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Physical interaction between calcineurin and Cav3.2 T-type Ca^{2+} channel modulates their functions

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ABSTRACT

$Ca_v3.2$ T-type Ca^{2+} channel is required for the activation of calcineurin/NFAT signaling in cardiac hypertrophy. We aimed to investigate how $Ca_v3.2$ and calcineurin interact. We found that Ca^{2+} and calmodulin modulate the $Ca_v3.2$/calcineurin interaction. Calcineurin binding to $Ca_v3.2$ decreases the enzyme's phosphatase activity and diminishes the channel's current density. Phenylephrine-induced hypertrophy in neonatal cardiac myocytes is reduced by a cell-permeable peptide with the calcineurin binding site sequence. These data suggest that $Ca_v3.2$ regulates calcineurin/NFAT pathway through both the Ca^{2+} influx and calcineurin binding. Our findings unveiled a reciprocal regulation of Ca^{2+} signaling which contributes to our understanding of cardiac hypertrophy.

Structured summary of protein interactions:
$Ca_v3.2$ physically interacts with **cn** by anti tag coimmunoprecipitation (View interaction)

1. Introduction

Low-voltage activated (T-type) Ca^{2+} channels are present in various cell types and implicated in important cellular events such as hormone secretion, gene expression, cell proliferation and differentiation, and development of neuronal and cardiac diseases [1–4]. The pore-forming subunits of T-channels are encoded by three genes: *cacna1g*, *cacna1h* and *cacna1i*, for the $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$ subunits [5–7]. T-channels are expressed abundantly at embryonic and neonatal stages; particularly, the $Ca_v3.2$ subunit is downregulated after birth in rodent hearts [8,9]. Normally T-channels are absent in normal adult cardiac myocytes; however, T-channels are re-expressed during development of pathological hypertrophy, post-infarction heart and after certain hormonal stimulation [10,11]. Moreover, our previous study shows that $Ca_v3.2$ T-channels plays an important role in the development of pathological cardiac hypertrophy by activating calcineurin–NFAT signaling pathway [12].

Calcineurin is a heterodimer serine/threonine phosphatase composed of a catalytic CnA subunit and a Ca^{2+} sensing regulatory subunit, CnB. When the level of intracellular Ca^{2+} increases, the binding of Ca^{2+}-bound calmodulin to calcineurin triggers the conformational changes of the auto-inhibitory domain, and results in enzyme activation [13]. One of the best characterized calcineurin substrates is NFAT. Dephosphorylation of NFAT by calcineurin induces translocation of NFAT into nucleus and activates hypertrophic genes expression in cardiac myocytes [14]. Multiple calcium channels including transient receptor potential channels (TRPC1, TRPC3, and TRPC6), $Ca_v1.2$ L-channels and $Ca_v3.2$ T-channels are known to regulate the calcineurin/NFAT-mediated signaling pathway [12,15–19]. However, the mechanism of how cardiac myocytes distinguish the "pathological" (signaling) Ca^{2+} from the "physiological" (contractile) Ca^{2+} influx remains unclear.

In this study, we investigated the biochemical and functional interactions between $Ca_v3.2$ and calcineurin. We report here that the interaction between $Ca_v3.2$ and calcineurin is inhibitory to both the $Ca_v3.2$ T-current and the calcineurin activity.

2. Materials and methods

2.1. Construction of plasmids

Human $Ca_v3.2$ and calcineurin Aβ cDNA were amplified by PCR and cloned to make deletion or truncation mutants. Different mutant constructs were cloned into pCMV-3tag-1a (Strategen), pcDNA4-myc-his-A (Invitrogen), pGEX-4T-1 (Invitrogen) and pEGFP-C3 plasmid (Promega).

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2.2. Preparation of rat neonatal cardiac myocytes

Cardiac myocytes were prepared from ventricles of new born rats using Neonatal Cardiomyocyte Isolation System (Worthington). Isolated myocytes were treated with 5 μM of 11R-PCISVE peptide, 11R-AAAAAE peptide (Kelowna) or vehicle for 2 h, followed by the treatment of 10 μM PE or vehicle for 48 h. Cells were stained with anti-α-actinin (Sigma–Aldrich) and Alexa Fluor 594-conjugated anti-mouse IgG (Invitrogen) antibodies and observed by fluorescence Microscopy.

2.3. Immunoprecipitation and GST pull-down assays

For immunoprecipation, transfected cells were harvested and the cell lysates were incubated with anti-FLAG antibody-conjugated beads (Sigma–Aldrich) at 4 °C overnight with rotation. For GST-pull down, recombinant GST–$Ca_v3.2$ fusion protein, purified bovine calcineurin A (Millipore), recombinant calmodulin (Millipore) and/or recombinant human calcineurin B (Millipore) were incubated overnight at 4 °C with rotation. GST protein complex were pulled-down with glutathione Sepharose beads (GE Healthcare). After wash, the bound proteins were eluted with protein sample buffer and analyzed by Western blotting assays.

2.4. Electrophysiological measurement

pEGFP-C3 and FLAG-$Ca_v3.2$, FLAG-$Ca_v3.2$-ΔA or FLAG-$Ca_v3.2$-9A plasmids were cotransfected into HEK293 cells. Whole cell patch clamp technique was conducted as described previously [12].

2.5. NFAT-luciferase reporter assay

NFAT-luciferase (pGL4.30) was co-expressed with $Ca_v3.2$ in HEK293 cells and the NFAT luciferase activities were determined with Dual Luciferase Reporter Assay System (Promega). $Ca_v3.2$ calcium currents were induced as described previously [12].

2.6. Calcineurin activity assay

The calcineurin activity was determined with calcineurin activity assay kit (BIOMOL) according to manufacturer's instruction.

2.7. Statistical analysis

Results are expressed as mean ± S.E.M. Student's *t*-test was used for comparison of two groups. One-way ANOVA with Turkey–Kramer test was used for comparison among three or more groups. Data were considered statistically different if $P < 0.05$.

For detailed information see Supplementary materials online.

3. Results

3.1. $Ca_v3.2$ interacts with the calcineurin with NFAT-like binding sites PCISVE and LTVP

Previously, we demonstrated that overexpressed $Ca_v3.2$ interacts with calcineurin in HEK293 cells [12]. Since calcineurin is a cytosolic protein, the interacting domain may be located at the intracellular region of the channel. We overexpressed FLAG-tagged $Ca_v3.2$ cytoplasmic domains in HEK293 cells, and found that only the C-terminus was co-immunoprecipitated with calcineurin (Fig. 1A). To determine the $Ca_v3.2$ binding site in calcineurin, we co-expressed Myc-tagged calcineurin variants with $Ca_v3.2$ C-terminus (FLAG-C-WT) in HEK293 cells. We found that both Cn-WT and CnN bind to FLAG-C-WT, while the CnC and CndM did not, which suggests the NFAT-binding domain (NBD) (327–333) of calcineurin is essential for $Ca_v3.2$ binding. Two calcineurin binding motifs, PXIXIT and LXVP, have been reported [13,20–22]. We examined and found the highly conserved PCISVE (2190–2195) and LTVP (2261–2264) motifs at the $Ca_v3.2$ C-terminus (Supplementary Table 1). To test the role of these motifs in calcineurin binding, we overexpressed $Ca_v3.2$ C-terminus variants in HEK293 cells and conducted immunoprecipitation. CIId1 showed reduced interaction with calcineurin when compared to CIId2 and C-WT, while C-9A completely lost the ability to bind calcineurin (Fig. 1C). To test whether there are other calcineurin binding sites within $Ca_v3.2$, weintroduced mutations to full length $Ca_v3.2$. $Ca_v3.2$-9A (PCISVE to AAAAAE and LTVP to AAAA) and $Ca_v3.2$-ΔA (without the 2081–2249 region) could not bind to endogenous calcineurin (Fig. 1D). To determine which motif is more important in calcineurin binding, we conducted in vitro GST pull-down assays with recombinant $Ca_v3.2$ C-terminal protein (GST–CM, 2173–2277) with mutations at PCISVE and LTVP motifs (Fig. 2E). Compared to wild type (CM), both CM-5A and CM-4A mutants showed reduced calcineurin binding ability. Interestingly, more calcineurin were pulled down by CM-4A than CM-5A (Fig. 2E). To test whether $Ca_v3.2$/calcineurin interaction can take place in cardiac myocytes, we conducted GST pull-down assays with mouse heart lysate. The results showed that GST–CII but not GST–CII5A or GST can bind to cardiac calcineurin (Supplementary Fig. 1). Our data demonstrate that $Ca_v3.2$ directly interacts with calcineurin through the PCISVE and LTVP motifs, and the PCISVE motif plays a more dominant role in calcineurin binding.

3.2. The interaction between $Ca_v3.2$ and calcineurin is calcium/calmodulin dependent

The calcineurin/NFAT interaction and calcineurin enzymatic activity are Ca^{2+}-, calmodulin- and calcineurin B (CnB)-dependent [23,24]. The binding between $Ca_v3.2$ and calcineurin was enhanced by calmodulin and 0.1 mM Ca^{2+}, and completely abolished by 2 mM EGTA, but not changed by CnB (Fig. 2A). Next, we examined the Ca^{2+}-dependence at physiological range of [Ca^{2+}]. The GST–CII/calcineurin interaction behaved in a Ca^{2+}-independent manner without calmodulin. In contrast, in the presence of calmodulin, $Ca_v3.2$/calcineurin interaction changed dose-dependently by Ca^{2+} (Fig. 2B and C). These results demonstrate that $Ca_v3.2$ interacts with calcineurin in a Ca^{2+}/calmodulin-dependent manner.

3.3. Calcineurin-binding deficient $Ca_v3.2$ T-channels display larger current densitie

We then investigated the effect of $Ca_v3.2$/calcineurin interaction on $Ca_v3.2$ Ca^{2+} currents. We examined the current properties of $Ca_v3.2$, $Ca_v3.2$-9A and $Ca_v3.2$-ΔA in HEK293 cells by whole-cell patch-clamp technique. The current densities of both $Ca_v3.2$-9A and $Ca_v3.2$-ΔA were significant larger than that of $Ca_v3.2$ (Fig. 3A). Supplementary Fig. 2A showed the average peak current traces of the three constructs elicited at −40 mV from a holding potential of −90 mV ($n = 3$). The peak current amplitude of $Ca_v3.2$-ΔA (−161.6 ± 17.2 pA/pF, $n = 10$) and $Ca_v3.2$-9A (−133.3 ± 18.6 pA/pF, $n = 12$) were significantly larger than that of wild type $Ca_v3.2$ (−83.7 ± 11.7 pA/pF, $n = 16$, $P < 0.05$). We conducted biotinylation assay to examine whether the effect is due to the increase of $Ca_v3.2$ on the cell surface. There is no significant difference in the surface channel proteins between mutant and wild type $Ca_v3.2$ (Supplementary Fig. 2B). However, we found a leftward shift in the voltage-dependent activation curves of $Ca_v3.2$-ΔA and $Ca_v3.2$-9A (Fig. 3C). The half maximal voltages ($V_{1/2}$) of the activation curves in $Ca_v3.2$, $Ca_v3.2$-9A and $Ca_v3.2$-ΔA are −40.4 ± 0.9 ($n = 17$), −46.3 ± 1.6 ($n = 12$, $P < 0.05$ vs $Ca_v3.2$) and

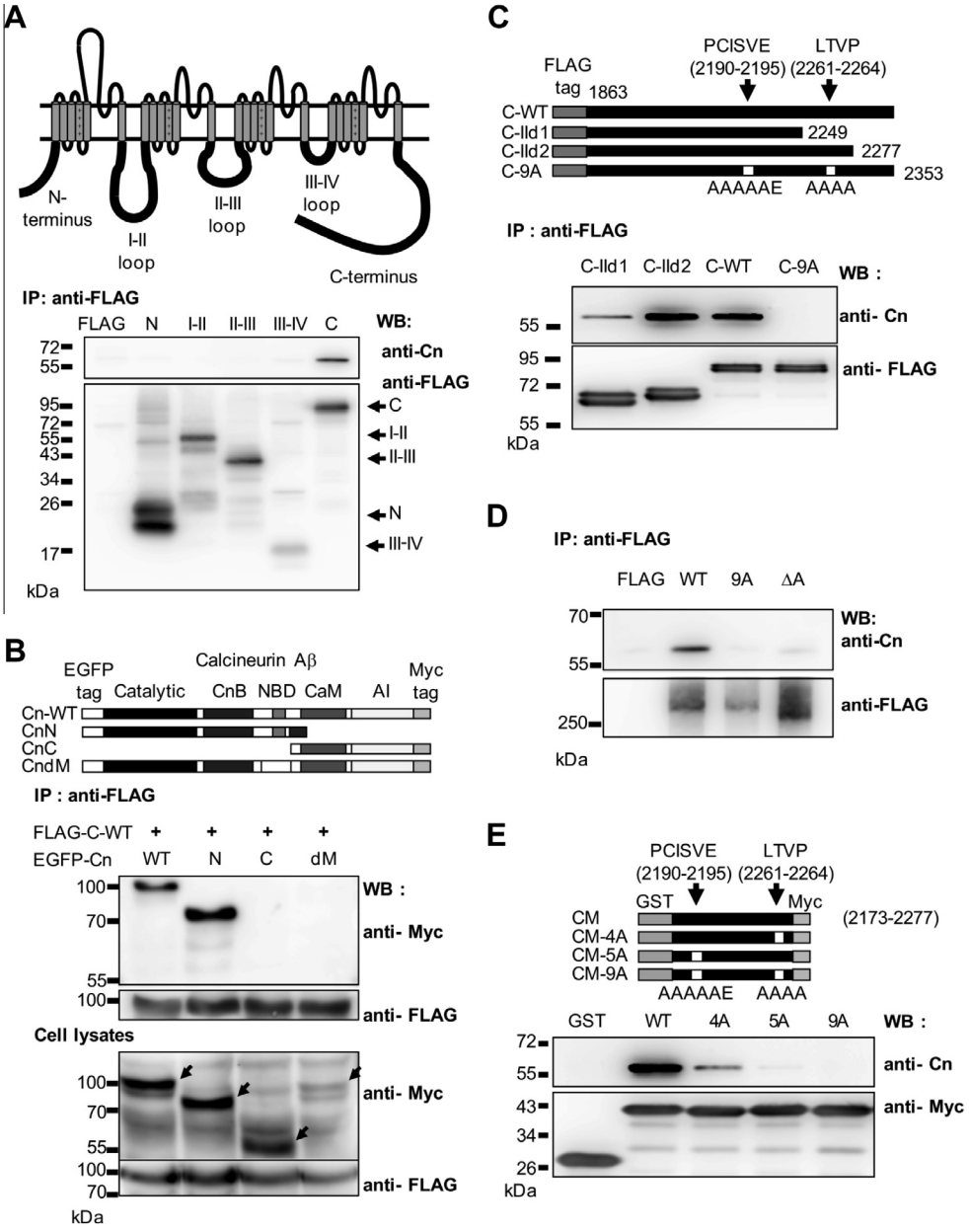

Fig. 1. C-terminal region of $Ca_v3.2$ interacts with the NFAT binding domain of calcineurin. (A–D) $Ca_v3.2$/calcineurin interaction in HEK293 cells was analyzed by coimmunoprecipitation assays. (A) Upper: schematic representation of $Ca_v3.2$ T-type Ca^{2+} channels. Lower: Interaction between endogenous calcineurin and FLAG-tagged $Ca_v3.2$ variants. N, N-terminus. C, C-terminus. I–II, II–III, and III–IV, indicated cytoplasmic loops. FLAG, parental vector. (B) Interaction between $Ca_v3.2$ C-terminus (FLAG-C-WT) and Myc-tagged wild type calcineurin (Cn-WT) or its deletion mutants (CnN, CnC and CndM). (C) Interaction between endogenous calcineurin and C-WT or its mutants (CIId1, CIId2 and C-9A). (D) Interaction between endogenous calcineurin and full length $Ca_v3.2$ (WT) or calcineurin binding deficient mutants (9A and ΔA). (E) Direct binding between calcineurin and $Ca_v3.2$ was determined by GST pull-down assays with Myc-tagged recombinant GST-$Ca_v3.2$ fusion protein (GST–CM) and mutants (CM-4A, CM-5A and CM-9A). The eluents of IP and GST pull-down assays were examined by Western blotting assays with antibodies against calcineurin, FLAG and Myc, respectively.

−49.0 ± 1.5 mV ($n = 13$, $P < 0.05$ vs $Ca_v3.2$), respectively. We hypothesized that the changes of current density and voltage-dependent activation curve in $Ca_v3.2$ mutants were caused by the dissociation of calcineurin. Because NFAT XIXIT peptides inhibit calcineurin/NFAT binding without affecting calcineurin enzymatic activity [20], we co-expressed GFP–VIVIT plasmid with $Ca_v3.2$. GFP–VIVIT reduced the binding of calcineurin to FLAG-C-WT (Supplementary Fig. 2C), and increased the current density of $Ca_v3.2$ by 52% ($n = 15$, $P < 0.05$ vs $Ca_v3.2$ control ($n = 35$), Fig. 3B and E). GFP–VIVIT induced a leftward shift in the activation curve of $Ca_v3.2$ (Fig. 3D). The $V_{1/2}$ of the activation curve for $Ca_v3.2$ + -VIVIT is −45.7 ± 1.4 mV ($n = 14$, $P < 0.05$ vs $Ca_v3.2$ control ($n = 17$)). Interestingly, co-expression of GFP–VIVIT did not change the current density nor the activation curves of $Ca_v3.2$-ΔA and $Ca_v3.2$-9A (Fig. 3D and E). These results suggest that dissociation of calcineurin from $Ca_v3.2$ indeed increase the current density of $Ca_v3.2$ and induce a leftward shift of the activation curve.

3.4. $Ca_v3.2$–calcineurin interaction inhibits calcineurin activity

We next investigated the role of calcineurin/$Ca_v3.2$ interaction on calcineurin/NFAT signaling. We measured calcineurin phosphatase activity in the presence of recombinant GST–CII or GST–CII5A proteins in vitro. GST–CII effectively inhibited calcineurin activity ($P < 0.05$, $n = 4$), while the GST–CII5A had no effect on the calcineurin activity (Fig. 4A). We have shown previously that $Ca_v3.2$ win-

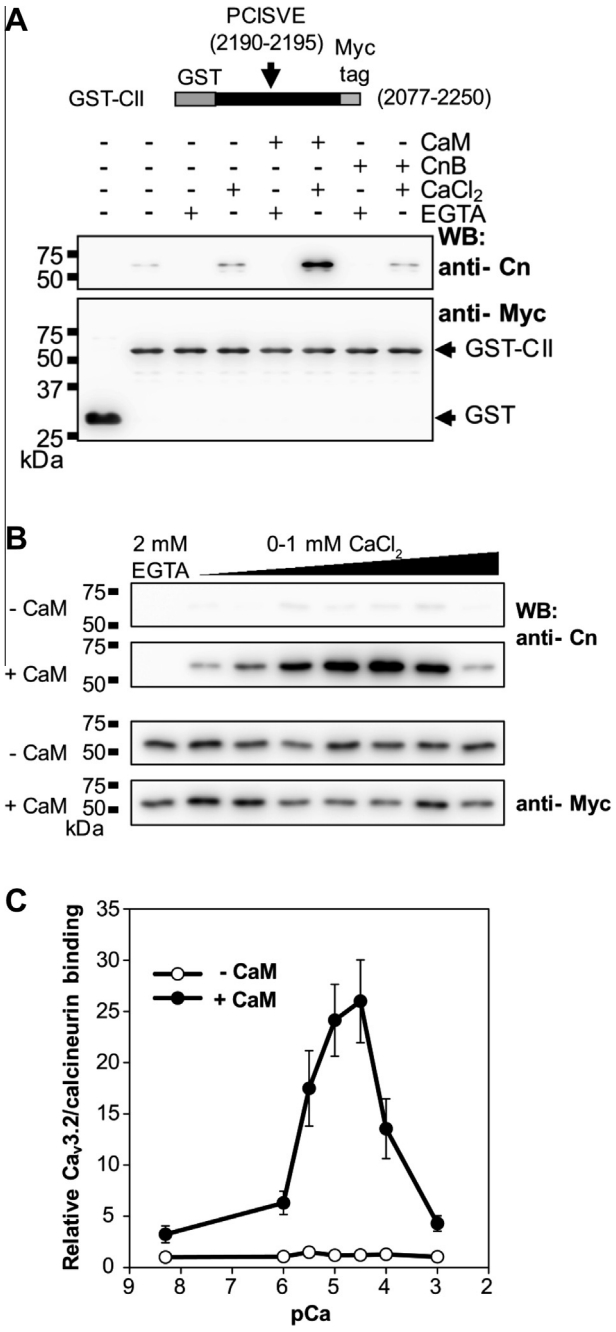

Fig. 2. $Ca_v3.2$–calcineurin interaction is dependent on calmodulin and calcium concentration. Interaction between $Ca_v3.2$ and calcineurin was analyzed by GST pull-down assays. (A) $Ca_v3.2$–calcineurin binding in the presence or absence of calmodulin, calcineurin B (CnB), 2 mM EGTA or 1 mM $CaCl_2$. (B) $Ca_v3.2$–calcineurin binding with or without calmodulin at different concentration of calcium: 0, 10^{-6}, 3×10^{-6}, 10^{-5}, 3×10^{-5}, 10^{-4} and 10^{-3} M $CaCl_2$. Protein levels were verified by anti-calcineurin or anti-Myc antibodies. C, The quantification result of the $Ca_v3.2$/calcineurin binding with (●) or without (○) calmodulin ($n = 3$). The densities of calcineurin were normalized with the density of GST–CII of each sample. pCa indicates the value of $-\log[Ca^{2+}]$.

dow currents can induce NFAT activation in HEK293 cells [12]. To further confirm that $Ca_v3.2$/calcineurin interaction can modulate calcineurin/NFAT signaling pathway, we co-expressed C-WT or C-9A with $Ca_v3.2$ and measured the Ca^{2+}-induced NFAT-luciferase activity. We found that C-WT reduced NFAT activation ($P < 0.05$, $n = 4$), while C-9A showed no inhibitory effect (Fig. 4B). Next, we tested the role of $Ca_v3.2$/calcineurin binding in cardiac hypertrophy with a cell permeable PCISVE peptide (Supplementary Fig. 3A) [25,26]. Similar to the GST–CII and GST–CM, 11R-PCISVE also inhibited calcineurin phosphatase activity in vitro, while the calcineurin binding-deficient peptide (11R-AAAAAE) did not (Supplementary Fig. 3B). We used PE-induced cellular hypertrophy of rat neonatal cardiac myocytes (rNCMs) as our model (Supplementary Fig. 3C). PE treatment induced a 35% increaseof cell surface area ($P < 0.05$, PE. ($n = 187$) vs control ($n = 201$)). 11R-PCISVE treatment prevented the hypertrophic effect of PE ($n = 192$) vs 11R-PCISVE alone ($n = 198$)). When cells were treated with 11R-AAAAE, PE treatment still induced a significant increase in cell size ($P < 0.05$, 11R-AAAAAE + PE ($n = 215$) vs 11R-AAAAAE alone ($n = 121$), Fig. 4C and D). These data suggest that the calcineurin binding motif PCISVE of $Ca_v3.2$ negatively regulates calcineurin activity and cardiac hypertrophy.

4. Discussion

The interaction between calcineurin and NFAT has been well studied during past decades [13]. Biochemical studies revealed that the linker region between catalytic and auto inhibitory domains of calcineurin is essential for NFAT binding [27]. Crystal structures later identified essential residues responsible for the interaction between calcineurin and the VXIXIT motif, which was found in various calcineurin binding molecules [21,28]. In addition to the major role of the VXIXIT motif, another LXVP motif locates near the C-terminus of NFAT was also suggested as a calcineurin binding site [13,22]. In this study, we show that calcineurin directly interacts with $Ca_v3.2$ at the PCISVE and LTVP motifs on its C-terminus and both sites are similar to those of NFAT [13]. Most of the reported calcineurin interacting proteins, including NFAT, TWIK-related spinal cord K channel (TRESK), A-kinase anchoring proteins (AKAP79/150), have a consensus PXIXIT motif [13]. The LXVP motif was found only in a few calcineurin substrates, such as NFAT and KSR2 [13]. In mammals, NFATs are the only known calcineurin binding partners bearing both consensus binding sites. Here we demonstrate that $Ca_v3.2$ is a new calcineurin interacting molecule containing both NFAT-like motifs.

Only a small number of membrane proteins, including cardiac $Ca_v1.2$ and Na^+/Ca^{2+} exchanger (NCX), interact with calcineurin [29–32]. However, unlike $Ca_v3.2$, neither $Ca_v1.2$ nor NCX binds calcineurin via an NFAT-like site. The only known ion channel with NFAT-like calcineurin binding site is TRESK [33]. By literature research, we found one PSILIQ motif and one LMVP motif at the C-terminus of Kir6.1. Kir6.1 is the pore-forming subunit of ATP-sensitive K^+ (K_{ATP}) channels, which has been linked to cardiac hypertrophy and post-infarction heart failure [34]. Calcineurin directly dephosphorylates the PKA-mediated phosphorylation site of Kir6.1 and attenuates K_{ATP} currents [35]. Calcineurin also binds to the PVITID sequence of Na^+/H^+ exchanger 1 (NHE1), which is involved in cardiac hypertrophy and heart failure. The high pH membrane microenvironment generated by NHE1 enhances calcineurin phosphatase activity and promotes the activation of NFAT signaling during cardiac hypertrophy [36–38]. It is possible that calcineurin regulates cardiac remodeling not only through the activation of NFAT, but also via direct interaction to those ion channels containing NFAT-like binding sites.

Another novel finding of our study is that $Ca_v3.2$/calcineurin interaction reduces the channel activity. We showed that $Ca_v3.2$-ΔA and $Ca_v3.2$-9A exhibited higher current density than wild type $Ca_v3.2$. Co-expression of VIVIT also increased the current density of $Ca_v3.2$ but not $Ca_v3.2$-ΔA or $Ca_v3.2$-9A. These results suggest that VIVIT and the lack of calcineurin binding are probably acting through similar pathways to increase $Ca_v3.2$ current amplitude. We observed a leftward shift in the voltage-dependent activation

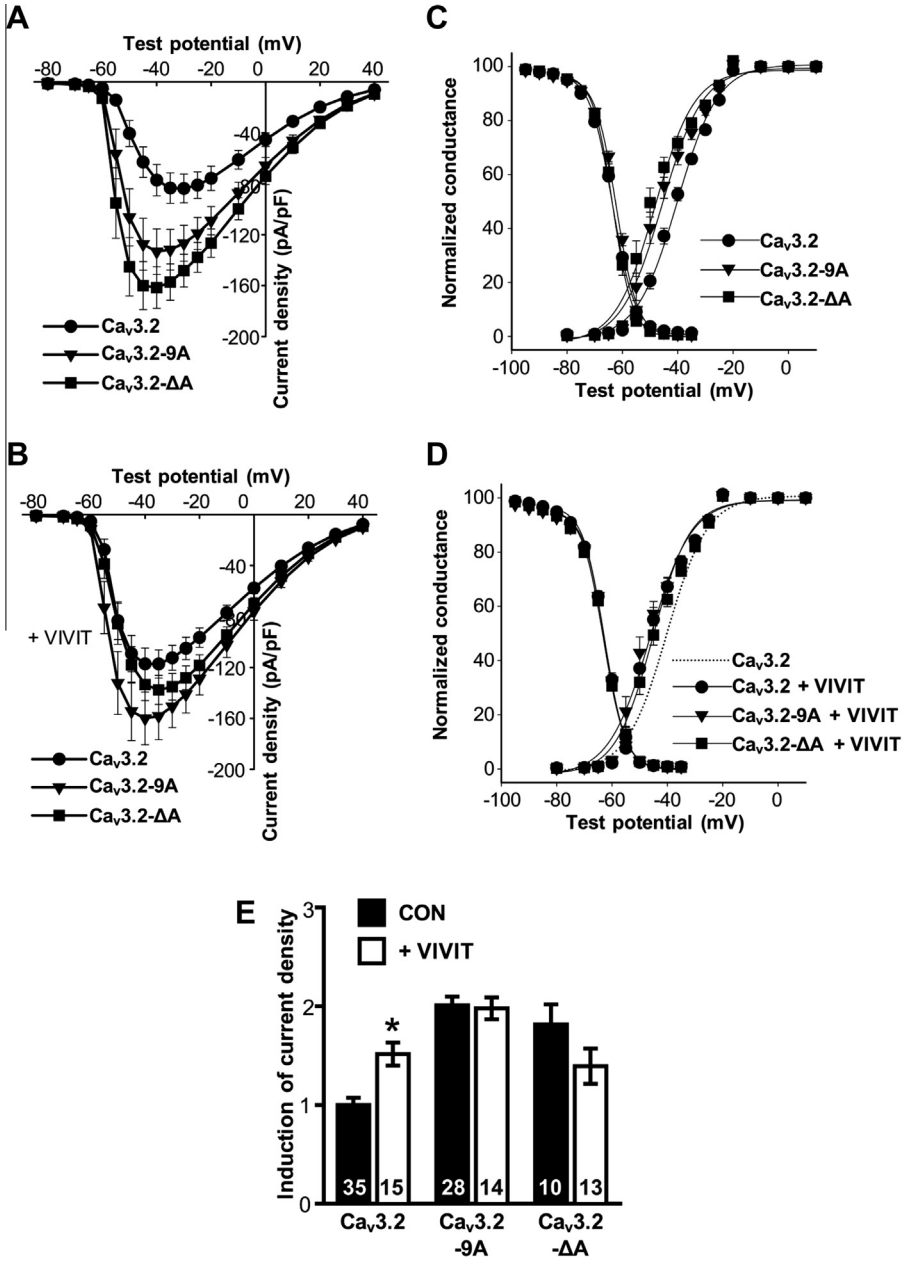

Fig. 3. Calcineurin-binding deficient $Ca_v3.2$ T-channels display larger current densities. Calcium currents generated by overexpressed $Ca_v3.2$ and calcineurin binding deficient mutants were measured in HEK293 cells using whole cell patch clamp technique. (A) The current density–voltage plots of $Ca_v3.2$ (●), $Ca_v3.2$–9A (▼) and $Ca_v3.2$-ΔA (■). (B) Current density–voltage plots in the presence of GFP–VIVIT. (C) Steady-state activation and inactivation curves. (D) Steady-state activation and inactivation curves in the presence of GFP–VIVIT. Dashed line in panel D represents the steady-state activation curve of wild type $Ca_v3.2$ shown in panel C. (E) Normalized peak current densities. $^*P < 0.05$ compared to control group. Numbers of cells for each group are shown.

curves of $Ca_v3.2$-ΔA, $Ca_v3.2$-9A, and wild type $Ca_v3.2$ when co-expressed with VIVIT. The inhibitory regulation of $Ca_v3.2$ by calcineurin binding could be contributed by the dephosphorylation of $Ca_v3.2$ by calcineurin, or the physical interaction, or both. Currently, we could not rule out either possibility.

We found that the interaction between $Ca_v3.2$ and calcineurin is calcium dependent in vitro. In addition, the enhancement of the interaction exhibited a bell shaped Ca^{2+}-dependence. The interaction is weak at pCa > 6 ($[Ca^{2+}]_i$ < 1 μM), increases dose-dependently at 4.5 < pCa < 6.0, and peaks at pCa 4.5, and then declines at pCa < 4.5 (Fig. 2C). It is unclear whether this calcium dependence of $Ca_v3.2$/calcineurin interaction occurs in vivo. Although the intracellular Ca^{2+} ($[Ca^{2+}]_i$) is normally under μM range (pCa > 6), local $[Ca^{2+}]_i$ around Ca^{2+} channel pores can increase acutely when the channels open. A computer modeling study that L-type Ca^{2+} current increases $[Ca^{2+}]_i$ to mM range at a distance of 25 nm from the channel pore in cardiac myocytes [39]. Another study predicts the local $[Ca^{2+}]_i$ is about 76–139 μM at a distance of 10–20 nm from the channel pore after the open of a L-channel in presynaptic neurons [40]. The distance from $Ca_v3.1$ C-terminus to the channel pore is estimated to be less than 5 nm [41]. Since the length of the C-terminus is similar in $Ca_v3.1$ and $Ca_v3.2$, it is possible that the local $[Ca^{2+}]_i$ around the calcineurin binding sites of $Ca_v3.2$ can reach 1 mM (pCa 3.0). Therefore, the $[Ca^{2+}]_i$ around $Ca_v3.2$ C-terminus may raise from pCa < 6.0 to pCa > 3.0 when the channels open. Moreover, both the phosphatase activity and calmodulin binding ability of calcineurin are also calcium dependent and peak at 4.5 < pCa < 3.0 [42], a range that $Ca_v3.2$/calcineu-

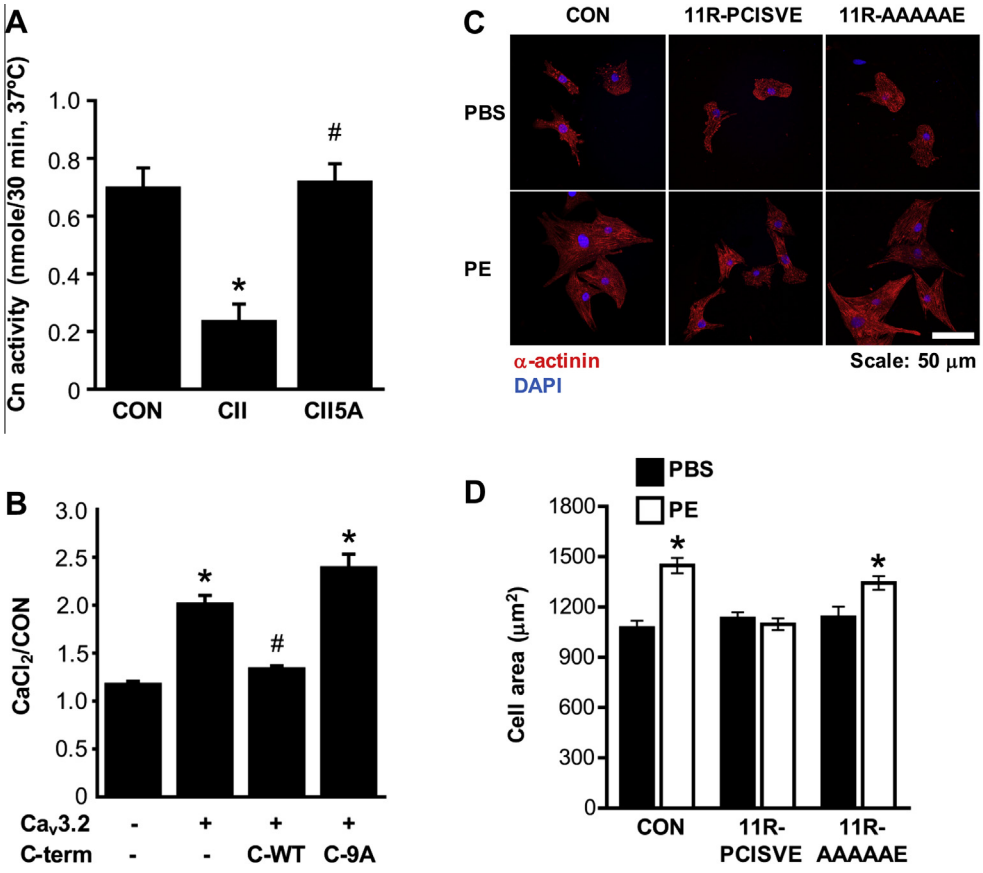

Fig. 4. $Ca_v3.2$–calcineurin interaction reduces calcineurin activity. (A) Calcineurin phosphatase activities were determined using in vitro calcineurin activity assays with GST (CON), GST–CII (CII) or GST–CII5A (CII5A) proteins, respectively. $^{*}P < 0.05$ compared to control. $^{\#}P < 0.05$ compared to GST–CII ($n = 4$). (B) $Ca_v3.2$ C-terminal domain (C-WT), binding-deficient C-9A constructs (C-9A) or vehicle vector were co-expressed with full-length $Ca_v3.2$ as indicated. $Ca_v3.2$ current-induced NFAT activation were induced by 10 mM Ca^{2+} for 6 h, and NFAT-luciferase activity was measured by luciferase assays. $^{*}P < 0.05$ compared to control. $^{\#}P < 0.05$ compared to $Ca_v3.2$ only group ($n = 4$). (C) Representative immunofluorescence images of rNCMs treated with 5 μM peptides in the presence of 10 μM phenylephrine (PE) or PBS for 2 days. Myocytes were identified by anti-α-actinin staining. Red: α-actinin. Blue: DAPI. Scale: 50 μm. (D) Cell surface area quantification of rNCMs. $^{*}P < 0.05$ compared to PBS control in the same group ($n = 121$–201).

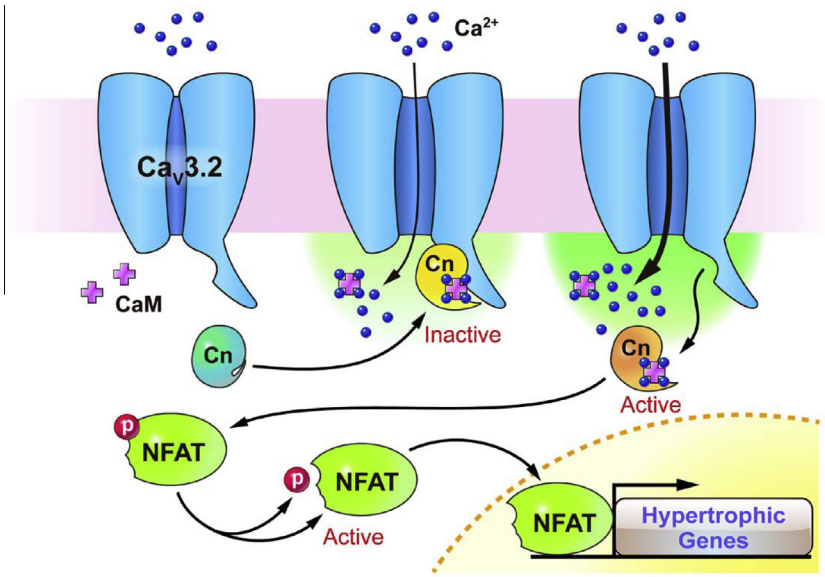

Fig. 5. Hypothetical model of $Ca_v3.2$/calcineurin/NFAT signaling pathway. During cardiac hypertrophy, $Ca_v3.2$-mediated Ca^{2+} influx activates Ca^{2+}/CaM complex, and induces calcineurin binding to $Ca_v3.2$ C-terminus. The binding inhibits calcineurin activity and partially reduces the Ca^{2+} influx at lower $[Ca^{2+}]_i$ (nM to sub-mM, light green). When the local Ca^{2+} concentration exceeds a certain level (mM, dark green), calcineurin dissociates from $Ca_v3.2$ and becomes fully activated, and subsequently dephosphorylates NFAT, leading to NFAT nuclear translocation and downstream gene activation.

rin interaction decreases dramatically. It is conceivable that when $Ca_v3.2$ is re-expressed during cardiac hypertrophy, the calcium influx via $Ca_v3.2$ T-channel initiates the binding of calcineurin to the C-terminus of $Ca_v3.2$ at pCa < 6. The activity of bound calcineurin is partial inhibited (Fig. 4A) and with more calcium influx (pCa < 4.5) the binding between calcineurin and $Ca_v3.2$ is reduced and the dissociated calcineurin will reach its optimal phosphatase activity at 4.5 < pCa < 3. The activated calcineurin can then dephosphorylate downstream substrates like NFAT.

Although the role of calcineurin/NFAT pathway in cardiac hypertrophy is well known, it remains unclear how this pathways is activated only by "pathological" but not "physiological" Ca^{2+} changes. The $[Ca^{2+}]_i$ oscillates from nM to μM at each heart beat in cardiac myocytes; however, this regular $[Ca^{2+}]_i$ oscillation does not activate the calcineurin/NFAT pathway. One hypothesis is that Ca^{2+} activates calcineurin/NFAT pathway specifically through location-dependent microdomains and macromolecular complexes in caveolaes [43,44].Recently, $Ca_v3.2$ T-channels were shown to colocalize with caveolin-3 in caveolaes in adult cardiac myocytes [45]. Several studies have suggested that calcineurin may be colocalized with caveolin-1 and caveolin-3 [44,46]. It is possible that calcineurin may be regulated by $Ca_v3.2$ T-channels in microdomains like caveolaes during cardiac hypertrophy. Our previous study demonstrates that $Ca_v3.2$ is required for NFAT activation during cardiac hypertrophy [12]. The inhibitory interaction between calcineurin and $Ca_v3.2$ shown in this study ensures that the activation of calcineurin only takes place at high $[Ca^{2+}]_i$, possibly in caveolaes. As Fig. 5 shows, calcineurin can be recruited to re-expressed $Ca_v3.2$ in hypertrophic myocytes. The phosphatase activity of calcineurin is inhibited when $[Ca^{2+}]_i$ is ranged from nM to sub-mM (light green in Fig. 5). With more calcium influx, the local $[Ca^{2+}]_i$ increases to mM range (dark green in Fig. 5) which leads to dissociation and fully activation of calcineurin. This mechanism can prevent the calcineurin/NFAT pathway from being activated by systolic Ca^{2+} concentration oscillates in physiology conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.04.040.

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