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ARTICLE ADDENDUM

The HOOK region of β subunits controls gating of voltage-gated Ca^{2+} channels by electrostatically interacting with plasma membrane

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ABSTRACT

Recently, we showed that the HOOK region of the β2 subunit electrostatically interacts with the plasma membrane and regulates the current inactivation and phosphatidylinositol 4,5-bisphosphate (PIP_2) sensitivity of voltage-gated Ca^{2+} (Ca_V) 2.2 channels. Here, we report that voltage-dependent gating and current density of the $Ca_V2.2$ channels are also regulated by the HOOK region of the β2 subunit. The HOOK region can be divided into 3 domains: S (polyserine), A (polyacidic), and B (polybasic). We found that the A domain shifted the voltage-dependent inactivation and activation of $Ca_V2.2$ channels to more hyperpolarized and depolarized voltages, respectively, whereas the B domain evoked these responses in the opposite directions. In addition, the A domain decreased the current density of the $Ca_V2.2$ channels, while the B domain increased it. Together, our data demonstrate that the flexible HOOK region of the β2 subunit plays an important role in determining the overall Ca_V channel gating properties.

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Introduction

Voltage-gated Ca^{2+} (Ca_V) channels adjust the Ca^{2+} influx in excitable cells. The channels contribute to the diverse physiologic responses, including neurotransmission, hormone secretion, muscle contraction, and gene transcription.[1-3] Dysregulation of Ca_V channels causes various neurologic disorders, such as autism, migraine, and pain.[4-7] A high-voltage activated (HVA) Ca_V channel consists of the pore-forming α1 subunit and the auxiliary β and $\alpha2\delta$ subunits. Surface expression of the α1 subunit requires the auxiliary subunits, where they also play critical roles in modulating the biophysical properties of Ca_V α1 channel gating.[8-10] Among the auxiliary subunits, the β subunit is particularly important in regulating the gating and membrane expression of Ca_V channels.[11-13] The β subunit can be divided broadly into 5 regions, such as the N and C terminus, the Src homology 3 (SH3), guanylate kinase (GK) domains, and the flexible HOOK region connecting the 2 domains.[12-18] Among the 5 regions of β subunit, N-terminus plays a key role in determining the subcellular localization of the β subunit, which is principally engaged in modulating the gating kinetics and membrane phospholipid sensitivity of Ca_V channels.[19-23] The Ca_V channel with membrane-anchored β subunit, such as β2a or β2e, exhibits relatively slow current inactivation and low sensitivity to the depletion of phosphatidylinositol 4,5-bisphosphate (PIP_2), whereas channels with cytosolic β subunit, such as β2b or β3, exhibit the opposite responses.

In our recent works, we found that the HOOK region of the β subunit acts as an important regulator for inactivation kinetics and membrane PIP_2 sensitivity of the Ca_V2 channels via dynamic electrostatic interaction of the β subunit with the plasma membrane.[24] The HOOK region can be further divided into 3 domains on the basis of amino acid composition: S (polyserine), A (polyacidic), and B (polybasic). Acidic residues within the A domain are needed for the increase of channel inhibition by PIP_2 depletion and the fast inactivation of $Ca_V2.2$ current like cytosolic β subunits, while basic amino acids within the B region are needed for the decrease in PIP_2 sensitivity and the slow inactivation like membrane-localized β subunits. Therefore, the regulatory mechanism of the

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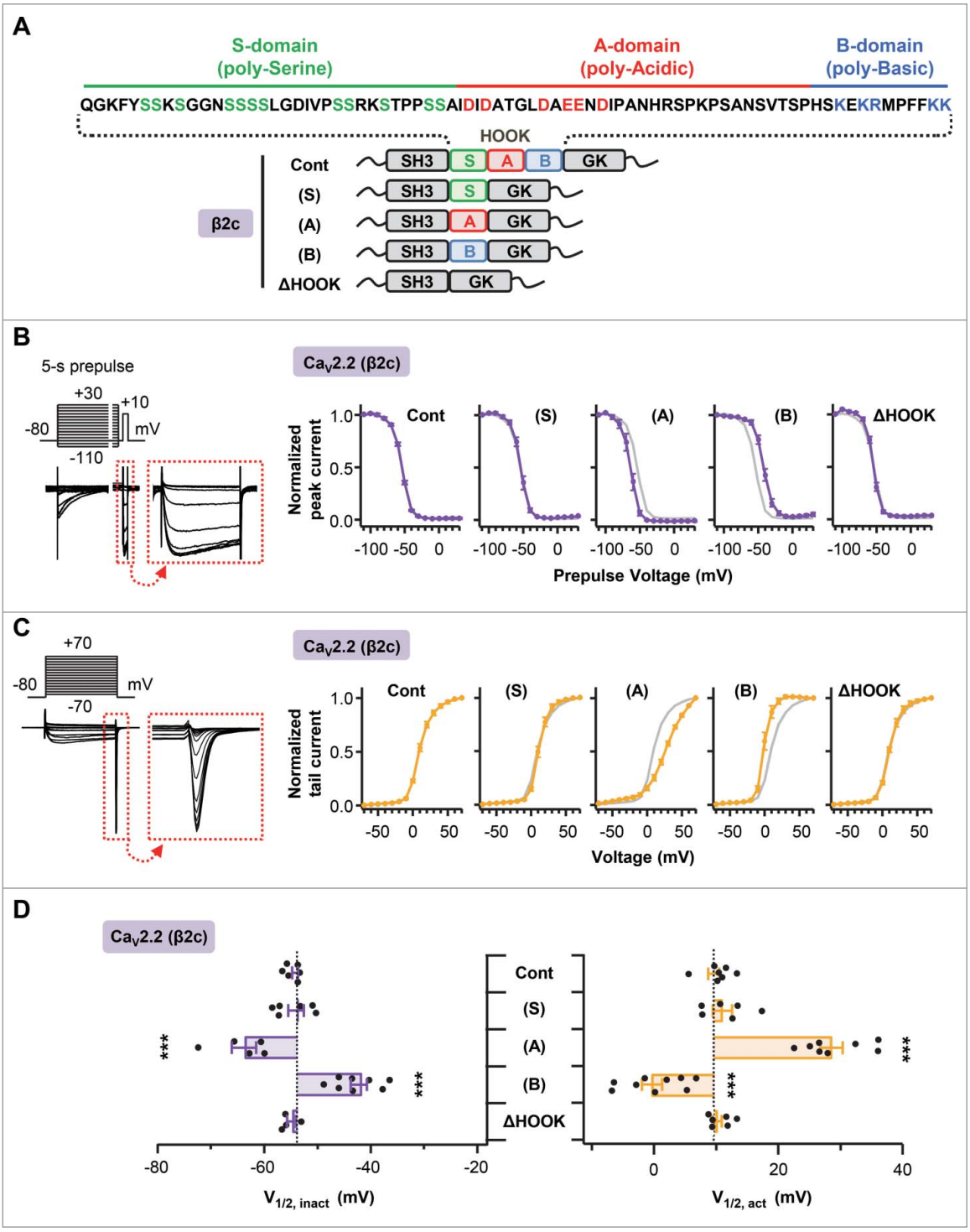

Figure 1. (For figure legend, see page 469.)

Ca_V2 channels by the HOOK region looks very similar to the gating regulation by N-terminus-dependent subcellular localization of the β subunit.

It has been known that the subcellular localization of the β subunit is important for determining the current density of the Ca_V channels.[19] The $Ca_V2.2$ channels with the cytosolic $\beta3$ subunit show significantly lower current density than channels with the membrane-tethered $\beta2a$ subunit. The Lyn-$\beta3$ subunit which is located at the plasma membrane by adding the membrane-targeting Lyn sequence to the cytosolic $\beta3$ increases the current density of the $Ca_V2.2$ channels, whereas mutant $\beta2a$(C3,4S) which localizes in the cytosol by disabling the 2 N-terminal

palmitoylation sites in the β2a subunit decreases the current density of the channels. This suggests that the gating properties of the Ca_V channels including the inactivation kinetics, PIP_2 sensitivity, and current density, are commonly regulated by N-terminus-dependent membrane interaction of the β subunit with the plasma membrane. Here, we further investigated the functional roles of the HOOK region of the β subunit on the gating of $Ca_V2.2$ and $Ca_V1.3$ channels. Our data demonstrate that through the interaction with the plasma membrane, the HOOK region of the β2c subunit also determines both voltage-dependent gating properties and the current density of the Ca_V channels.

Results

The HOOK region of the β2c subunit affects the voltage-dependent gating of the $Ca_V2.2$ channels

We recently showed that the HOOK region of the β2 subunit is crucial for determining the inactivation kinetics and PIP_2 sensitivity of the $Ca_V2.2$ channels through dynamic interaction with the plasma membrane.[24] Here, we examined whether the HOOK region of the β subunit also affects the voltage-dependent inactivation (VDI) and voltage-dependent activation (VDA) of the Ca_V channels. Fig. 1A shows the deletion mutants of the β2c HOOK region. When the β2c derivatives were expressed with N-type $Ca_V2.2$ channels, the VDI of the $Ca_V2.2$ channels with β2c(B) shifted to more positive voltages compared with the β2c control, whereas those channels with β2c(A) shifted to more negative voltages (Fig. 1B and D Left). In contrast, the VDA of the $Ca_V2.2$ channels with β2c (B) shifted to more negative voltages compared with the β2c control, whereas those channels with β2c(A) shifted to more positive voltages (Fig. 1C and D Right). Channels with β2c(S) and β2cΔHOOK did not show any significant changes in VDI and VDA (Fig. 1B–D). Those results suggest that the charged amino acids in the HOOK region plays a key role in determining the voltage-dependent gating properties of the $Ca_V2.2$ channels.

Charged amino acids in the HOOK region of β subunit determines the current density of the $Ca_V2.2$ channels

Because the subcellular localization of the Ca_Vβ subunit is important in determining the current density of the Ca_V channels,[19] we examined whether the HOOK region of the β2c subunit also affects the current density of the $Ca_V2.2$ channels. Fig. 2 shows that the relationship between voltage and current density of the $Ca_V2.2$ channels with β2c(S) or β2cΔHOOK was almost the same as those of channels with the β2c control. However, the $Ca_V2.2$ channels with β2c(A) showed dramatically lower current density, whereas the channels with β2c(B) showed relatively higher density (Fig. 2A and C).

Since charged amino acids of the HOOK region are mainly important in regulating inactivation kinetics and PIP_2 sensitivity of $Ca_V2.2$ channels,[24] we examined whether the charged amino acids of the HOOK region also influence the current density of the $Ca_V2.2$ channels. As shown in Fig. 3A and B,

Figure 1. (see previous page) The HOOK region of β2c subunit is important in determining the voltage-dependent gating of $Ca_V2.2$ channels. (A) Sequence of the HOOK region of β2c (Top) and topological illustration of HOOK region deletion constructs of β2c (Bottom). Full length of β2c consists of the N- and C-terminus, the highly conserved SH3 and GK domain (gray) and HOOK region (green, blue and red). The HOOK region of β2c was separated into 3 domains, S (poly-serine), A (poly-acidic) and B (poly-basic). Serine residues within S-domain are marked in green, acidic residues within A-domain are in blue, and basic residues within B-domain are in red, respectively. (B) The pulse protocol was composed of a 5-s prepulse from -110 mV to 30 mV in 10 mV steps, followed by a 40-ms test pulse to 10 mV. Holding potential is -80 mV (Left, Top). Representative Ba^{2+} current traces were elicited by the pulse protocol (Left, Bottom). Peak point of test pulse currents following 5-s prepulse were normalized to the largest current in each series of test pulse. The curves were fitted by a Boltzmann equation. The voltage dependence of normalized steady-state inactivation for $Ca_V2.2$channels with β2c mutant derivatives (purple lines, Right). Data from control β2c (gray trace) are reproduced to facilitate visual comparison. (C) The pulse protocol was composed of a 40-ms test pulse from −70 mV to 70 mV in 10 mV steps. The membrane potential was held at -80 mV (Left, Top). Representative Ba^{2+} current traces were elicited by the pulse protocol (Left, Bottom). Tail currents following test pulses were normalized to the largest tail current in each series of test pulse. The curves were fitted by a Boltzmann function. The voltage dependence of normalized steady-state activation for $Ca_V2.2$channels with β2c mutant derivatives (orange lines, Right). Data from control β2c (gray trace) are reproduced to facilitate visual comparison. (D) The magnitude of changes in the V1/2 of normalized steady-state inactivation (purple bars, left) and activation (orange bars, right) from the mean of β2c control in cells expressing $Ca_V2.2$channels with β2c mutant derivatives. Dot lines indicate the β2c control level. Dots display the individual data points for each experiment. $^{***}P < 0.001$, one-way ANOVA followed by Dunnett's post-hoc test. Data are mean ± SEM.

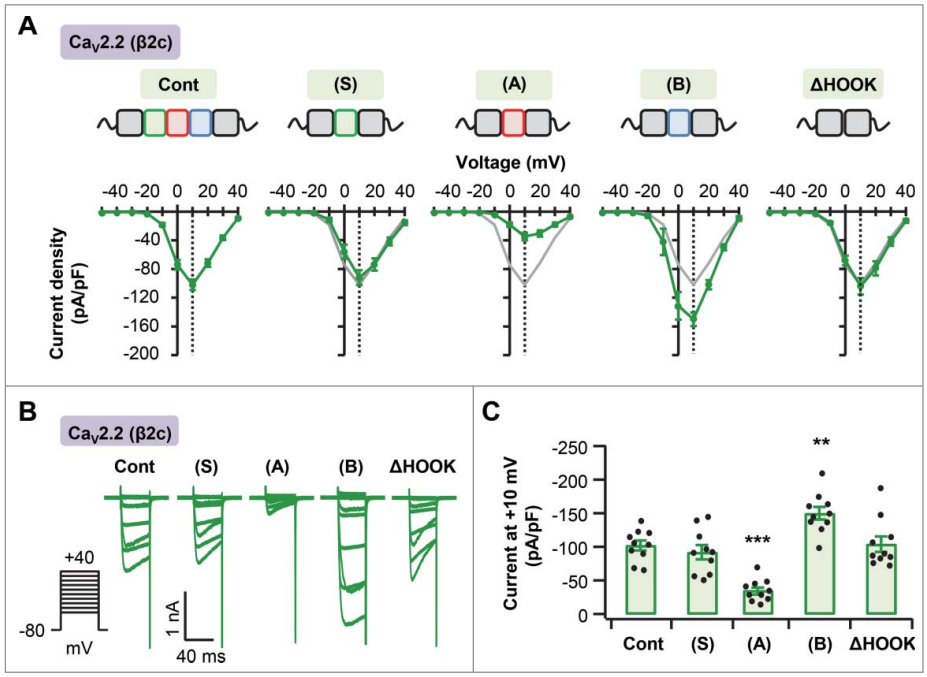

Figure 2. The HOOK region of β2c subunit regulates current density of Ca$_V$2.2 channels. (A) Topological illustration of HOOK region deletion constructs of β2c (Top). S domain is marked in green, A domain is marked in red and B domain is marked in blue, respectively. Population current density versus voltage relations for Ca$_V$2.2 channels with β2c mutant derivatives in tsA-201 cells (Bottom). Data from β2c control (gray trace) are reproduced for visual comparison. (B) Representative Ba^{2+} current traces elicited between -50 and +40 mV in 10 mV steps (see pulse protocol) in cells expressing Ca$_V$2.2 channels with β2c mutant derivatives. Holding potential is -80 mV. (C) Summary of current density (pA/pF) of Ca$_V$2.2 channels with β2c mutant derivatives. Cells were transfected with the same amount of cDNA. Dots display the individual data points for each experiment. $^{**}P < 0.01$, $^{***}P < 0.001$, one-way ANOVA followed by Dunnett's post-hoc test. Data are mean ± SEM.

channels with complete or partial deletion of the A domain and charge-neutralizing mutations in A domain of the β2c subunit showed significantly higher current density than the β2c control, whereas channels with complete deletion of the B domain and charge-neutralizing mutations in B domain showed lower current density (Fig. 3C and D). These results suggest that acidic amino acids of the A domain play a role for decreasing the current density of the Ca$_V$2.2 channels, whereas basic amino acids of the B domain are important for increasing the response. We recently found that 2 hydrophobic Phe residues in the B domain are needed for decreasing the inactivation kinetics and PIP_2 sensitivity of the Ca$_V$2.2 channels.[24] Consistently, the Phe-mutated form (β2cPhe$_{Ala}$) also slightly decrease the current density of the channels (Fig. 3C and D). Together with the findings of our recent study, the present results indicate that dynamic interaction between the HOOK region and the plasma membrane through charged amino acids in the A and B domains are crucial for determining the current density of the Ca$_V$2.2 channels.

The HOOK region of the β subunit modulates the PIP_2 sensitivity and inactivation kinetics of the Ca$_V$1.3 channels

We reported that N-type Ca$_V$2.2 channel gating is tightly modulated by the electrical properties of the HOOK region in the β2c subunit.[24] We then examined whether the HOOK region also influences the PIP_2 sensitivity and inactivation kinetics of L-type Ca$_V$1.3 channels. As shown in Fig. 4A, B, and C, Ca$_V$1.3 channels with β2c(A) show faster current inactivation, whereas channels with β2c(B) show slower inactivation. The fast inactivation kinetics of the Ca$_V$1.3 channels with β2c(S) and β2cΔHOOK were slightly decreased but not significantly different compared with the Ca$_V$1.3 channels with the β2c control. The Ca$_V$1.3 channels with β2c(A) showed higher sensitivity to PIP_2 depletion mediated by the activation of zebrafish form of voltage-sensing phosphatase (Dr-VSP), whereas channels with β2c(B) showed lower inhibition to PIP_2 depletion (Fig. 4D and E). These results demonstrate that the current inactivation

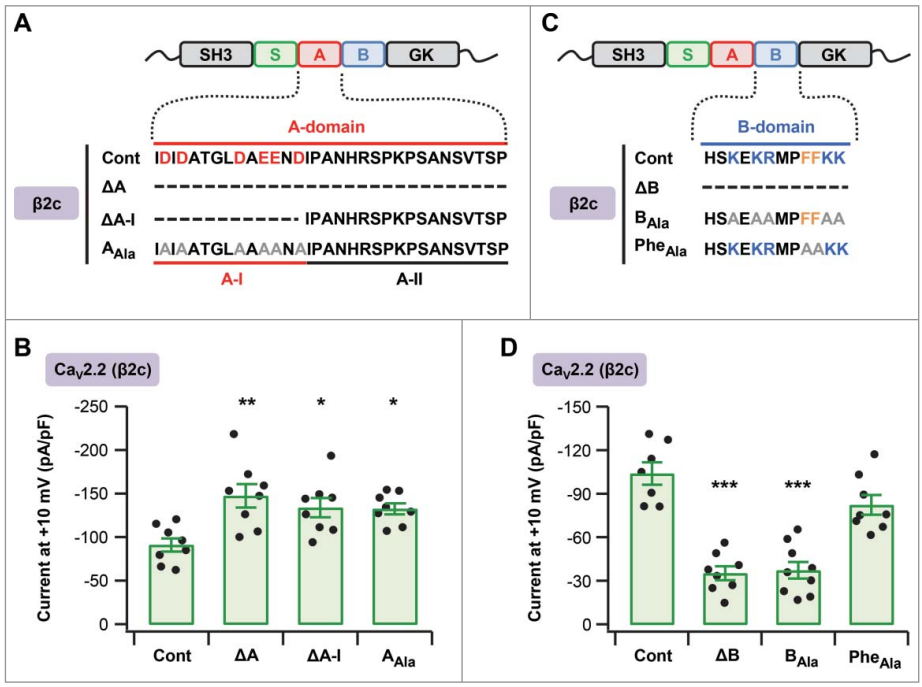

Figure 3. Charged amino acids in HOOK region perform crucial roles in determining the current density of $Ca_V2.2$ channels. (A) Topological illustration of β2c displaying the amino acid sequence of wild type A domain (control), A domain-deleted form (ΔA), A-I domain-deleted form (ΔA-I) and acidic residues replaced by alanine within the A-I domain (A_{Ala}). (B) Summary of current density (pA/pF) of $Ca_V2.2$ channels with A domain derivatives of β2c subunit. Cells were transfected with the same amount of cDNA. (C) Topological illustration of β2c displaying the amino acid sequence of wild type B domain (control), B domain-deleted form (ΔB), basic residues substituted by alanine within B domain (B_{Ala}) and 2 phenylalanine residues substituted with alanine (Phe_{Ala}). (D) Summary of current density (pA/pF) of $Ca_V2.2$ channels with B domain derivatives of β2c subunit. Cells were transfected with the same amount of cDNA. Dots display the individual data points for each experiment. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, one-way ANOVA followed by Dunnett's post-hoc test. Data are mean ± SEM.

and PIP_2 sensitivity of the $Ca_V1.3$ channels are commonly regulated by the flexible HOOK region of the β subunit.

Discussion

We recently found that the HOOK region of the β2 subunit regulates the inactivation kinetics and PIP_2 sensitivity of $Ca_V2.2$ channels via the electrostatic interaction with phospholipids in the plasma membrane. Here, our results enlarge our understanding for functional effects of the HOOK region on Ca_V channel regulation. (a) The HOOK region of the β2c subunit regulates the voltage-dependent gating of $Ca_V2.2$ channels. The β2c subunit containing only the A domain in the HOOK region shifted the VDI and VDA to more negative and positive voltages, respectively, whereas the β2c subunit with only the B domain triggered the responses in the opposite directions. (b) The charged amino acids of the A and B domains are also crucial in determining the current density of the $Ca_V2.2$ channels. Acidic residues within the A domain plays an important role in decreasing the current density of the $Ca_V2.2$ channels, whereas basic residues within the B domain are important in increasing it. (c) The inactivation kinetics and PIP_2 sensitivity of the $Ca_V1.3$ channels are also regulated by the HOOK region of the β2c subunit. Together, our results suggest that the HOOK region determines the channel gating and current density of HVA α1 types.

It has been reported that subcellular localization of Ca_V β subunits is important in regulating the biophysics and PIP_2 sensitivity of the Ca_V channels.[19] Our recent data showed that the net surface charge of dynamic HOOK region of β subunits also performs similar functions in regulating the Ca_V channel gating.[24] We found that the net charge of HOOK region is mainly determined by the exposure of either A- or B-domain to the β-subunit surface. In resting state, A- and B-domains of HOOK region seem to be masking

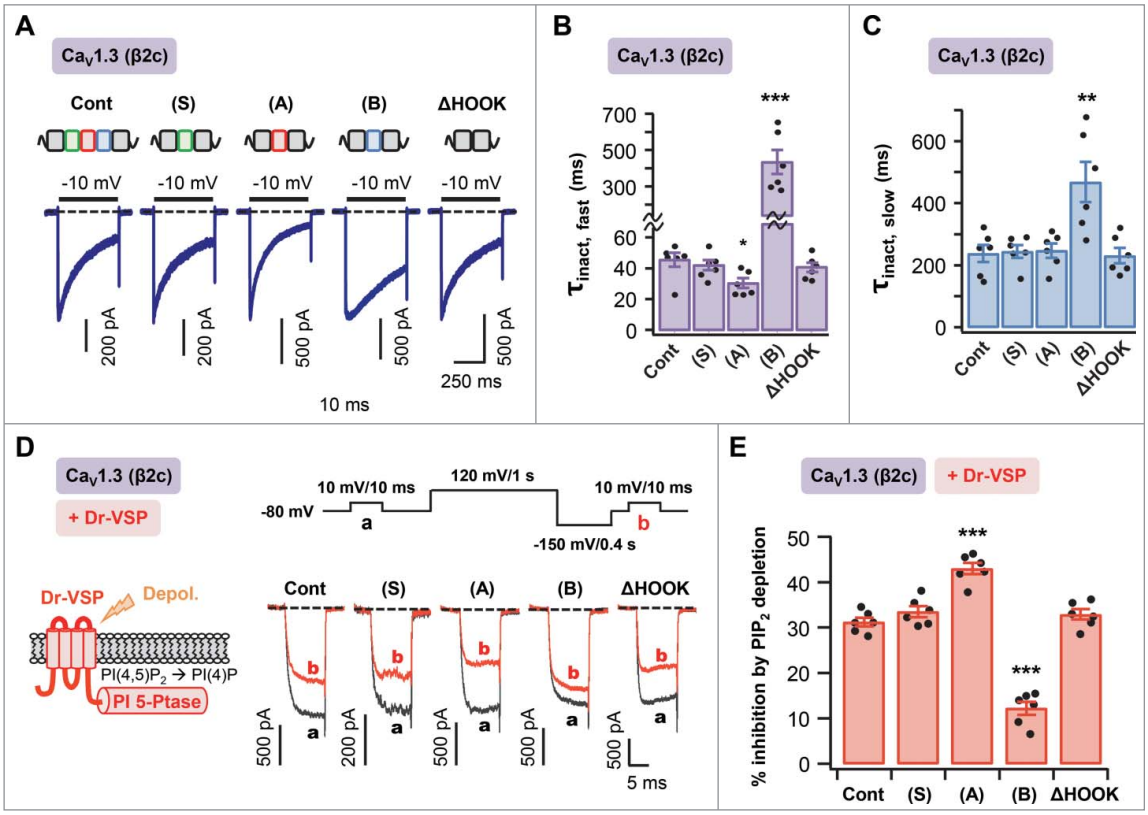

Figure 4. The HOOK region of β2c subunit is crucial in determSining the current inactivation and PIP_2 regulation of $Ca_V1.3$ channels. (A) Schematic illustration of HOOK region deletion constructs of β2c (Top). Current inactivation was measured during 500-ms test pulses to -10 mV in cells expressing $Ca_V1.3$ channels with β2c mutant derivatives (Bottom). (B and C) The current decay of $Ca_V1.3$ channels were fitted to a double exponential function. Summary of time constants of fast ($\tau_{inact,\ fast}$; B) and slow ($\tau_{inact,\ slow}$; C) current inactivation. (D) Current inhibition of $Ca_V1.3$ channels with β2c mutant derivatives by Dr-VSP-mediated PIP_2 depletion. Schematic diagram of Dr-VSP and test protocol (Top). The currents before (a) and after (b) the depolarizing pulse to 120 mV were superimposed (Bottom). (E) Summary of the Dr-VSP-induced current inhibition (percentage) of $Ca_V1.3$ channels with β2c mutant derivatives. Dots display the individual data points for each experiment. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, one-way ANOVA followed by Dunnett's post-hoc test. Data are mean ± SEM.

each other through electrostatic interaction, making the HOOK region be more neutral. When the net charge of HOOK region is changed to the basic by exposing B-domain, the β subunit can further move to the plasma membrane and electrostatically interact with phospholipids via the basic HOOK region. When the net charge of HOOK region, meanwhile, is changed to acidic due to A-domain exposure, the β subunit will move toward the cytosol by repulsion between acidic phospholipids in the plasma membrane and the acidic HOOK region. We found that membrane-interacting β subunit of the Ca_V channels through either N-terminus or HOOK region commonly slows inactivation, enhances PIP_2 sensitivity, and increases the current density of the Ca_V channels. The reason why the Ca_V channels with membrane–interacting β subunit exhibit higher current density than the channels with cytosolic β subunit remains unclear. There may be 2 possibilities. First, the membrane-interacting β subunit enhances the expression level of the Ca_V channel complex in the plasma membrane. Recently, it was reported that the other auxiliary $\alpha 2\delta$ subunit elevates the current density by enhancing the channel trafficking to the plasma membrane.[8] Second, the membrane-tethering β subunit alters the kinetics of channel gating and thus increases the open time of the channel gate. However, in the presence of α1B and $\alpha 2\delta$1, the diverse β2c-deletion mutants are present in the plasma membrane,[24] suggesting that the HOOK region of the β2c subunit does not influence the formation of heteromeric Ca_V channel complex in the plasma membrane. Further studies are needed to define which is important in the gating control of the Ca_V channels by the membrane-interacting β subunit. It is also reported that N-terminus-dependent subcellular localization of the β subunit

regulates the gating of L-type $Ca_V1.3$ and P/Q-type $Ca_V2.1$ channels.[19] Similarly, the phenomena also appeared in our experiments using the mutants of the HOOK region in the $Ca_V1.3$ channels, suggesting that the regulation of the Ca_V channel gating by the N-terminus or HOOK region of the β subunit seems to be a general mechanism for all types of HVA $\alpha1$ subunit.

In conclusion, our findings provide another modulatory mechanism of Ca_V channel gating through the dynamic interaction of the HOOK region of the β subunit with the plasma membrane. Recently, it has been reported that the Ca_V channel gating can be regulated by induced anchoring of intracellular loops of channels to the plasma membrane.[26,27] Those studies suggested a possibility that by interacting with the β subunit, the intracellular I-II loop of the Ca_V channel plays a significant role in regulating the Ca_V channel gating. Further studies are needed to investigate whether the conformational change of the I-II loop using the interacting membrane via the β subunit determines the Ca_V channel gating in cells.

Materials and Methods

Cell culture and transfection

TsA-201 cells were cultured in a Dulbecco Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen) and 0.2% penicillin/streptomycin (Invitrogen) in 100-mm culture dishes at 37°C with 5% CO_2. For Ca_V channel expression, the cells were transiently co-transfected with $\alpha1$, $\alpha2\delta1$ and $\beta2$ subunit in a 1:1:1 molar ratio using Lipofectamine 2000 (Invitrogen). Transfected cells were plated onto Poly-L-lysine-coated coverslip chips 24–36 h after transfection, 12 h before the electrophysiological experiments, as described previously.[24]

Solutions

The bath solution used to record Ba^{2+} currents contained 10 mM $BaCl_2$, 150 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, and 8 mM glucose (adjusted to pH 7.4 with NaOH). The pipette solution contained 175 mM $CsCl_2$, 5 mM $MgCl_2$, 5 mM HEPES, 0.1 mM 1,2-bis(2-aminophenocy)ethane N,N,N',N'-tetraacetic acid (BAPTA), 3 mM Na_2ATP, and 0.1 mM Na_3GTP (adjusted to pH 7.4 with CsOH), as described previously.[24]

Patch clamp recording

Whole-cell Ba^{2+} currents were recorded at room temperature (22–25°C) using patch clamp amplifier EPC10 with pulse software (HEKA). Electrodes pulled from glass micropipette capillaries (Sutter Instrument) had resistances of 2–4 MΩ. Series-resistance errors were compensated for >60%. For all recordings, cells were held at -80 mV, as described previously.[24]

Data analysis

Pulse/Pulse Fit 8.11 software and an EPC-10 patch clamp amplifier (HEKA) were used for data acquisition and analysis. Further data processing used Microsoft Excel, WaveMetrics Igor Pro, and GraphPad Prism version 5.01, as described previously.[24] Voltage dependence of steady-state activation and inactivation was fitted by the Boltzmann function of the form $1/(1+\exp[-(V-V_{1/2})/k])$, where $V_{1/2}$ is the half-maximal voltage for activation or inactivation and K is a slope factor. The time course of current inactivation was fitted by the double exponential function of the form as described previously.[24] All quantitative data were presented as the mean ± SEM. Statistical significance was analyzed using one-way ANOVA, followed by Dunnett's post-hoc test ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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