indeed, caveolin-binding domain sequences vary greatly among different proteins, but all share a similar pattern of aromatic alternating with non aromatic amino acid residues.[37] We observe a putative caveolin-binding sequence within the N terminus of AC_V. This stretch of amino acids is absent in the AC_{VI} isoform (Figure 5**a**). Inhibitory peptides generated from the putative binding sequences are able to disrupt the interaction between caveolin and AC_V and unmask the effects of AC_V in AC_{VI} KO animals without PDE blockers. Taken together, the data support

our notion that this region within the N terminus of AC$_V$ is responsible for the association between AC$_V$ and caveolin, thus, the specific compartmentalization of AC$_V$ within the t-tubules.

The location of PDE4 isoforms along the z-line region was previously reported.[16] Two isoforms of PDE, PDE4b and 4d, have been shown to regulate $I_{Ca,L}$ in response to βAR stimulation.[16,38,39] We observe a putative caveolin-binding domain within the C termini of PDE4b and 4d isoforms which is highly conserved across species. In fact, the sequence is conserved in more than 20 different splice variants of PDE4b and 4d, suggesting its biological significance. Finally, inhibitory peptides derived from this putative binding domain unmask the effects of AC$_V$ isoform, suggesting that the binding domain is responsible for the interaction between caveolin and PDEs leading to the compartmentalization of PDE and AC$_V$ isoform within the t-tubules. The schematic representation is presented in Figure 6**c**.

Potential clinical importance and future directions

Our findings support the divergent subcellular localization of the two main isoforms of AC in ventricular myocytes. The data further suggest that the two isoforms represent distinct downstream effector molecules for β$_1$AR *vs.* β$_2$AR signaling pathways. Due to its specialized location, the effects of AC$_V$ isoform are masked by the action of PDE. Finally, the specific protein-protein interactions with the scaffolding protein, caveolin-3, is responsible for the compartmentalization of AC$_V$ isoform and PDE4b and 4d within the t-tubules of cardiomyocytes.

Our data further provide prediction for the disparate function of the two isoforms of AC in the heart. Disruption of AC$_V$ and PDE4b and 4d could promote βAR signaling. Indeed, an increase in β$_2$AR reactivity has been reported in heart failure, which was explained by the loss of t-tubules in ventricular cardiomyocytes and the redistribution of β$_2$ARs.[19] Based on our findings, the loss of t-tubules may also be predicted to disrupt the compartmentalization of the AC$_V$/PDE interaction which may further result in β$_2$AR's contribution in cardiac toxicity. Finally, our findings help explain the domain organization of βARs in the ventricular cardiomyocytes and suggest that the AC$_V$ isoform and PDE4b and 4d isoforms work in concert. Since caveolin-3 represents common scaffolding protein essential for the formation of caveolae in different cell types, our findings may be important not only for cardiac physiology and pathophysiology, but also for the organization of cellular compartmentalization in other cell types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations

AC	Adenylyl cyclase
PDE	Phosphodiesterase
AR	Adrenergic Receptor
CAV3	Caveolin-3
$I_{Ca,L}$	L-type Ca^{2+} current

Novelty and Significance

What Is Known?

- Adenylyl cyclase (AC) represents one of the principal effector molecules in the β-adrenergic receptor (βAR) signaling pathway.

- AC type 5 (ACV) and type 6 (ACVI) are the two main isoforms in the heart.

- Caveolin-3 is a scaffolding protein, integrating many intracellular signaling molecules in specialized areas called caveolae.

What New Information Does This Article Contribute?

- The two AC isoforms involved in the regulation of L-type Ca2+ current (ICa,L) in ventricular myocytes show distinct compartmentalization.

- ACV compartmentalized within the t-tubule enhances ICa, L via regulatory signaling restricted by phosphodiesterase (PDE).

- ACVI, enhances Ica, L localized to the outside of t-tubules.

- ACVI isoform is responsible for β1AR signaling-mediated enhancement of the L-type Ca2+ current (ICa,L).

- The interaction between caveolin-3 with ACV and PDE is responsible for the compartmentalization of ACV signaling.

AC represents one of the principal effector molecules in the βAR signaling pathway. Even though ACV and ACVI have been identified as the two main isoforms in the heart, it is not known whether these two isoforms have distinct subcellular localization. Here we report that enhancement of ICa,L via ACV isoform is compartmentalized within the t-tubule and is mediated by both β1AR and β2AR. In contrast, the effects of ACVI are coupled to L-type Ca2+ channels which are localized outside of the t-tubules and are mediated mainly through β1AR. We also found that a specific protein-protein interaction with caveolin-3 is responsible for the compartmentalization of ACV isoform and PDE4b and 4d within the t-tubules of cardiomyocytes. Hence, loss of t-tubules, which has been reported in heart failure, could disrupt the compartmentalization of the ACV and PDE, resulting in an increase in the contribution of βAR to cardiac toxicity.
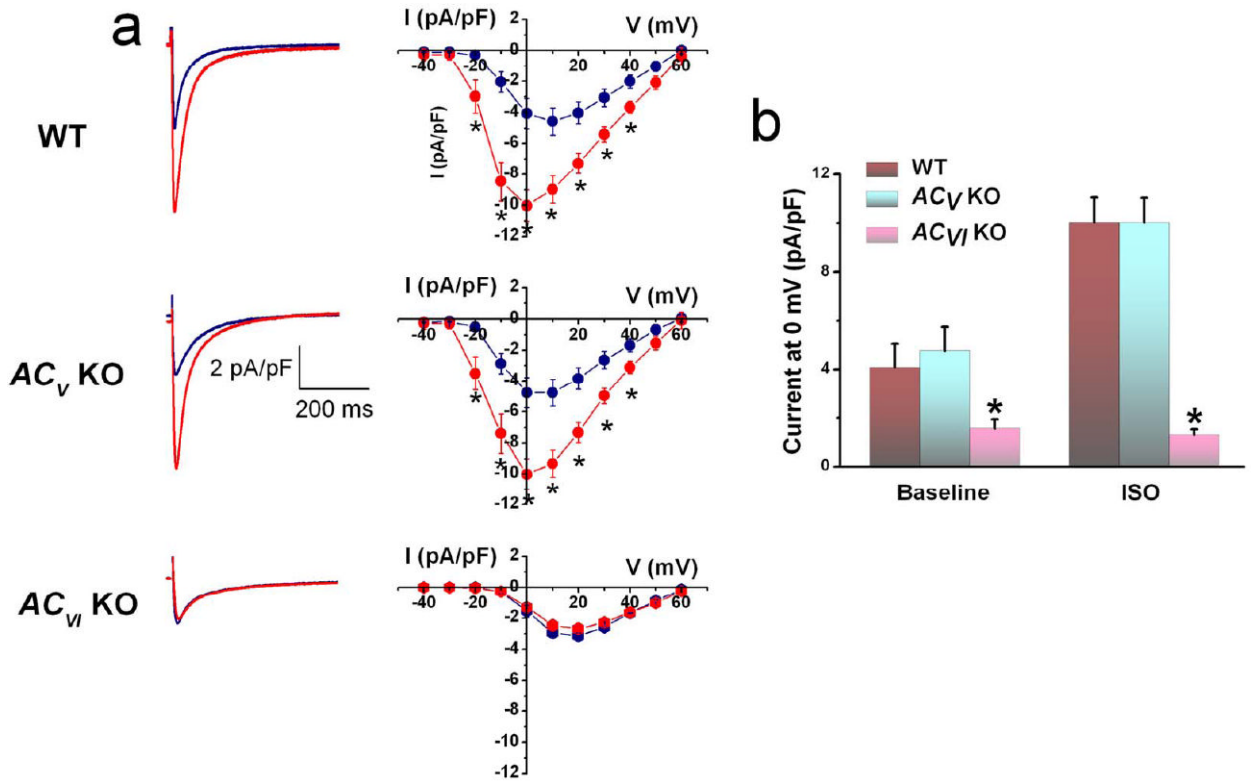
Figure 1. β-AR enhances $I_{Ca,L}$ in mouse ventricular cardiomyocytes *via* the activation of AC$_{VI}$
(**a**) Representative $I_{Ca,L}$ recorded from ventricular myocytes isolated from WT, AC_V KO, and AC_{VI} KO, respectively. Current traces were elicited using a voltage step of 0 mV for 500 ms from a holding potential of -55 mV at baseline (blue) and after 20 minutes of ISO (red). The corresponding currentvoltage (IV) relations elicited using a family of voltage steps from -40 to +60 mV from a holding potential of -55 mV are shown to the right. (**b**) Schematic representation of the known regulation of $I_{Ca,L}$ by β-AR stimulation. (**c**) Summary data of $I_{Ca,L}$ density (in pA/pF) from the three groups of mice (n=8 for each group, *$P<0.05$).
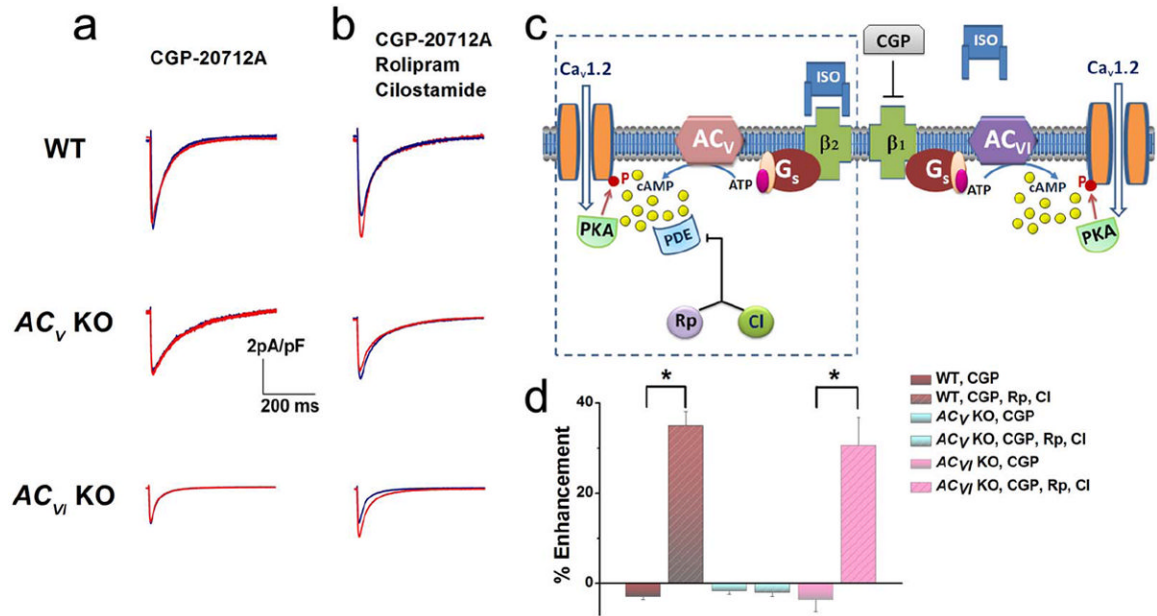
Figure 2. AC$_V$ isoform is critical for β_2AR enhancement of $I_{Ca,L}$ in the mouse ventricular myocytes

(**a**) Representative $I_{Ca,L}$ recorded from ventricular myocytes isolated from WT, AC_V KO, and AC_{VI} KO, respectively. Current traces were elicited using a voltage step of 0 mV for 500 ms from a holding potential of -55 mV at baseline (blue) and after 20 minutes of ISO (red). β_1ARs were blocked by CGP-20712A (CGP). No significant effect of β_2AR stimulation was observed. (**b**) Application of a PDE3 blocker (rolipram, Rp) and a PDE4 blocker (cilostamide, CI) revealed the effect of β_2AR stimulation only in WT and AC_{VI} KO animals. (**c**) Schematic representation of the experimental results suggesting that AC$_V$ is localized within the same compartment as β_2ARs and PDE shown in the box as outlined, separated from β_1ARs. (**d**) Summary data of the percentages of $I_{Ca,L}$ enhancement by β_2AR stimulation in the three groups of mice with and without PDE blockers (n=7-8 for each group, *$P<0.05$).
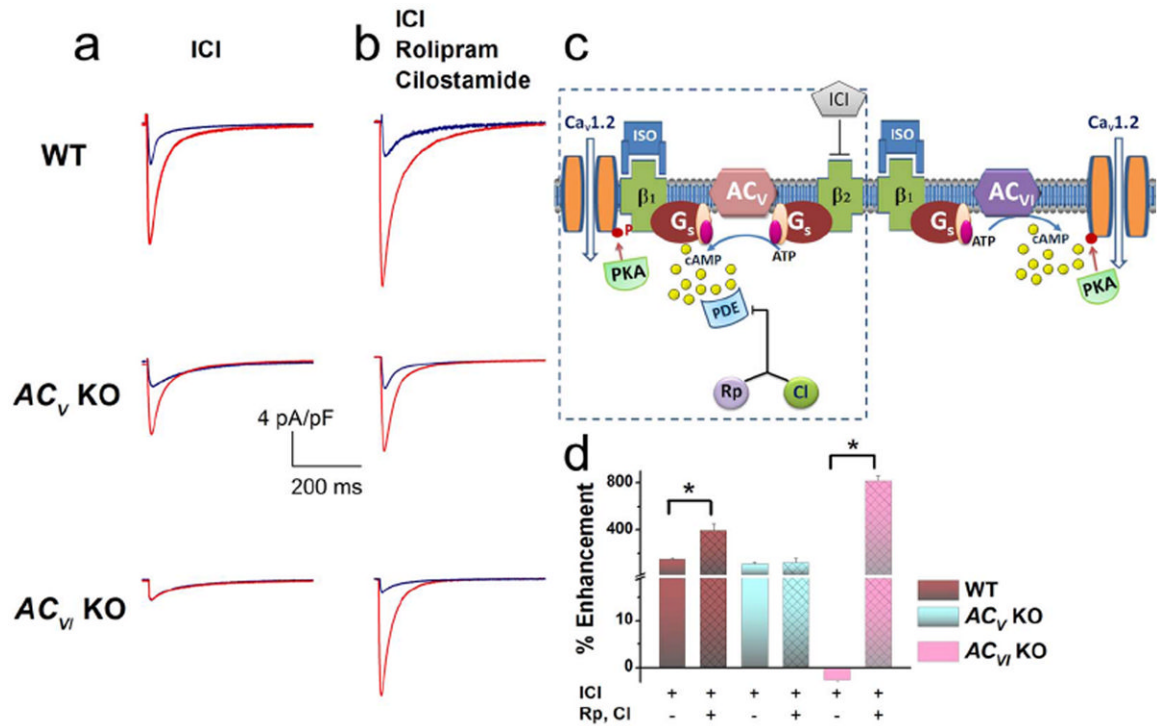
Figure 3. β₁ARs mediate the enhancement effects on $I_{Ca,L}$ *via* both AC$_V$ and AC$_{VI}$ isoforms
(**a**) Representative $I_{Ca,L}$ recorded from ventricular myocytes isolated from WT, AC_V KO, and AC_{VI} KO, respectively. Current traces were elicited using a voltage step of 0 mV for 500 ms from a holding potential of -55 mV at baseline (blue) and after 20 minutes of ISO (red). β2-ARs were blocked by ICI- 118,551 (ICI). No β₁AR stimulation of the $I_{Ca,L}$ was observed in the AC_{VI} KO group. (**b**) Application of a PDE3 blocker (Rp) and a PDE4 blocker (CI) revealed the stimulatory effect of β₁AR in the AC_{VI} KO mice and further enhanced the effects of β₁AR in the WT mice. (**c**) Schematic representation of the experimental results which suggest that β₁ARs mediate the enhancement effects on $I_{Ca,L}$ *via* both AC$_V$ and AC$_{VI}$ isoforms. Moreover, only the effect through the AC$_V$ isoform is restricted by PDE. (**d**) Summary data of the percentages of $I_{Ca,L}$ enhancement by β₁AR stimulation in the three groups of mice with and without PDE blockers (n=6-8 for each group, *$P<0.05$).
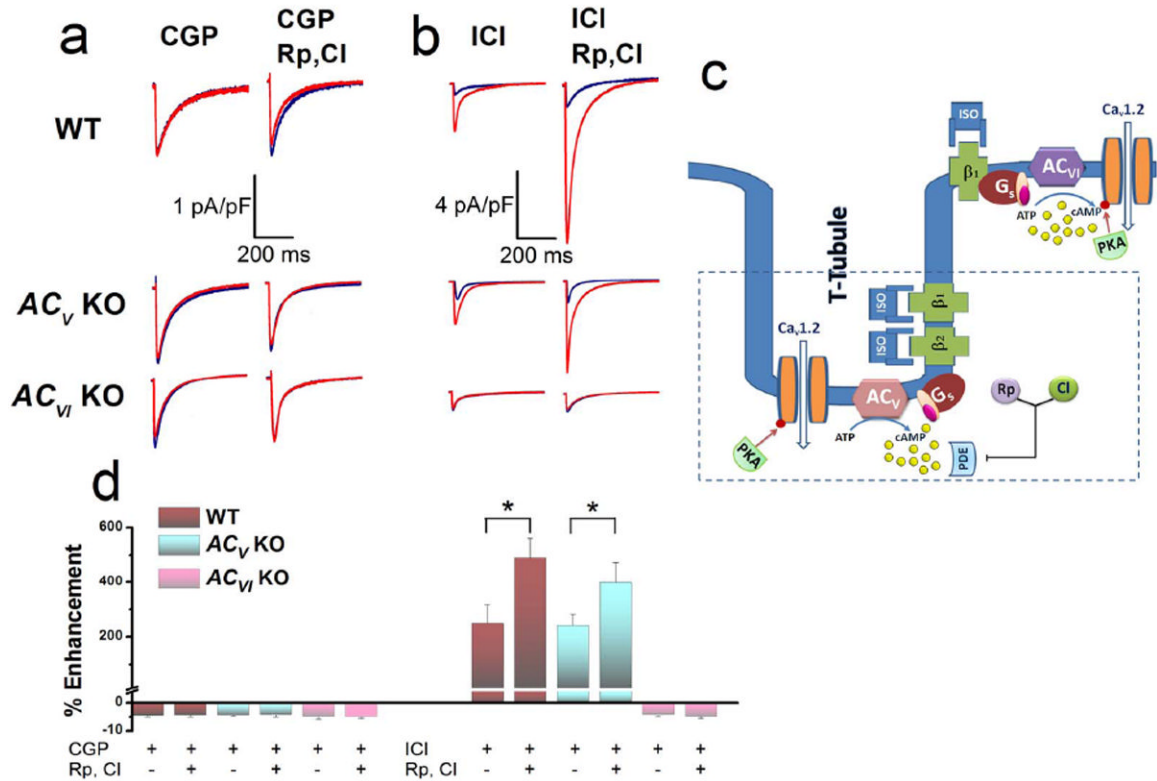
Figure 4. Detubulation abolished the enhancement effects of β₂AR stimulation on $I_{Ca,L}$ via AC_V isoform

(a) Representative $I_{Ca,L}$ recorded from ventricular myocytes after detubulation from WT, AC_V KO, and AC_{VI} KO, respectively. Current traces were elicited using a voltage step of 0 mV for 500 ms from a holding potential of -55 mV at baseline (blue) and after 20 minutes of ISO (red) in the presence of a β₁AR blocker (CGP) without PDE blockers (left column) and with PDE blockers (right column). Compared to Figure 2, after detubulation, PDE blockers failed to enhance $I_{Ca,L}$ in the WT or AC_{VI} KO mice. (**b**) Similar experiments conducted in the presence of a β₂AR blocker (ICI) without PDE blockers (left column) and with PDE blockers (right column). Compared to Figure 3, after detubulation, PDE blockers failed to enhance $I_{Ca,L}$ in the AC_{VI} KO group. (**c**) Schematic representation of the experimental results which support the compartmentalization of AC_V signaling within the t-tubule. (**d**) Summary data of the percentages of $I_{Ca,L}$ enhancement in the three groups of mice with and without PDE blockers after detubulation in the presence of β₁AR or β₂AR blockers (n=6-8 for each group, *$P<0.05$).
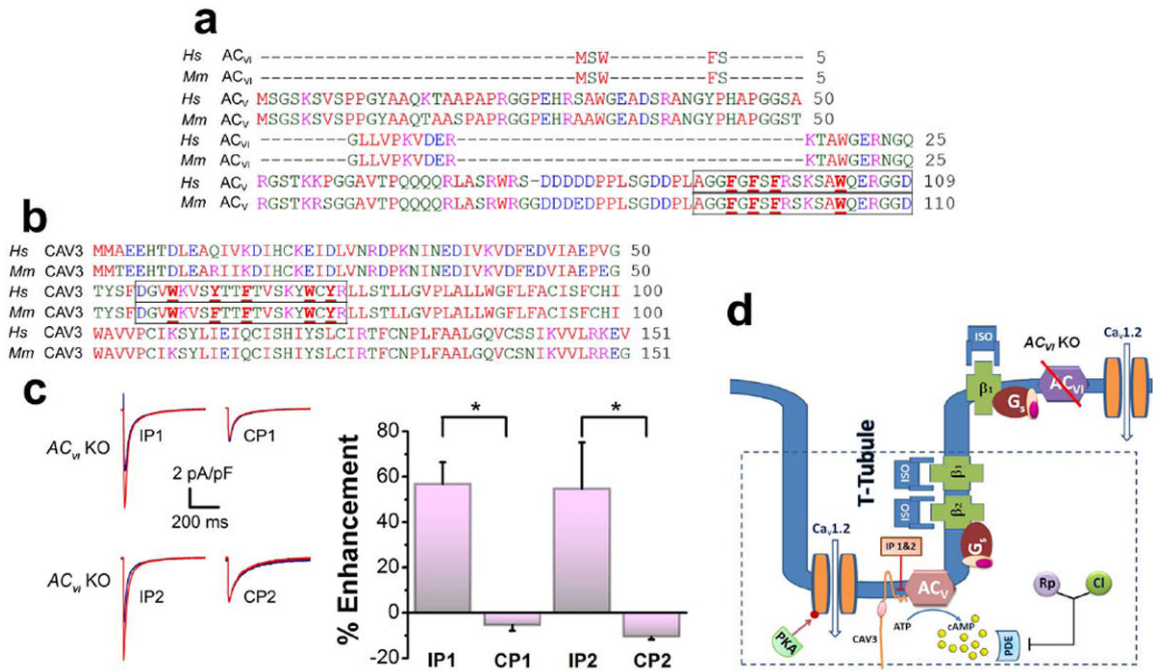
Figure 5. Caveolin-3 interacts and anchors AC$_V$ isoform within t-tubules in ventricular myocytes

(**a, b**) Amino acid sequence alignment of the N-termini of human (*Hs*) and mouse (*Mm*) AC$_V$ and AC$_{VI}$ isoforms in (**a**) and the N-termini of human (*Hs*) and mouse (*Mm*) caveolin-3 (CAV3) in (**b**). Two inhibitory peptides were generated encompassing the conserved region of AC$_V$ (outlined in the box, IP1) and the scaffolding domain in CAV3 (outlined in the box, IP2), respectively. The corresponding control peptides (CP1, CP2) were generated by mutating the aromatic amino acids (shown in bold and underlined in **a** & **b**) into alanine. Note that the AC$_{VI}$ isoform does not contain the putative caveolin-binding sequence. (**c**) Representative $I_{Ca,L}$ recorded from AC_{VI} KO in the presence of the inhibitory and control peptides in control (blue) and after 20 minutes ISO application (red). Both inhibitory peptides (IP1 and IP2) revealed the significant stimulatory effects of ISO. Summary data of the percentages of $I_{Ca,L}$ enhancement in AC_{VI} KO cardiomyocytes in the presence of the inhibitory or control peptides (n=6 for each group, *P<0.05). (**d**) Schematic representation of the experimental results which suggest a caveolin binding domain on N-terminus of the AC$_V$ isoform. Caveolin-3 interacts and anchors the AC$_V$ isoform within the t-tubules of the cardiac myocytes. A pink ellipse represents the caveolin scaffolding domain.
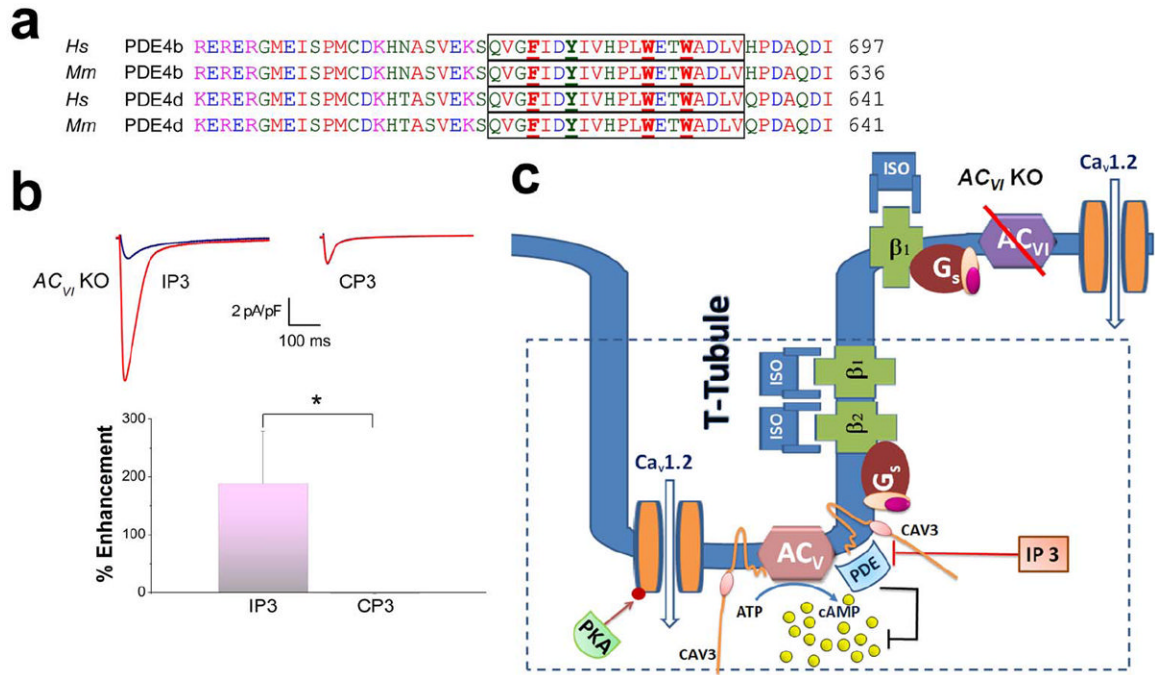
Figure 6. Cavelin 3 associates with PDE and localizes PDE to AC$_V$ compartment within t-tubules in ventricular myocytes

(**a**) Amino acid sequence alignment of human (*Hs*) and mouse (*Mm*) PDE4b and d. Both isoforms contain putative caveolin-binding domains. A 20-amino-acid inhibitory peptide was generated encompassing the putative caveolin-binding domain (outlined in the box, IP3). A corresponding control peptide (CP3) was generated by mutating the aromatic amino acids (shown in bold and underlined) to alanine. (**b**) Representative $I_{Ca,L}$ recorded from AC_{VI} KO cardiomyocytes in the presence of IP3 (left) and CP3 (right) in control (blue) and after 20 minutes ISO application (red). Lower panel shows the summary data of the percentages of $I_{Ca,L}$ enhancement in AC_{VI} KO cardiomyocytes in the presence IP3 compared to CP3 (n=6 for each group, *P<0.05). (**c**) Schematic representation of the experimental results showing the association between caveolin-3 and PDE and the compartmentalization of PDE and AC$_V$ isoform within the t-tubules in ventricular myocytes.