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Interaction of T-Type Calcium Channel $Ca_V3.3$ with the β-Subunit

Jinhee Bae[1], Eui Jin Suh[1,2], and Cheolju Lee[1,2,*]

The β-subunit of high-voltage-activated (HVA) calcium channels is essential for the regulation of expression and gating. On the other hand, various reports have suggested that β subunits play no role in the regulation of low-voltage-activated T-type channels. In addition there has been no clear demonstration of a physical interaction between the α-subunit of T-type channel with β-subunit. In this study, we systematically investigated the interaction between $Ca_V\alpha$ and $Ca_V\beta$. The four $Ca_V\beta$ isoforms were expressed in a bacterial system and purified into homogeneity, whereas the ten types of $Ca_V\alpha$ alpha interaction domain (AID) peptides were chemically synthesized. All possible combinations of $Ca_V\alpha$ and $Ca_V\beta$ were then tested for by *in vitro* immunoassays. We describe here the identification of a new interaction between $Ca_V3.3$ and $Ca_V\beta$ proteins. This interaction is of low affinity compared to that between the AID of the HVA α-subunit and the alpha-binding pocket (ABP) site of the β-subunit. The AID peptide of HVA channel exerted no effect on the $Ca_V3.3$-$Ca_V\beta$ interaction, thus demonstrating that another site not in the ABP of $Ca_V\beta$ protein played a role in binding with $Ca_V3.3$. This is the first demonstration of an α-β subunit interaction in a T-type calcium channel.

INTRODUCTION

Voltage-dependent calcium channels (VDCCs) are classified into five essential groups: L-type [$Ca_V1.1$ (1S), 1.2 (1C), 1.3 (1D), 1.4 (1F)], N-type [$Ca_V2.2$ (1B)], T-type [$Ca_V3.1$ (1G), 3.2 (1H), 3.3 (1I)], P/Q-type [$Ca_V2.1$ (1A)], and R-type [2.3 (1E)] (Ellinor et al., 1993). P/Q-, N-, and R-type channels are all found in the brain, where they are primarily responsible for the initiation of synaptic transmission at fast synapses in the nervous system (Jun et al., 1999). L-type channels are ubiquitous, particularly in skeletal and cardiac muscle, where they play an essential role in excitation–contraction coupling (Catterall, 2000; Mikami et al., 1989). The pores of all these channels are gated by high voltage and therefore are referred to as high-voltage-activated (HVA) calcium channels. In contrast, low-voltage-activated (LVA) channels are comprised of the T-type family only (Nowycky et al., 1985). T-type channels are localized to the brain, kidney, and heart, where they play important roles in a wide variety of physiological functions, including neuronal firing, neuropathic pain development, hormone secretion, smooth muscle contraction, cell proliferation of cardiac tissue, and myoblast fusion (Bean, 1989; Huguenard, 1996; Na et al., 2008). In general, T-type currents recorded using various cell types are similar in their electrophysiological properties, but differences have been noted in their activation and deactivation as well as in their pharmacology. Such heterogeneity can be explained in part by the existence of three T-type channels that are encoded on three separate genes (Perez-Reyes, 2006).

Calcium channels are multi-subunit complexes containing the main pore-forming α_1 subunit, and the auxiliary subunits $\alpha_2\delta$ ($Ca_V\alpha_2\delta$), β ($Ca_V\beta$), and sometimes γ1 ($Ca_V\gamma1$) (Arikkath and Campbell, 2003). Four β subunit isoforms ($Ca_V\beta1$-$Ca_V\beta4$) have been cloned so far, all of which are hydrophilic, nonglycosylated, and located within the cell. Recent structural modeling and X-ray crystallography have revealed that β subunits contain a conserved Src homology 3 (SH3) domain and a conserved guanylated kinase (GK) domain linked together by a flexible HOOK region. (Hanlon et al., 1999). The β subunits bind to the 18-amino acid α1-interaction domain (AID) of the cytoplasmic linker between internal repeats I and II of the α1 subunit (Van Petegem et al., 2004). The AID is conserved between the Ca_V1 and Ca_V2 calcium channel subfamilies, but not in the Ca_V3 subfamily. AID interacts at a stoichiometry of 1:1 with the β subunit through an extensive, conserved hydrophobic cleft aptly named the alpha-binding pocket (ABP) (De Waard et al., 1995). The α subunits of HVA channels most likely associate with the β and $\alpha_2\delta$ subunits, whereas the subunit composition of LVA channels is not yet known.

A few studies have suggested that the β and $\alpha_2\delta$ subunits might modulate T-type calcium channels (Dolphin et al., 1999), whereas other studies found that β subunits do not regulate T-type current density and expression. Expression of $Ca_V3.1$ alone leads to the induction of robust currents with all of the typical properties of LVA T-type currents (Perez-Reyes et al., 1998). In contrast, coexpression with any β subunit has no significant effect on T-type channel expression, voltage dependence, or kinetics (Arias et al., 2005; Dubel et al., 2004). Most of these studies were carried out on $Ca_V3.1$ and not $Ca_V3.3$, which was cloned more recently. In this study, we in-

[1]Life Sciences Division, Korea Institute of Science and Technology, Seoul 136-791, Korea, [2]Department of Biomolecular Science, University of Science and Technology, Daejeon 305-333, Korea
*Correspondence: clee270@kist.re.kr

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vestigated the interaction between $Ca_V\alpha$ and $Ca_V\beta$ in a more systematic way. The four $Ca_V\beta$ isoforms were expressed and purified into homogeneity, and the ten types of AID peptides were chemically synthesized. All possible α-β combinations were then tested for by *in vitro* immunoassays. Our results show that the AID of $Ca_V3.3$ interacts with the β-subunit.

MATERIALS AND METHODS

Subcloning, expression, and purification of $Ca_V\beta$ proteins

Full-length human $Ca_V\beta1$ (NM_199247.1), $Ca_V\beta2$ (NM_000724.2), and $Ca_V\beta3$ (NM_000725.2) were purchased from OriGene Tech (USA). Full-length mouse $Ca_V\beta4$ (NM_146123.2) was purchased from 21C Human Gene Bank (Korea). Core domains of $Ca_V\beta$ isoforms were amplified by PCR and subcloned into a pET28a vector. The plasmids were transformed in *Escherichia coli* Rosetta cells, after which positive cells were grown for 4-6 h at 37°C in 2 L of LB kanamycin media. Upon reaching an A_{600} of 0.6, the expression of His-tagged $Ca_V\beta$ proteins was induced with 1 mM isopropyl β-D-galactopyranoside (Sigma-Aldrich, USA) at 18°C for 16 h. The bacteria were then centrifuged (4,000 × *g* for 15 min) at 4°C and resuspended in 50 ml of lysis buffer [50 mM Tris-HCl, 100 mM NaCl, 2% glycerol, 10 mM $MgCl_2$, 1 mM DTT, and protease inhibitor cocktail (Roche Diagnostics, USA), pH 8.0]. After cell lysis by French press, cell debris was removed by centrifugation at 12,000 × *g* for 50 min at 4°C. The soluble fraction was loaded onto a HisTrap column (GE Healthcare, USA) equilibrated with binding buffer (10 mM Na-HEPES, 250 mM NaCl, 1 mM DTT, 1% glycerol, pH 7.2). The column was washed with binding buffer containing 20 mM imidazole at a flow rate of 5 ml/min until a stable base line was achieved, after which proteins were eluted with a linear gradient of 20-500 mM imidazole. Five milliliters of the protein eluate was then injected into a gel filtration column equilibrated with the above binding buffer at a flow rate of 0.5 ml/min. Fractions 1.2 ml in volume were collected during the elution. The protein concentration of each fraction was determined by Bradford assay.

Avidin-agarose pull-down assays

Purified His-tagged $Ca_V\beta$ protein was incubated with biotin-tagged AID peptides (b-AIDs) for 1 h at 4°C in 300 μl of binding buffer supplemented with 1% NP-40. The reaction solution was then transferred to a new tube containing 20 μl avidin-agarose resin (Pierce Biotec Co., USA), followed by incubation for an additional 1 h. Agarose beads were washed 4 times with the above binding buffer. Bound proteins were recovered by boiling the beads in 1× SDS-PAGE sample buffer.

Western blotting

Protein samples were analyzed by 10% SDS-PAGE and electrotransferred onto PVDF membranes using a Mini Trans-Blot® cell (Bio-Rad, USA). The PVDF membrane was incubated with a blocking buffer (5% skimmed milk in TBST) for 1 h at 25°C and then with a monoclonal anti-His antibody (Roche Diagnostics, USA; diluted 1:500) overnight at 4°C. After washing three times, the membrane was reacted with anti-mouse IgG horseradish peroxidase (GE Healthcare; diluted 1: 2000) for 1 h at 25°C. Chemiluminescence was detected using an ECL™ Western Blotting Detection system (GE Healthcare).

Surface plasmon resonance analysis

Surface Plasmon Resonance (SPR) experiments were performed using a Biacore 2000 Instrument (Biacore Inc., Sweden). A streptavidin sensor chip (SA chip; Biacore, Inc., Sweden) was cleaned with three consecutive 2 min injections of regeneration solution (1 M NaCl and 50 mM NaOH) at a rate of 50 μl/min. b-AID diluted in running buffer (10 mM Na-HEPES, 250 mM Nacl, 2 mM DTT, 1% glycerol, 0.5% NP-40, pH 7.4) to 50 μg/ml was injected for 7 min at a flow rate of 5 μl/min. To measure the maximum immobilization of b-AID to streptavidin, multiple injections were performed. To remove any non-specific weak binders and to test whether or not the regeneration process harmed or affected the capture activity of the SA chip, the regeneration solution was injected twice for 1 min at a flow rate of 30 μl/min. Running buffer containing the $Ca_V\beta$ protein was then injected onto the AID immobilized sensor chip for 4-8 min at a flow rate of 30 μl/min, during which the association data were collected. The dissociation data were collected over 10 min and the SA chip was regenerated. Sensograms were collected at various concentrations of $Ca_V\beta$ proteins.

Competition ELISA assay

The $Ca_V\beta$ proteins were pre-incubated with the b-AIDs at 4°C for 1 h in 100 μl of reaction buffer (1% NP40, 1% glycerol, 10 mM HEPES, 250 mM NaCl and 1 mM DTT, 0.1% BSA pH 7.2). The mixture was then transferred to a 96-well Reacti-Bind Streptavidin High Binding Capacity plate (Pierce Biotec Co., USA), which was shaken at room temperature for 1 h. After washing three times with reaction buffer, enzyme-labeled anti-His antibody (NOVUS Biologicals, USA; diluted 1:1000) was added to the plate, which was shaken at room temperature for 1 h. After washing three times, reaction with horseradish peroxidase was performed using 100 μl of 1-Step™ Turbo TMB-ELISA (Pierce Biotec Co., USA). After a 15 min incubation, 100 μl of Stop Solution (Pierce Biotec Co., USA) was added, after which the absorbance was measured at 450 nm using an ELISA reader.

RESULTS

Purification of His-$Ca_V\beta$ proteins and synthesis of $Ca_V\alpha$ subunit AID peptides

Sequence alignment of human $Ca_V\beta1$, $Ca_V\beta2$, $Ca_V\beta3$ and mouse $Ca_V\beta4$ is shown in Fig. 1A. The core region encompassing the SH3 and GK domains was subcloned in an *E. coli* overexpression vector. His-tagged $Ca_V\beta$ proteins were induced and purified into electrophoretic homogeneity by metal affinity and gel filtration chromatography (data not shown). Similar to a previous study (Geib et al., 2002), purified protein was fully functional for *in vitro* binding assays. AIDs of 10 different $Ca_V\alpha$ proteins were chemically synthesized with 6 additional amino acids at both the N- and C-termini (Fig. 1B). For the pull-down, SPR and ELISA assays, a biotin tag was attached to the N-terminus of each AID peptide.

$Ca_V\beta$ interacts with AID of $Ca_V3.3$

Using biotinylated AID peptides and His-tagged $Ca_V\beta$ proteins, we examined all possible combinations of the α and β subunits of VDCCs. The biotin tag provides an attachment site to the solid matrix, whereas the His-tag was used for chemiluminence detection (Fig. 2A). The AID peptides of the Ca_V1 and Ca_V2 subfamilies interacted with the $Ca_V\beta$ proteins, as revealed by pull-down assay followed by Western blot analysis (Fig. 2B). In the same experimental setup, T-type channels showed a different interaction pattern. Hardly any interaction was detected between the $Ca_V\beta$ proteins and the AID of $Ca_V3.1$ or $Ca_V3.2$, similar to what is already known. However, the analysis clearly revealed that the third T-type calcium channel $Ca_V3.3$ had

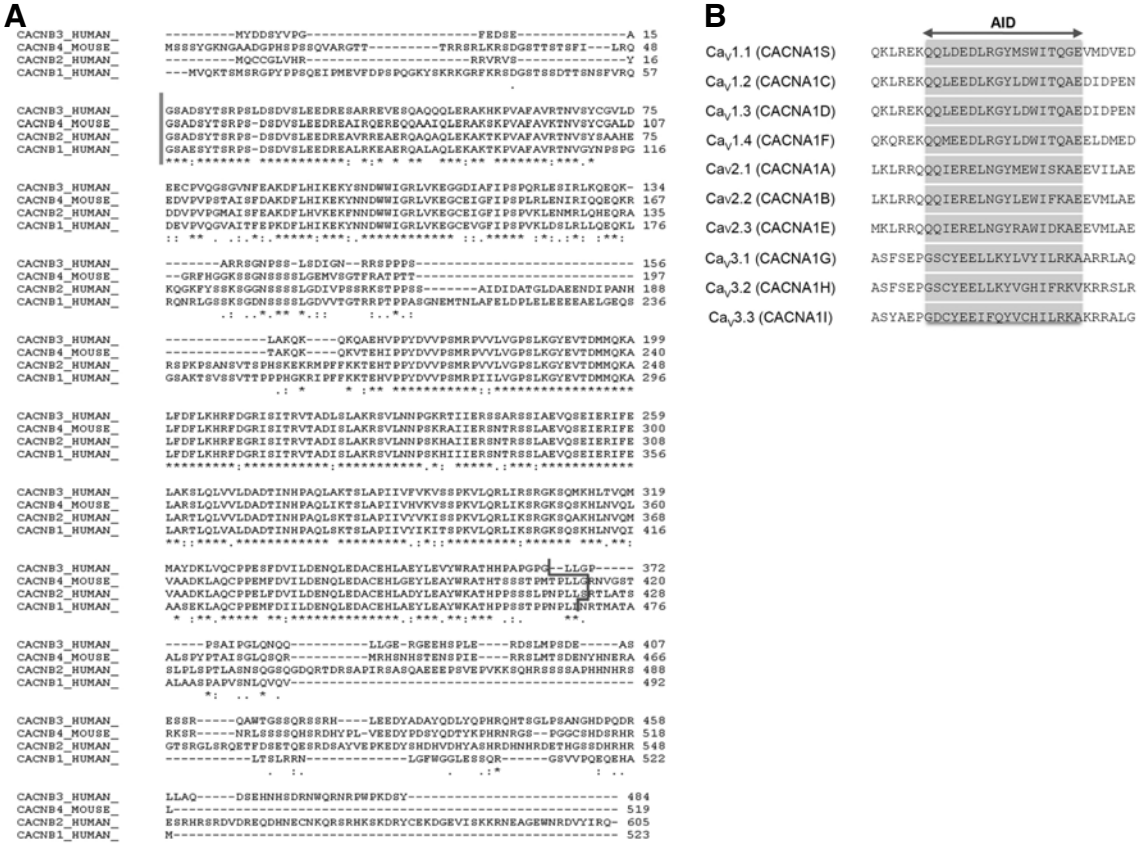

Fig. 1. (A) Sequence alignment of the human Ca$_V$β1, Ca$_V$β2, Ca$_V$β3 and mouse Ca$_V$β4 proteins. The two vertical grey lines are the boundaries between the N- and C- termini of cloned recombinant proteins. All proteins were cloned with an N-terminal His-tag. (B) Sequence alignment of the synthesized Ca$_V$α peptides. The peptides include AID sequences plus 12 neighboring sequences (6 for N-terminus and 6 for C-termi-nus). AID sequences are high-lighted in grey. Web-based ClustalW2 program was used for multiple sequence alignment (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

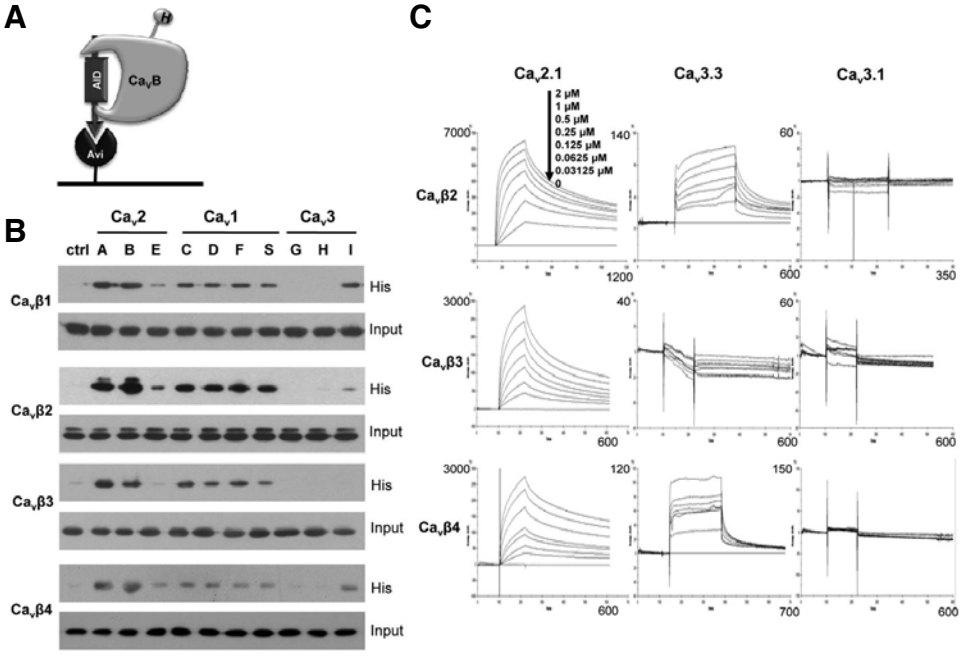

Fig. 2. Binding of Ca$_V$β proteins to AID peptides. (A) Schematic illustration of the avidin pull-down assay for Ca$_V$β-AID binding. Avi: avidin bead; AID: biotinylated AID peptide; Ca$_V$B: His-tagged Ca$_V$β protein. (B) His-tagged Ca$_V$β proteins (80 μg/ml) were incubated with biotin-tagged AID peptides (73 μg/ml) in 300 μl of binding buffer. Bound proteins were pulled-down with 20 μl of avidin-agarose resin and analyzed by Western blotting with anti-His$_6$ antibody. (C) Surface plasmon resonance assay of Ca$_V$β-AID binding. Biotin-AID peptides were immobilized on the surface of a streptavidin sensor chip, after which Ca$_V$β proteins at various concentrations were injected. The maximum response units (RU) for Ca$_V$β2, Ca$_V$β3 and Ca$_V$β4 binding to Ca$_V$2.1 were 6000 RU, 3500 RU and 2500 RU, respectively. The values for Ca$_V$β1-Ca$_V$3.3 and Ca$_V$β4-Ca$_V$3.3 were 120 RU and 100 RU, respectively. No binding occurred between Ca$_V$β3 and Ca$_V$3.3. (x-axis: time in second; y-axis: RU)

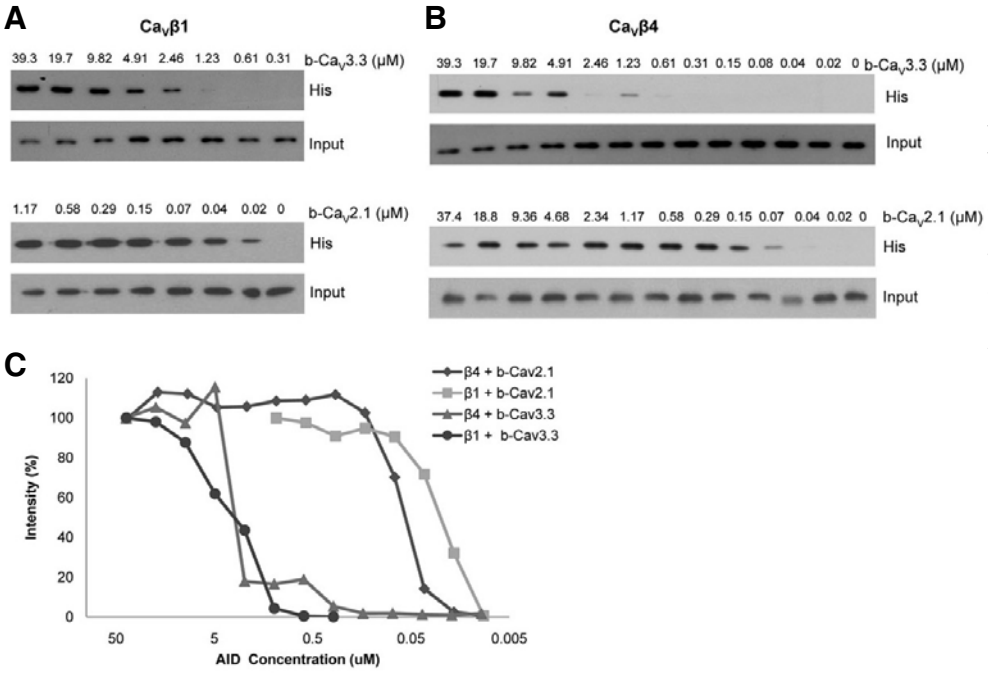

Fig. 3. Binding affinity of the $Ca_V3.3$ AID to $Ca_V\beta$ proteins. (A, B) Various concentrations of $Ca_V3.3$ and $Ca_V2.1$ AID peptides were assayed for binding to $Ca_V\beta1$ (0.43 μM) and $Ca_V\beta4$ (0.12 μM), and binding was quantified by Western blotting. (C) Band intensities of the Western blot data are plotted as a function of AID concentration. b-Ca_Vx.x: biotinylated Ca_Vx.x AID peptide

affinity toward all $Ca_V\beta$ proteins except $Ca_V\beta3$ (Fig. 2B). No binding was observed to $Ca_V\beta$ proteins by a control peptide with a scrambled amino acid sequence.

We also characterized the interaction of $Ca_V3.3$ with $Ca_V\beta$ using SPR technology. AID peptides were immobilized on a streptavidin sensor chip via biotin, after which $Ca_V\beta$ proteins (β2, β3 and β4) at various concentrations (2 μM to 31 nM) were injected onto the chip. $Ca_V\beta2$ and $Ca_V\beta4$ showed typical sensograms for association and dissociation, whereas $Ca_V\beta3$ did not (Fig. 2C). No binding was observed for the $Ca_V3.1$ AID. Dose-dependent interactions between the $Ca_V2.1$ and all of the $Ca_V\beta$ proteins were clearly observed (Fig. 2C). The SPR signal amplitude recorded for $Ca_V3.3$ was more than 10 times smaller than that of $Ca_V2.1$, implying that the $Ca_V3.3$-$Ca_V\beta$ interaction might be smaller than the well known $Ca_V2.1$-$Ca_V\beta$ interaction.

$Ca_V3.3$ has lower binding affinity to $Ca_V\beta$ than $Ca_V2.1$

The binding affinity between $Ca_V3.3$ and $Ca_V\beta$ was measured by Western blot analysis. Analysis of the binding of various concentrations of AID peptides to $Ca_V\beta1$ and $Ca_V\beta4$ (Fig. 3A) demonstrated that the binding was saturable; specific binding appeared at about 0.5 μM and became saturated at about 20 μM. The saturation curve of the binding of $Ca_V3.3$ AID to $Ca_V\beta1$ was fitted to a logistic function, producing a dissociation constant (K_d) of 3.4 μM for the AID peptide (Fig. 3C). A K_d value of 2.6 μM was acquired for $Ca_V\beta4$. Binding of the $Ca_V2.1$ AID peptide to $Ca_V\beta1$ and $Ca_V\beta4$ occurred at a concentration lower than that to $Ca_V3.3$ (Fig. 3B). The K_d values of the $Ca_V2.1$ AID were 24 nM and 62 nM for binding to $Ca_V\beta1$ and $Ca_V\beta4$, respectively. All together, these results reveal that $Ca_V3.3$ interacts with $Ca_V\beta$ with approximately 100-fold lower affinity compared to the interaction between $Ca_V2.1$ and $Ca_V\beta$.

$Ca_V3.3$ AID does not compete with $Ca_V2.1$ AID for $Ca_V\beta$ binding

We next tested whether or not the binding of $Ca_V3.3$ with $Ca_V\beta$ occurred via the typical AID-ABP interaction. For this, we set up an ELISA competition assay in which a streptavidin-coated 96-well plate was used to capture the AID-$Ca_V\beta$ complex. As seen in Figs. 4A and 4B, the ELISA showed specific binding for $Ca_V2.1$. However, we failed to detect specific binding between $Ca_V3.3$ and $Ca_V\beta$ using this assay system, presumably due to low binding affinity (data not shown). Hence, we tested the effect of non-biotinylated $Ca_V3.3$ peptide on the binding of $Ca_V2.1$ to $Ca_V\beta$ proteins. The interaction of $Ca_V2.1$ with $Ca_V\beta1$ was reduced by non-biotinylated $Ca_V2.1$ AID peptide in a dose-dependent manner. Specifically, an equal amount of non-biotinylated $Ca_V2.1$ diminished binding by about 50%, whereas a 10-fold molar excess almost completely inhibited the interaction (Fig. 4A). On the contrary, the interaction was not affected by the presence of $Ca_V3.3$ peptide even at 1,000-fold molar excess, which is equivalent to a 10-fold molar excess of $Ca_V2.1$ AID peptide if the K_d ratio of $Ca_V3.3$ over $Ca_V2.1$ was considered (Fig. 4A). $Ca_V3.3$ peptide did not compete with $Ca_V2.1$ for binding to $Ca_V\beta4$, either (Fig. 4B). The effect of non-biotinylated $Ca_V2.1$ peptide on the interaction between $Ca_V3.3$ and $Ca_V\beta$ was then examined by Western blot analysis. Non-biotinylated $Ca_V2.1$ peptide had no significant effect on the specific binding of $Ca_V3.3$ to either $Ca_V\beta1$ or $Ca_V\beta4$ (Figs. 4C and 4D).

DISCUSSION

We describe here the identification of a new interaction, at least *in vitro*, between the $Ca_V3.3$ and $Ca_V\beta$ proteins. This interaction is of low affinity compared to that between the AID of the HVA α-subunit and the ABP of the β subunit. The dissociation constants of the $Ca_V3.3$ AID were 3.4 μM and 2.6 μM for binding to $Ca_V\beta1$ and $Ca_V\beta4$, respectively, whereas those of the $Ca_V2.1$ AID were 24 nM and 62 nM, respectively (Fig. 3). The high affinity interaction of $Ca_V2.1$ with $Ca_V\beta1$ and $Ca_V\beta4$ is in good agreement with previous estimates (Canti et al., 2001; Opatowsky et al., 2003). The $Ca_V2.1$ AID peptide had no significant effect on the binding of the $Ca_V3.3$ AID to $Ca_V\beta1$ and $Ca_V\beta4$, which implies that the ABP of $Ca_V\beta$ protein does not play a role in binding to the $Ca_V3.3$ AID (Fig. 4). It has long been known that the β subunit regulates the expression and channel activity

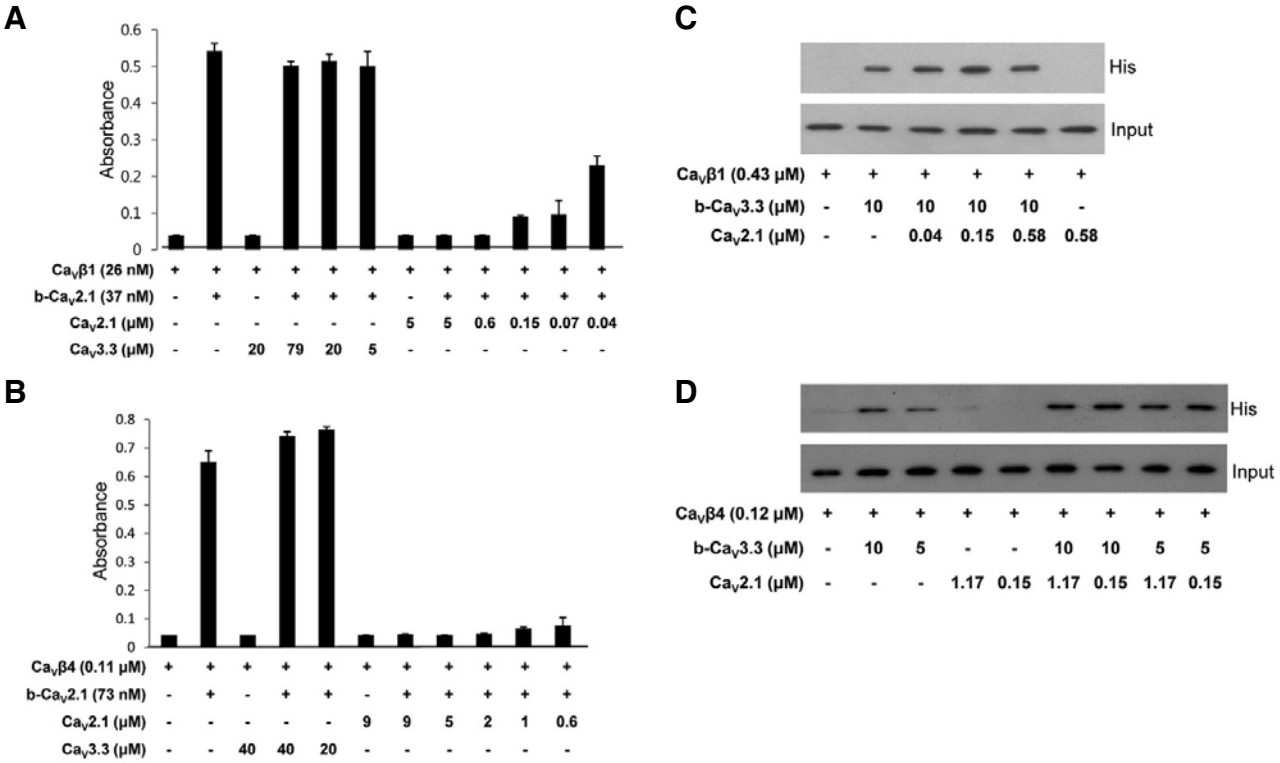

Fig. 4. Characterization of the $Ca_V3.3$-$Ca_V\beta$ interaction. (A) Effect of non-biotinylated $Ca_V3.3$ AID peptides on the $Ca_V2.1$-$Ca_V\beta$ interaction was measured by ELISA competition assay. Various concentrations of non-biotinylated AID peptides ($Ca_V2.1$ or $Ca_V3.3$) were mixed with 37 nM of biotinylated $Ca_V2.1$ AID peptide and 26 nM $Ca_V\beta1$ protein on a streptavidin-coated well plate. The amount of bound $Ca_V\beta$ protein was determined by ELISA using HRP-con-jugated anti-His antibody. Error bars represent standard deviations of three independent replicates (B) ELISA competition assay was performed with 0.11 μM of $Ca_V\beta4$ protein and 73 nM of biotinylated $Ca_V2.1$ AID peptide. (C, D) The effect of non-biotinylated $Ca_V2.1$ AID peptides on the $Ca_V3.3$-$Ca_V\beta$ interaction was measured by avidin pull-down assay. b-Ca_Vx.x: biotinylated Ca_Vx.x AID peptide; Ca_Vx.x: non-biotinylated Ca_Vx.x AID peptide.

of the Ca_V2 and Ca_V1 families, although the extent to which $Ca_V\beta$ influences calcium channel function appears to differ among different expression systems (Richards et al., 2004). The regulation is largely mediated via the AID-ABP interaction between the two subunits of HVA channels. However, such an interaction has not yet been clearly demonstrated in T-type channels (Perez-Reyes, 2006).

There remains a controversy regarding the role $Ca_V\beta$ proteins play in the regulation of expression and voltage gating of T-type channels. Expression of rat β subunits in *Xenopus* oocytes slowed the average current decay of endogenous T-type channels. (Lacerda et al., 1994). However, depletion of endogenous β subunits by RNAi in nodose ganglion neurons or neuroblastoma cell lines showed no effect on T-type currents (Lambert et al., 1997; Leuranguer et al., 1998). Coexpression of recombinant β subunits with $Ca_V3.1$ affected the gating parameters of the $Ca_V3.1$ channel in COS cells (Dolphin et al., 1999) but not in HEK-293 cells (Arias et al., 2005). Despite these functional studies, to the best of our knowledge, there has been no report on the AID-ABP interactions of T-type channels. Most noteworthy is that most of these previous electrophysiological studies on T-type channels were carried out using $Ca_V3.1$ or $Ca_V3.2$ (Chen et al., 2003). This leaves open the possibility of selective interaction of β subunit with $Ca_V3.3$. In our biochemical study, we also observed that none of the $Ca_V\beta$ proteins tested showed binding affinity toward the AIDs of $Ca_V3.1$ and $Ca_V3.2$ (Fig. 2). Even though $Ca_V3.3$ is classified as a T-type channel, it has not been investigated thoroughly compared to the other channels. Sequence similarity analysis revealed that the loop connecting repeats I and II of $Ca_V3.3$ is the most distinct among the three channels. Since the α-β interaction, which is separate from the typical AID-ABP interaction, was only observed in $Ca_V3.3$, one cannot generalize the effect of β subunits on T-type channels based only on the study of $Ca_V3.1$.

For the AID-ABP interactions, specific amino acids, including tryptophan and tyrosine, within the AID motif are crucial for binding to $Ca_V\beta$ subunits (Van Petegem et al., 2004). The AID sequence of the α subunit is well conserved across HVA calcium channels but poorly conserved among LVA calcium channels (Fig. 1B). Based on previous findings, the interactions we observed for the AIDs of Ca_V1 or Ca_V2 (Fig. 2) were likely mediated through the ABP of $Ca_V\beta$ protein. In addition to the AID site, $Ca_V\beta$ binds to secondary sites in α-subunits. Specifically, $Ca_V\beta2a$ binds to $Ca_V2.3$ near the middle of C-terminal tail (Qin et al., 1997) and $Ca_V\beta4$ binds to the C-terminus of $Ca_V2.1$ (Walker et al., 1998). In the latter case, the binding site of $Ca_V\beta4$ is not the ABP but actually the C-terminal region of $Ca_V\beta4$. Although the interaction occurs with almost 100-fold of lower affinity than the typical AID-ABP interaction, it still plays a role in the regulation of channel inactivation kinetics (Walker et al., 1998). The binding affinity observed between $Ca_V3.3$ and $Ca_V\beta$ in the current study was similar to that of $Ca_V2.1$ to the C-terminal region of $Ca_V\beta4$. Under physiological conditions, modular interaction domains bind their protein partners with disso-

ciation constants in the low nanomolar to high micromolar range (Bourgeas et al., 2010; Calakos et al., 1994; Pawson and Nash, 2003). Combined with our results, this suggests that there may be many secondary interactions between core channel proteins and β-subunits. Sequence alignment of the AID peptides shows that AIDs of HVA calcium channels are farther from that of $Ca_V3.3$ than either the other two LVA channels. Therefore, the *in vivo* effect of the β-subunit on the $Ca_V3.3$ channel will be different from the effect on HVA calcium channels.

Rat *in situ* hybridization and Northern blot analysis on human tissues showed that $Ca_V3.3$ is mainly distributed in the brain region, especially the olfactory bulb, straiatum, cerebral cortex, hippocampus, reticular nucleus, laberal habenula, and cerebellum (Monteil et al., 2000; Talley et al., 1999). We found that $Ca_V\beta1$, $Ca_V\beta4$, and obviously $Ca_V\beta2$ showed affinity for $Ca_V3.3$, whereas no interaction was detected for $Ca_V\beta3$ (Fig. 2). These differences in affinity suggest functional significance for the $Ca_V\beta$ proteins according to their tissue distribution. $Ca_V\beta1$ is widely expressed in a number of tissues, including the brain (Ludwig et al., 1997). The $Ca_V\beta2$ subunit is expressed at a low level in the brain but is also present in specific neuronal cell types, including cerebellar Purkinje cells, hippocampal pyramidal neurons, and photoreceptors (Ball et al., 2002; Ludwig et al., 1997). $Ca_V\beta4$ is strongly expressed in the brain, most prominently in the cerebellum (Ludwig et al., 1997). According to the information gathered thus far, the tissue distributions of $Ca_V3.3$ and $Ca_V\beta$ largely overlap. Therefore, it is worthwhile to investigate functional effect of $Ca_V\beta$ proteins on $Ca_V3.3$ in neurons by considering changes in electrophysiological properties as well as in relation to biogenesis. Our data contribute to the understanding of the general interaction between the α and β subunits of VDCCs. The discovery of a new interaction between $Ca_V3.3$ and $Ca_V\beta$ highlights the utility of combinatorial biochemical assays. Though the biochemical data cannot be directly extrapolated to an *in vivo* setup, they at least provide a basic starting point for further in-depth analysis.

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