



Ahnak1 interaction is affected by phosphorylation of Ser-296 on Cav β_2

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ABSTRACT

Ahnak1 has been implicated in protein kinase A (PKA)-mediated control of cardiac L-type Ca²⁺ channels (Cav1.2) through its interaction with the Cav β_2 regulatory channel subunit. Here we corroborate this functional linkage by immunocytochemistry on isolated cardiomyocytes showing co-localization of ahnak1 and Cav β_2 in the T-tubule system. In previous studies Cav β_2 attachment sites which impacted the channel's PKA regulation have been located to ahnak1's proximal C-terminus (ahnak1^{4889–5535}, ahnak1^{5462–5535}). In this study, we mapped the ahnak1-interacting regions in Cav β_2 and investigated whether Cav β_2 phosphorylation affects its binding behavior. *In vitro* binding assays with Cav β_2 truncation mutants and ahnak1^{4889–5535} revealed that the core region of Cav β_2 consisting of Src-homology 3 (SH3), HOOK, and guanylate kinase (GK) domains was important for ahnak1 interaction while the C- and N-terminal regions were dispensable. Furthermore, Ser-296 in the GK domain of Cav β_2 was identified as novel PKA phosphorylation site by mass spectrometry. Surface plasmon resonance (SPR) binding analysis showed that Ser-296 phosphorylation did not affect the high affinity interaction ($K_D \approx 35$ nM) between Cav β_2 and the α_{1C} I–II linker, but affected ahnak1 interaction in a complex manner. SPR experiments with ahnak1^{5462–5535} revealed that PKA phosphorylation of Cav β_2 significantly increased the binding affinity and, in parallel, it reduced the binding capacity. Intriguingly, the phosphorylation mimic substitution Glu-296 fully reproduced both effects, increased the affinity by ≈ 2.4 -fold and reduced the capacity by $\approx 60\%$. Our results are indicative for the release of a population of low affinity interaction sites following Cav β_2 phosphorylation on Ser-296. We propose that this phosphorylation event is one mechanism underlying ahnak1's modulator function on Cav1.2 channel activity.

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1. Introduction

Ca²⁺ influx through the L-type Ca²⁺ channel (Cav1.2) located in the T-tubular invaginations of the sarcolemma triggers Ca²⁺ release from the closely apposed ryanodine receptors on the sarcoplasmic reticulum leading to cardiac contraction [1]. The channel contains the pore-forming α_{1C} subunit along with accessory Cav β_2 and $\alpha_2\delta$ subunits [2]. Cav β_2 binds the alpha interaction domain (AID) located in the α_{1C} cytoplasmic linker connecting the first and second repeated domains (α_{1C} I–II linker). This binding is pivotal for channel trafficking to the T-tubular invaginations of the sarcolemma and for Ca²⁺ current modulation [3–5]. The increase of the Ca²⁺ current upon beta-adrenergic receptor stimulation is crucial for regulation of the heartbeat and is transmitted by protein kinase A (PKA) [6]. The causative phosphorylation sites have been recently defined to reside in the C-terminus of α_{1C} upstream of the proteo-

lytic cleavage site [7]. Their phosphorylation is supposed to relieve the inhibitory control exerted by the proteolytically cleaved distal C-terminal fragment (CCT). Albeit the auto-inhibitory function of CCT is well documented [8,9], it is presently not clear whether CCT is steadily available for Ca²⁺ channel regulation as the CCT is reported to translocate to the nucleus serving as transcription regulator [10,11]. Thus, alternative routes of beta-adrenergic signaling may exist acting in complementary or hierarchical manner. Especially phosphorylation of Ser-478/79 on Cav β_2 has been suggested to be critical for PKA-mediated upregulation of Ca²⁺ current [12,13].

Emerging evidence suggest that scaffold proteins cluster different signaling molecules leading to channel subpopulations with variable composition of modulator proteins [14]. Ahnak1 is a large membrane scaffold protein of 5890 amino acids providing docking sites for a number of proteins including cytoskeletal proteins and the Cav1 channel family [15–17]. We previously defined Cav β_2 binding sites within the C-terminal ~ 1000 amino acids of ahnak1 [18]. Interestingly, ahnak1 peptides encompassing the Cav β_2 docking sites were able to modulate Ca²⁺ channel activity [19–22]. Specifically, we found that ahnak1^{5462–5535} mimicked the action of the beta-adrenergic agonist isoprenaline on Ca²⁺ current. These

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findings led us to suggest that the proximal C-terminus of ahnak1 acts as repressor in basal conditions by masking Cav β_2 functionality and that this effect could be relieved by PKA phosphorylation of the interaction partners [18,20].

In the present work we focused on Cav β_2 since recent studies revealed a more complex picture for ahnak1's implication in Ca $^{2+}$ current regulation [21,22]. We found that ahnak1-deficient cardiomyocytes responded normally to isoprenaline excluding ahnak1 as a key regulator for beta-adrenergic increase in Ca $^{2+}$ current [22]. But, ahnak1 emerged as additional player for Ca $^{2+}$ channel activity regulation [21,22]. Here, we addressed the questions which Cav β_2 regions confer binding to ahnak1's proximal C-terminus and whether it is modulated by Cav β_2 phosphorylation. We identified Ser-296 in the GK domain of Cav β_2 as novel PKA phosphorylation site that reduces Cav β_2 –ahnak1 interaction by eliminating and/or masking an attachment site but it does not modify the Cav β_2 – α_{1C} I–II linker interaction.

2. Materials and methods

2.1. Immunocytochemistry

Cardiomyocytes from adult mouse hearts were seeded onto laminin-coated coverslips in chamber slides (NUNC-4) and incubated for 2 h at 37 °C and 5% CO $_2$. Cells were washed twice with PBS, fixed for 10 min with 3.7% formaldehyde, permeabilized in PBS with 0.5% Triton X-100 for 10 min, and subsequently blocked for 30 min in PBS containing 0.2% fish skin gelatine (Sigma–Aldrich). For colocalization, the primary antibodies mouse anti-ahnak1-NT (Abnova, 5 μ g/ml) and rabbit anti-Cav β_2 (5 μ g/ml) were used in combination with secondary Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L, 2 μ g/ml, Invitrogen) and Alexa Fluor 568-conjugated goat anti-rabbit IgG (H + L, 2 μ g/ml, Invitrogen), respectively. Complete z-stacks of labeled cells were acquired using fluorescence laser scanning microscopy (LSM 510 Meta, Zeiss) and center slices were selected for display.

2.2. Recombinant proteins

Cav β_2 constructs Cav β_2^{1-59} , Cav β_2^{1-228} , Cav β_2^{1-408} , Cav $\beta_2^{195-606}$ and Cav β_2 (GenBank ID: X64297.1, *Oryctolagus cuniculus*), Cav1.2 I–II linker and ahnak1 $^{4889-5535}$ were expressed as GST fusion proteins and purified as described [15]. Ahnak1 $^{5462-5535}$ was expressed as HIS fusion protein and purified using Ni–NTA Agarose (GE Healthcare, Freiburg) as described in [20]. The mutations S296A and S296E were introduced using QuickChange site-directed mutagenesis kit (Stratagene Europe, Amsterdam, The Netherlands). For binding studies between Cav β_2 and the Cav1.2 I–II linker the GST-tag was digested from Cav β_2 using biotinylated thrombin allowing fast and efficient removal of the protease by streptavidin-agarose (Merck, Darmstadt).

2.3. Antibodies

The antibodies against ahnak1 C-terminus, Cav β_2 –pSer-296, and Cav β_2 –pSer-478/pSer-479 were generated in rabbits and affinity-purified onto coupled ahnak1 C-terminus, the synthetic peptides SNTRS–pS-LAEVQ and QHRS–pS–pS-SAPH (Biogenes, Berlin, Germany), respectively. The ahnak1-CT antibody preparation was depleted from GST-directed antibodies.

2.4. Immunocapture assay

Wells ($d = 13.2$ mm) of microtiter plates (MaxiSorp, Nunc) were immobilized with 100 nM GST-tagged Cav β_2^{1-59} , Cav β_2^{1-228} ,

Cav β_2^{1-408} , Cav $\beta_2^{195-606}$ and Cav β_2 variants in 50 mM NaHCO $_3$ buffer, pH 9.6, for 1 h at 4 °C. The surfaces were blocked with 1 ml of blocking buffer (Candor) at 4 °C overnight followed by two washing steps with buffer A (PBS, 0.1% Tween 20). Interaction with 250 ng of recombinant ahnak1 $^{4889-5535}$ was performed in buffer A containing 1% BSA and protease inhibitors (Sigma and Roche) for 3 h. After three washing steps with buffer A the proteins were harvested and run on an 8% SDS gel followed by western blot.

2.5. In vitro PKA phosphorylation

Recombinant Cav β_2 was phosphorylated in a buffer containing 300 mM NaCl, 50 mM Na $_2$ HPO $_4$ (pH 7.4), 20 mM MgCl $_2$, 0.25 mM ATP, 100 μ g purified PKA catalytic subunit [15] at 30 °C for 10 min. For the control reaction MgCl $_2$ and ATP was replaced by 2 mM EDTA to inhibit phosphorylation. The reaction was stopped by exchanging to buffer A (50 mM Hepes, 150 mM NaCl, 50 mM Na $_4$ P $_2$ O $_7$, 25 mM NaF, 10 mM EDTA, 0.2 mM DTT, 0.05% Tween-20) using PD-10 Sephadex columns (GE Healthcare).

2.6. Identification of the phosphorylation sites by NanoLC-MS/MS

An aliquot of phosphorylated GST-tagged Cav β_2 subunit was adjusted to pH 8.0 with 1 M Tris-buffer and digested with trypsin at 37 °C for 12 h. An UltiMate 3000 HPLC system (Dionex, Idstein, Germany) was coupled on-line to a 4000 QTRAP Mass Spectrometer (Applied Biosystems/MDS Sciex). The sample (5 μ l) was loaded onto a precolumn (PepMap C18, 5 μ m, 100 Å, 5 mm \times 300 μ m i.d., Dionex) using the autosampler of the Ultimate 3000 system. Peptides were eluted onto an analytical column (PepMap C18, 3 μ m, 100 Å, 150 mm \times 75 μ m i.d., Dionex) and separations were performed at an eluent flow rate of 300 nL/min. Mobile phase A was 0.06% formic acid in water and B was 0.05% FA in acetonitrile–water (8:2, v/v). Runs were performed using a gradient of 1–50% B in 30 min. MS/MS analyses were conducted by using collision energy profiles chosen on the basis of the m/z value and the charge state of the parent ion. The Bioanalyst software (version 1.4.1, Applied Biosystems) was used for processing and to submit the data to the MASCOT server (version 2.1, Matrix Science Ltd., London, UK) for in-house search against the NCBI nr protein database [23]. The mass tolerance of precursor ions and sequence ions was set to 0.4 Da. The searches included variable modifications of STY-phosphorylation and methionine oxidation. One missed cleavages using trypsin in a separate search cleavage without an enzyme was allowed.

2.7. Western Blot analysis

Protein samples were separated on 10% SDS polyacrylamide gels and transferred to nitrocellulose for 2 h at 300 mA. The transfers were incubated with affinity purified antibodies anti-ahnak1-CT, anti-Cav β_2 , anti-Cav β_2 –pSer-296 and anti-Cav β_2 –pSer-478/pSer-479 followed by peroxidase-coupled anti-rabbit IgG (Pierce, Rockford, IL, USA). Immunoreactive proteins were visualized by chemiluminescence (ECL) reaction (Millipore, Schwalbach).

2.8. Surface plasmon resonance (SPR) spectroscopy

SPR binding studies were performed using CM5 sensor chips in a Biacore 2000/X100 instrument (Biacore, Uppsala, Sweden) as described [22]. Ahnak1 served as ligand for the interaction with the Cav β_2 analyte. For Cav β_2 – α_{1C} I–II linker binding anti-GST antibodies were immobilized on the chip surface to capture GST-tagged I–II linker and Cav β_2 was applied as analyte.

2.9. Statistics

Statistical difference between mean values were calculated using one-way ANOVA followed by Bonferroni post hoc test and Student's *t*-test for paired values and were considered significant at *p*-values of <0.05.

3. Results and discussion

3.1. Ahnak1 and Cavβ₂ co-localize in cardiac myocyte T-tubules

We first addressed the question whether ahnak1 and Cavβ₂ co-localize in adult cardiomyocytes by immunocytochemistry. Confocal images of isolated cardiomyocytes double labeled for ahnak1 and Cavβ₂ reveal a cross-striated and punctual intracellular staining for both proteins (Fig. 1, upper panel). The merged image indicates a co-localization of ahnak1 and Cavβ₂ along T-tubular structures that is confirmed by line scan analysis (Fig. 1, lower panel). Ahnak1 localization in T-tubules appears to be specific for cardiac muscle since in skeletal muscle ahnak1 located along the peripheral sarcolemma and to costameres, but not to T-tubules [24,25]. In cardiomyocytes, we found that ahnak1 and Cavβ₂ co-localized also to perinuclear regions (Fig. 1, Merge). This is to our knowledge a novel observation offering the possibility that ahnak1 and Cavβ₂ may play a role in excitation–transcription coupling. Importantly, solely ahnak1 shows a pronounced and distinctive labeling of the peripheral sarcolemma. This localization likely reflects the role of ahnak1 in cytoarchitecture and in membrane resealing and repair [26–28]. Colocalization of ahnak1 and Cavβ₂ in the T-tubular invaginations of the sarcolemma provides the structural basis for ahnak1's cross-talk with the Cav1.2 channel and its role in cardiac-excitation–contraction coupling.

3.2. Mapping of ahnak1 interacting sites on Cavβ₂

We addressed the question, which sites on Cavβ₂ interact with ahnak1's proximal C-terminus (ahnak1^{4889–5535}) (Fig. 2C). Cavβ₂ belongs to the membrane-associated guanylate kinase (MAGUK) protein family and contains two highly conserved interacting domains, namely src-homology 3 (SH3) and guanylate kinase (GK) linked by a variable HOOK region as illustrated in Fig. 2A [4,29,30]. We created five GST-fused Cavβ₂ truncation mutants

spanning the variable and conserved regions (Fig. 2B), and performed capture assays followed by Western Blot analyses using an ahnak1-directed antibody. The intensity of the ahnak1 signal captured by the full length Cavβ₂ was set to 100% and served as reference. The binding assays revealed no specific binding between the N-terminus of Cavβ₂ (Cavβ₂^{1–59}) and ahnak1^{4889–5535} when compared to the negative control GST alone (Fig. 2D). The constructs Cavβ₂^{1–228} and Cavβ₂^{1–408} show interaction signals similar to the full-length Cavβ₂. Both consist of SH3 and HOOK, whereas the longer Cavβ₂^{1–408} additionally covers the GK region. While Cavβ₂^{1–228} lacking GK shows a slightly reduced binding (87%), Cavβ₂^{1–408} reaches 100% indicating that the C-terminus is dispensable for ahnak1^{4889–5535} interaction. Interestingly, Cavβ₂^{195–606} that lacks SH3-HOOK and encompasses only GK and the C-terminus has a significantly reduced binding (58%). This result points to the SH3-HOOK region as playing a major role in ahnak1^{4889–5535} interaction, which is supportively enhanced by the neighboring GK domain. In an additional experiment, an isolated His-tagged SH3 domain exhibited only minor binding, suggesting multivalent interactions with negative cross-talk in the SH3-HOOK-GK module to achieve the complete binding potential for ahnak1^{4889–5535}. It highlights the variable HOOK region that may be crucial for ahnak1 binding. Since the SH3 and GK module are highly conserved within Cavβ isoforms, they provide a basis for ahnak1 association. But, it remains to be investigated if Cavβ isoforms (e.g. Cavβ₃ and Cavβ₄) whose HOOK domain varies differ in their capability to interact with ahnak1.

3.3. Identification of Ser-296 in Cavβ₂ as novel PKA phosphorylation site

Phosphorylation of Ser-478/79 in Cavβ₂ were suggested to be critical for PKA-mediated upregulation of Ca²⁺ current in HEK cells transfected with a truncated α_{1C} and Cavβ₂ [12,13]. The mechanism was questioned by Miriyala et al. [5]. Thus, we searched for additional phosphorylation sites in Cavβ₂ and their potential impact for ahnak1 interaction. Recombinant Cavβ₂ subunit was phosphorylated by PKA and analyzed using mass spectrometry (MS). Probed on SDS–PAGE (Fig. 3A) phosphorylated Cavβ₂ revealed a migration shift of about 10 kD compared to non-phosphorylated Cavβ₂. A similar shift was observed with the truncated Cavβ₂ subunit. By MS analysis we identified the peptides ²⁹⁵SSLAEVQSEIER³⁰⁶ and ⁴⁷⁸SSSSAPHNNHR⁴⁸⁸ to be phosphorylated at Ser-296 and

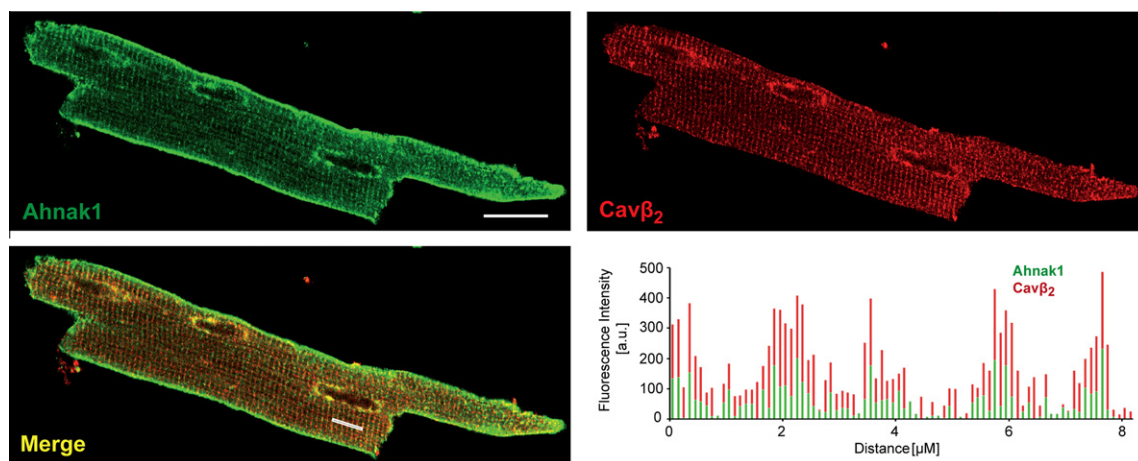


Fig. 1. Immuno-localization of ahnak1 and Cavβ₂ in mouse ventricular myocytes. Fluorescence confocal images of longitudinal sections through isolated exemplary cardiomyocytes labeled for ahnak1 (green) and Cavβ₂ (red) as indicated. The intracellular striated pattern displayed by both proteins indicates a co-localization in T-tubular structures (Merge) which is confirmed by the line scan plot demonstrating analyzed pixel intensities along the white line of the merged image. Note, a co-localization of ahnak1 and Cavβ₂ in the perinuclear compartment is observed. Ahnak1 does not co-localize with Cavβ₂ in the peripheral sarcolemma as the myocytes are outlined in green (Merge). Scale bar 30 μm.

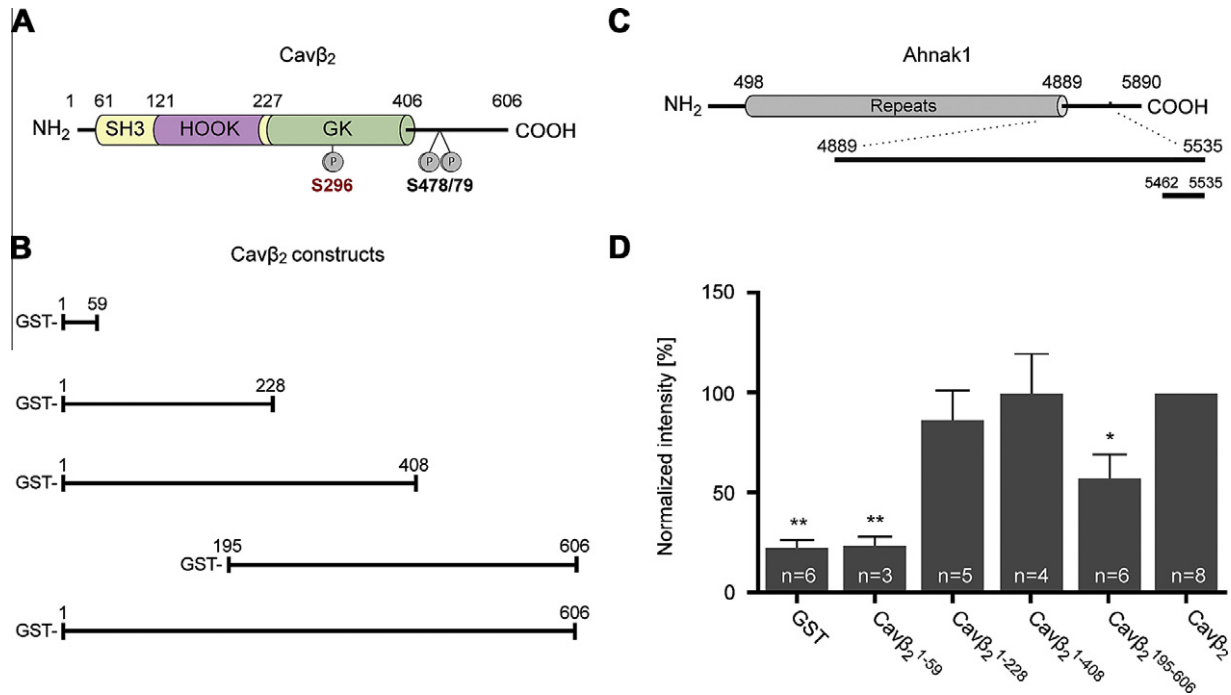


Fig. 2. (A) Molecular structure of Cavβ₂. (B) GST-Cavβ₂ constructs for ahnak1^{4889–5535} interaction site mapping are shown. The Cavβ₂^{1–59} construct consists of the N-terminus whereas Cavβ₂^{1–228} further covers the SH3-HOOK region of Cavβ₂. Cavβ₂^{1–408} spans the N-terminus, SH3-HOOK-GK, and lacks the C-terminus whereas Cavβ₂^{195–606} encompasses only the GK domain and the C-terminus. (C) Molecular structure of ahnak1 and its proximal C-terminus applied in binding assays. (D) ELISA approach analysis of ahnak1^{4889–5535} interaction with Cavβ₂ truncation mutants reveals significant differences, $F(5,26) = 13.13$, $p < 0.0001$; ** $p < 0.001$, * $p < 0.05$ compared to reference Cavβ₂ (one-way ANOVA followed by Bonferroni post hoc test).

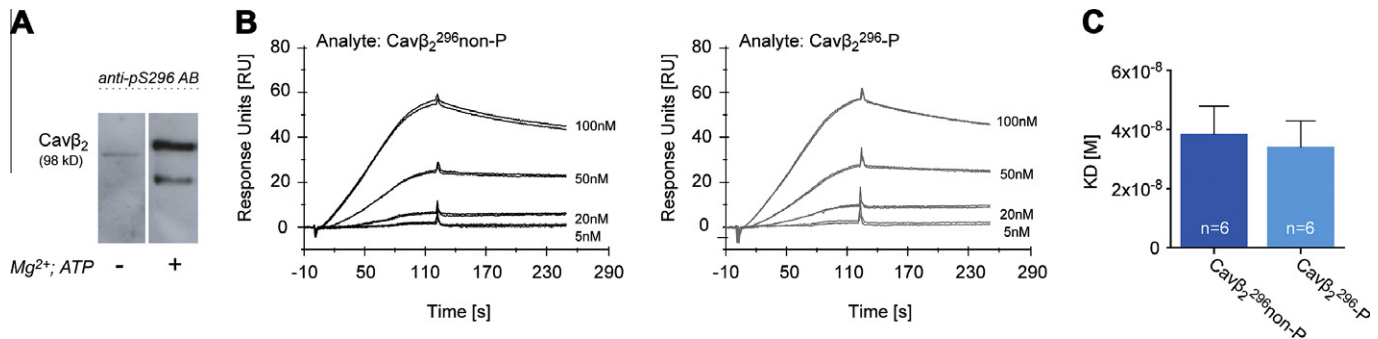


Fig. 3. (A) Western blot analysis of non-phosphorylated Cavβ₂²⁹⁶ (Cavβ₂²⁹⁶ non-P) and PKA phosphorylated Cavβ₂²⁹⁶ (Cavβ₂²⁹⁶-P) demonstrates the phosphorylation at Ser-296. (B) Representative SPR measurements between the I-II linker of Cav1.2 and Cavβ₂²⁹⁶ non-P (black curves) and Cavβ₂²⁹⁶-P (gray curves), respectively, are shown. (C) Bar graph displays K_D values of Cavβ₂²⁹⁶ non-P and Cavβ₂²⁹⁶-P indicating no significant difference.

Ser-479, respectively. We developed phospho-epitope specific anti-bodies directed against pSer-296 and pSer-478/pSer-479 and employed them to assess the specificity of the phosphorylation sites of Cavβ₂ (Fig. 3A). Importantly, Ser-296 is a novel PKA phosphorylation site within Cavβ₂ which, to our knowledge, is described here for the first time. Notably, the site resides within the GK domain (Fig. 2A), known to interact with the I-II linker of Cav1.2 to regulate Ca²⁺ channel activity [4,31] and ahnak1 mapped in this study.

3.4. Ser-296 phosphorylation has no effect on the interaction with the I-II linker of Cav1.2

As Ser-296 resides in the GK domain of Cavβ₂ (Fig. 2A) we first analyzed the potential impact of Ser-296 phosphorylation on its interaction with the I-II linker of Cav1.2. We performed real time interaction analysis by surface plasmon resonance spectroscopy (SPR). PKA was present in both cases and phosphorylation was induced by the addition of Mg and ATP. To focus only on Ser-296

we mutated Ser-479 to Ala allowing only PKA phosphorylation at Ser-296. The western blot result shows the phosphorylation of Cavβ₂²⁹⁶ (Fig. 3A). Applying SPR binding experiments non-phosphorylated and phosphorylated Cavβ₂²⁹⁶ bound the I-II linker with similar attraction (Fig. 3B, C). The high affinity values of ≈ 35 nM coincide well with data from previous studies [32,33] and confirm functionality of the used recombinant proteins I-II linker and Cavβ₂. It was reported that the high affinity interaction between both is crucial for channel trafficking; once the channel complex is incorporated into the plasma membrane, the I-II linker-Cavβ₂ affinity decreases [34].

3.5. Phosphorylation of Ser-296 on Cavβ₂ reduces its binding potential to ahnak1^{5462–5535}

To dissect whether phosphorylation of Ser-296 and/or Ser-479 on Cavβ₂ affects ahnak1^{5462–5535} interaction we performed SPR binding experiments. Recombinant Cavβ₂ was phosphorylated by

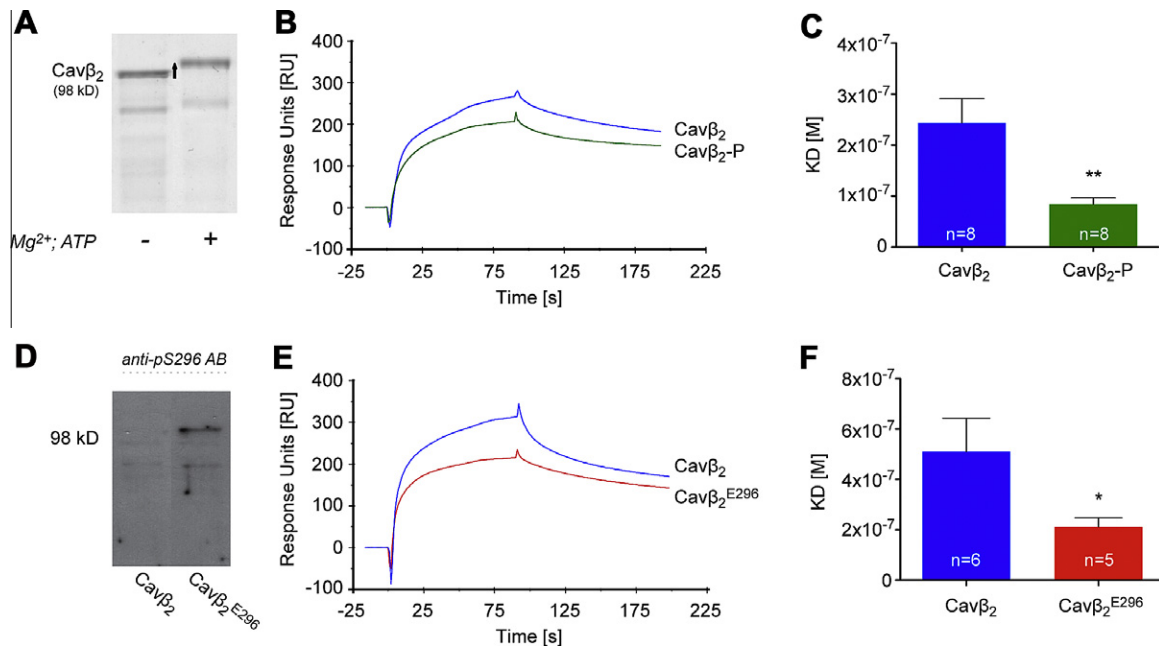


Fig. 4. (A) SDS–PAGE of Cav β_2 and PKA phosphorylated Cav β_2 . For the phosphorylation reaction PKA in the presence or absence of Mg²⁺ and ATP, respectively, was applied. (B) Representative real-time association (0–90 s) and dissociation (90–190 s) of 4 μ M of non-phosphorylated Cav β_2 (Cav β_2 , blue) and phosphorylated Cav β_2 -P (Cav β_2 -P, green) proteins as analytes to on the sensor surface immobilized ligand ahnak1^{5462–5535}. (C) Bar graphs demonstrating binding affinities (K_D) of Cav β_2 and Cav β_2 -P with ahnak1^{5462–5535}. (D) Western Blot analysis shows detection of Cav β_2 ^{E296} by a phospho-specific antibody against Ser-296. (E) Representative SPR signals demonstrating association and dissociation of Cav β_2 (blue) and Cav β_2 ^{E296} (red) with ahnak1^{5462–5535}. (F) Dissociation constant (K_D) analysis for Cav β_2 and Cav β_2 ^{E296}, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PKA representing a phosphorylation of both sites (Ser-296 and Ser-479) and subsequently passed over the ahnak1^{5462–5535} protein immobilized on a biosensor chip. Real time binding revealed a significantly lower K_D value for Cav β_2 -P compared to non-phosphorylated Cav β_2 indicating a 3-fold higher affinity between Cav β_2 -P and ahnak1^{5462–5535} (Fig. 4B). However, the binding capacity displayed as relative binding units (RU) of ahnak1^{5462–5535} and Cav β_2 -P is reduced by 34% compared to non-phosphorylated Cav β_2 (Fig. 4C). These effects provide the first evidence that Cav β_2 phosphorylation by PKA modifies ahnak1^{5462–5535} interaction in a complex manner. To further clarify the role of the novel identified phosphorylation site Ser-296 concerning the affected ahnak1–Cav β_2 binding we substituted Ser to Ala serving as control and to Glu (Cav β_2 ^{E296}) mimicking phosphorylation, respectively. Using the phosphospecific antibody against Ser-296 in western blot it was possible to detect the Cav β_2 ^{E296} variant and to discriminate it from the non-mutated Cav β_2 protein (Fig. 4D). SPR binding studies gave K_D values of 510 nM and 210 nM for Cav β_2 and Cav β_2 ^{E296}, respectively, implying a 2.4-fold higher binding affinity for the Cav β_2 ^{E296} mutant (Fig. 4F). At the same time, ahnak1^{5462–5535}'s binding capacity for Cav β_2 ^{E296} is decreased by 60% compared to Cav β_2 (Fig. 3E). Thus, phosphorylation mimic Cav β_2 ^{E296} reproduced the same effects of the PKA phosphorylated Cav β_2 on ahnak1 interaction. Given these results we assume that Cav β_2 has different populations of binding sites on ahnak1^{5462–5535}. PKA phosphorylation of Ser-296 may induce a conformational change to Cav β_2 that eliminates or masks one set of affinity sites. It is likely that a population of lower affinity sites is lost while the high affinity site population remains. Recent studies described a phosphorylation-dependent ahnak1–Cav β_2 interaction which was released after PKA phosphorylation of both binding partners accompanied with Ca²⁺ current upregulation [18,20]. We now could determine one specific phosphorylation site within Cav β_2 that is responsible for a reduced binding capability to ahnak1. We suggest that phosphorylation of Ser-296 on Cav β_2 may play an important role in the underlying complex mechanism of beta-adrenergic modulation by ahnak1 [22]. In summary, the

phosphorylation of Ser-296 Cav β_2 affects ahnak1 interaction in a complex manner and is a leading candidate for optional PKA-mediated modulation of L-type Ca²⁺ channel activity in the heart.

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