The HOOK region of voltage-gated Ca^{2+} channel β subunits senses and transmits PIP_2 signals to the gate

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The β subunit of voltage-gated Ca^{2+} (Ca_V) channels plays an important role in regulating gating of the α1 pore-forming subunit and its regulation by phosphatidylinositol 4,5-bisphosphate (PIP_2). Subcellular localization of the Ca_V β subunit is critical for this effect; N-terminal–dependent membrane targeting of the β subunit slows inactivation and decreases PIP_2 sensitivity. Here, we provide evidence that the HOOK region of the β subunit plays an important role in the regulation of Ca_V biophysics. Based on amino acid composition, we broadly divide the HOOK region into three domains: S (polyserine), A (polyacidic), and B (polybasic). We show that a β subunit containing only its A domain in the HOOK region increases inactivation kinetics and channel inhibition by PIP_2 depletion, whereas a β subunit with only a B domain decreases these responses. When both the A and B domains are deleted, or when the entire HOOK region is deleted, the responses are elevated. Using a peptide-to-liposome binding assay and confocal microscopy, we find that the B domain of the HOOK region directly interacts with anionic phospholipids via polybasic and two hydrophobic Phe residues. The β2c-short subunit, which lacks an A domain and contains fewer basic amino acids and no Phe residues in the B domain, neither associates with phospholipids nor affects channel gating dynamically. Together, our data suggest that the flexible HOOK region of the β subunit acts as an important regulator of Ca_V channel gating via dynamic electrostatic and hydrophobic interaction with the plasma membrane.

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INTRODUCTION

Voltage-gated Ca^{2+} (Ca_V) channels play essential roles in adjusting Ca^{2+} influx upon membrane depolarization. These channels are important for various physiological responses, such as neurotransmitter release, muscle contraction, tumorigenesis, hormone secretion, gene expression, and cell death. Dysfunctional regulation of Ca_V channels is associated with numerous diseases, including epilepsy, autism, chronic pain, and migraine (Catterall, 2011).

Ca_V channels can be classified into two subgroups according to their activation threshold: low-voltage activated (LVA) and high-voltage activated (HVA) channels. HVA Ca_V channels require auxiliary α2δ and β subunits for proper trafficking and gating, whereas LVA Ca^{2+} channels can perform their function without any other subunits. Among the auxiliary subunits, the Ca_V β subunit is broadly involved in regulating the surface expression and fine-tuning the gating of Ca_V channels (Lacinová, 2005; Buraei and Yang, 2010). The Ca_V β subunit is composed of five distinct regions: the variable N and C termini, the highly conserved Src homology 3 (SH3) and guanylate kinase (GK) domains, and the flexible and variable HOOK region connecting the SH3 and GK domains (Birnbaumer et al., 1998; Hanlon et al., 1999; Colecraft et al., 2002; Chen et al., 2004; Opatowsky et al., 2004; Van Petegem et al., 2004; Buraei and Yang, 2010). The middle three regions of the β subunit (i.e., SH3, HOOK, and GK) are called the Ca_V β core (De Waard et al., 1994; McGee et al., 2004; Opatowsky et al., 2004; Chen et al., 2009). Of the five regions in the β subunit, the N terminus is key for determining the subcellular localization of β subunits and the inactivation kinetics of Ca_V channels, where N-terminal targeting of the β subunit to the plasma membrane generally slows the current inactivation (Olcese et al., 1994; Chien et al., 1996; Qin et al., 1998). In contrast, the HOOK region is also known to be largely engaged in modulating the inactivation kinetics of Ca_V channels (Qin et al., 1996; Takahashi et al., 2003; Stotz et al., 2004; Richards et al., 2007). For example, swapping the HOOK regions of the β1b and β2a cores made the inactivation of Ca_V channels like those of β2a and β1b, respectively (He et al., 2007). In addition, deletion of the HOOK region from the β2a subunit increased the inactivation rate of $Ca_V2.2$ channels (Richards et al., 2007). Recently, when the HOOK region of the Ca_V β subunit was divided into two segments, polyserine and polybasic, the polybasic segment was responsible for slowing the inactivation rate of $Ca_V2.3$ channels (Miranda-Laferte et al., 2012).

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Abbreviations used: Ca_V, voltage-gated Ca^{2+}; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GK, guanylate kinase; HVA, high-voltage activated; LVA, low-voltage activated; PIP_2, phosphatidylinositol 4,5-bisphosphate; PJ, pseudojanin; PS, phosphatidylserine; Rapa, rapamycin; SH3, Src homology 3.

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All these studies suggest that the HOOK region of the β subunit is another key regulator of Ca_V channel gating. However, the molecular mechanism of the channel regulation by the HOOK region remains unclear.

Ca_V channels are dynamically modulated by receptor-dependent intracellular signals (Hille, 1994; Catterall, 2000). Here, we focus on the regulation of Ca_V channels by the plasma membrane phosphoinositide phosphatidylinositol 4,5-bisphosphate (PIP_2). Direct evidence for PIP_2 regulation of Ca_V channels was obtained using Dr-VSP, a voltage-sensing lipid phosphatase from zebrafish. Dr-VSP is useful for the analysis of PIP_2 regulation of ion channels independent of the activation of G_q-coupled receptors or the generation of downstream second messengers (Murata et al., 2005; Okamura et al., 2009; Falkenburger et al., 2010). By using this technique, it has been found that the activity of HVA, but not LVA, Ca_V channels is suppressed by membrane PIP_2 depletion (Suh et al., 2010; Hille et al., 2015; Jeong et al., 2016). It has also been reported that some of the receptor-mediated slow inhibition of Ca_V current in sympathetic neurons is attributed to the arachidonic acid released from PIP_2 hydrolysis (Liu and Rittenhouse, 2003; Roberts-Crowley et al., 2009).

Our recent studies showed that the subcellular localization of the Ca_V β subunit is important for determining the PIP_2 sensitivity of Ca_V channels as well as the kinetics of current inactivation (Suh et al., 2012; Keum et al., 2014; Kim et al., 2015a,b, 2016). $Ca_V2.2$ channels coexpressed with a membrane-localized β subunit, such as β2a or β2e, exhibit low PIP_2 sensitivity and slow inactivation, whereas channels with a cytosolic β subunit, such as β3, exhibit high PIP_2 sensitivity and fast inactivation (Suh et al., 2012; Kim et al., 2016). The β2a subunit is usually posttranslationally palmitoylated at two cysteine residues in the N terminus and therefore localized at the plasma membrane (Olcese et al., 1994; Chien et al., 1996; Qin et al., 1998; Hurley et al., 2000). When the palmitoylation sites cysteine 3 and 4 are substituted by serine, the mutant β2a(C3,4S) is present in the cytosol in the absence of the α1 pore subunit. $Ca_V2.2$ channels coupled with the mutated β2a(C3,4S) subunit show relatively fast inactivation and high PIP_2 sensitivity, which is similar to channels with a cytosolic β3 subunit. The addition of a membrane-targeting Lyn sequence to the N terminus of the β3 subunit reversibly changes the subunit, making it express in the plasma membrane and act more like a β2a subunit. This suggests that both PIP_2 sensitivity and the inactivation of Ca_V channels are commonly regulated by the subcellular localization of coupled β subunits (Suh et al., 2012; Keum et al., 2014). In addition, the extent of current inactivation and PIP_2 sensitivity are positively correlated with each other.

Here, we examined the molecular mechanism and functional effects of the HOOK region of the Ca_V β subunit on the regulation of HVA $Ca_V2.2$ channels. Our data demonstrate that the HOOK region actively participates in controlling Ca_V channel gating by interacting with the plasma membrane via electrostatic forces and that the coupling of the β subunit to the plasma membrane through the HOOK region slows the inactivation of these channels and decreases their PIP_2 sensitivity. Our results will shed light on how the receptor-mediated dynamic posttranslational modification of the HOOK region could influence Ca_V channel activity in physiological conditions.

MATERIALS AND METHODS

cDNAs

The following plasmids were given to us: the channel subunits α1B of rat $Ca_V2.2e[37b]$ (GenBank accession no. AF055477) and rat α2δ1 (GenBank accession no. AF286488) from D. Lipscombe (Brown University, Providence, RI); Dr-VSP (AB308476) from J.B. Jensen (University of Washington, Seattle, WA); mouse M_1R (GenBank accession no. NM_001112697) from N.N. Nathanson (University of Washington, Seattle, WA); and RFP-PJ, RFP-dead, and LDR from B. Hille (University of Washington School of Medicine, Seattle, WA).

Molecular cloning

Mouse cDNAs of β2a, β2c, and β2c-short were cloned by V. Flockerzi (Saarland University, Homburg, Germany). For the C-terminal fusion of green fluorescent protein (GFP) to each β2 subunit, the cDNAs encoding β2a, β2c, and β2c-short were amplified by PCR using nTaq DNA polymerase (Enzynomics), TA cloned into T-Easy Vector (Promega), and cloned in pEGFP-N1 vector (Takara Bio Inc.). The primers used for β2-GFP are listed in Table S1. For point and deletion mutants, β2-GFP was amplified by inverse PCR using Pfu Turbo DNA polymerase (Agilent Technologies), plasmid DNA was digested by Dpn I (Agilent Technologies), the PCR product was 5′-phosphorylated by T4 polynucleotide kinase (Enzynomics), and then PCR product was ligated by T4 DNA ligase (New England Biolabs, Inc.). The primers used for mutagenesis are listed in Table S2. The mutants were verified by DNA sequencing.

Cell culture and transfection

Human embryonic kidney tsA-201 cells were obtained from B. Hille. The cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS (Invitrogen) and 0.2% penicillin and streptomycin (Invitrogen) in 100-mm culture dishes at 37°C with 5% CO_2. For transfection, Lipofectamine 2000 (Invitrogen) was used when the confluency of cells reached 50–70%. For Ca_V channel expression, cells were cotransfected with α1 of Ca_V, α2δ1, and various β2 subunits in a 1:1:1 molar ratio. Transfected cells were plated onto coverslip chip coated with 0.1 mg/ml poly-L-lysine (Sigma-Aldrich), and the fluorescent cells were studied in electrophysiological and confocal experiments 36–48 h after transfection, as described previously (Suh et al., 2012).

Solutions and materials

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, 3 mM Na_2ATP, and 0.1 mM Na_3GTP (adjusted to pH 7.4 with CsOH). The bath solution for confocal imaging contained 160 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 8 mM glucose (adjusted to pH 7.4 with NaOH).

Current recording

A whole-cell configuration was used to record Ba^{2+} currents using a HEKA EPC-10 amplifier with pulse software at room temperature (22–25°C). Electrodes pulled from glass micropipette capillaries (Sutter Instrument) had resistances of 2–4 MΩ. The whole-cell access resistance was of 2–6 MΩ, and series-resistance errors were compensated by 60%. Ba^{2+} currents were recorded with a membrane holding potential of −80 mV, applying a 10-ms or 500-ms test pulse. For Dr-VSP experiments, step depolarization to 120 mV for 1 s was applied to activate Dr-VSP and deplete PIP_2 in cells (Keum et al., 2014).

Confocal imaging

Images were taken with the LSM 700 confocal microscope (ZEISS) at room temperature (22–25°C). For time courses, images were obtained by scanning cells with a 40× (water) objective lens at 512 × 512 pixels using digital zoom. During time course experiments, images were taken every 5 s in imaging software (Zen; ZEISS). For a single image, 1,024 × 1,024 pixels were used. Analysis of line scanning and quantitative analysis of the plasma membrane or cytosolic fluorescence intensity was performed using the "profile" and the "measure" tools, respectively, for the region of interest in Zen 2012 lite imaging software (ZEISS). All images were transferred from LSM4 to JPEG format, and raw data from time-course experiments were processed with Excel 2010 (Microsoft) and Igor Pro (WaveMetrics), as described previously (Kim et al., 2016).

Preparation of liposomes

All lipids were purchased from Avanti Polar Lipids, Inc. Liposomes consisted of L-α-phosphatidylcholine, L-α-phosphatidylethanolamine, L-α-phosphatidylserine (PS), cholesterol, PIP_2, and Rhodamine-DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-lissamine rhodamine B sulfonyl ammonium salt; 48%:14%:10%:25%:2%:1% molar percentage). In case of no PS or PIP_2, L-α-phosphatidylcholine contents were adjusted accordingly. In brief, lipid mixture dissolved in chloroform/methanol mixture (2:1 ratio) was dried under a gentle stream of nitrogen in the hood, thereby generating lipid film. Lipid film was then dissolved with 100 µl buffer containing 150 mM KCl, 20 mM HEPES, pH 7.4, with KOH and 5% sodium cholate (Kim et al., 2015b). Detergent was removed using size exclusion column (Sephadex G50 in 150 mM KCl and 20 mM HEPES, pH 7.4).

Assay for peptide–liposome binding

Peptides were labeled at the N terminus with BODIPY 493/503 succinimidyl ester (D-2191; Invitrogen). Binding of peptide to liposomes was monitored using fluorescence resonance energy transfer (FRET) measurements in which rhodamine-DOPE incorporated in liposomes quenches the fluorescence of BODIPY 493/503. All measurements were performed at 37°C in 1 ml of buffer containing 150 mM KCl and 20 mM HEPES-KOH, pH 7.4; excitation at 490 nm and emission at 515 nm. FRET was normalized as F/F_0, where F_0 and F represent fluorescence before and after liposome addition, respectively.

Curve fitting

The time course of Ca_V current inactivation was fitted by the double exponential function of the form:

$$I = A_{fast} \times \exp\frac{(-t - t_0)}{\tau_{fast}} + A_{slow} \times exp\frac{(-t - t_0)}{\tau_{fast}} + C,$$

where τ_{fast} and τ_{slow} are the time constants of fast and slow, respectively. A_{fast} and A_{slow} are each the current amplitude of the time constants.

Data analysis

Data acquisition and analysis used Pulse/Pulse Fit 8.11 software in combination with an EPC-10 patch-clamp amplifier (HEKA). Additional data processing was performed with Igor Pro (WaveMetrics), Excel 2010 (Microsoft), and GraphPad Software Prism version 5.01. Time constants measured by double exponential fit. All quantitative data were expressed as the mean ± SEM and analyzed by Student's *t* test or one- or two-way ANO VA followed by Bonferroni or Dunnett's post-hoc test (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$).

Online supplemental material

Table S1 shows primers for tagging GFP. Table S2 shows primers for β2 mutagenesis.

RESULTS

The HOOK region of the β2 subunit regulates inactivation kinetics and PIP_2 sensitivity of $Ca_V2.2$ channels

The amino acid sequences of the five isoforms of the β2 subunit are the same, except for the N-terminal region because they are encoded by a single gene with multiple splicing (Takahashi et al., 2003; Link et al., 2009; Kim et al., 2015a). Here, we divided the HOOK region of the β2c subunit into three domains based on amino acid composition: S (polyserine), A (polyacidic), and B (polybasic; Fig. 1 A). The S domain retains the serine-rich sequence that includes possible phosphorylation sites. The A and B domains retain acidic and basic amino acid–rich sequences, respectively. β2c(S), β2c(A), and β2c(B) are β2c subunits with only S, A, and B domains in the HOOK region, respectively. β2cΔHOOK is a β2c subunit without the indicated whole HOOK region. In the presence of α1B and α2δ1, all the β2c mutant derivatives labeled with GFP were located at the plasma membrane (Fig. 1 A, inset). This suggests that the HOOK region of the β2c subunit does not affect heteromeric Ca_V channel complex formation or targeting of Ca_V channels to the plasma membrane (Opatowsky et al., 2004; Richards et al., 2007; Miranda-Laferte et al., 2012). Then, we compared the effects of β2c derivatives on the inactivation of $Ca_V2.2$ currents. As shown in Fig. 1 B, channels with β2c(A) showed faster inactivation than channels with control β2c, whereas channels with β2c(B) showed much slower inactivation. Interestingly, the current inactivation was also significantly faster in $Ca_V2.2$ channels complexed with β2c(S) or β2cΔ HOOK. The inactivation of $Ca_V2.2$ currents was fitted to a double exponential function. The fast component of inactivation ($\tau_{inact,\ fast}$) was dramatically altered by deletion mutations of the β2c HOOK region, whereas the slow component of inactivation ($\tau_{inact,\ slow}$) showed no change, except for β2c(B) (Fig. 1, C and D).

In control experiments without Dr-VSP, the current amplitude of $Ca_V2.2$ channels with β2c derivatives was not significantly different before and after 120-mV depolarization (Fig. 1, E and F, top). In contrast, PIP_2 depletion by Dr-VSP–mediated depolarization inhibited $Ca_V2.2$ channels with β2c(S) by 42 ± 1%, which was similar to channels with β2cΔHOOK (43 ± 1%) but more than channels with β2c control (37 ± 1%; Fig. 1, E and F, bottom). $Ca_V2.2$ channels with the β2c(A) subunit showed higher inhibition (58 ± 1%) after PIP_2 depletion, whereas channels with the β2c(B) subunit showed dramatically lower inhibition (9 ± 1%). These results indicate that the positively charged B domain of the HOOK region plays an important role in decreasing the PIP_2 sensitivity and inactivation rate of $Ca_V2.2$ channels, whereas the negatively charged A domain plays a role in increasing these responses. Because the HOOK-deleted β2cΔHOOK and A/B domain–deleted β2c(S) subunits acted more like β2c(A), the net charge of the intact HOOK region seems to be slightly positive. Together, the data suggest that the HOOK region of the β2c subunit serves as an important regulator of not only ion conductance but also PIP_2 regulation of $Ca_V2.2$ channels.

We also examined whether the membrane-tethered β2a subunit regulates channel gating through the HOOK region (Fig. 2 A, left). Because the N terminus of the β2a subunit contains two cysteines that can be palmitoylated, thus targeting the subunit to the plasma membrane, we also constructed several HOOK region mutant derivatives using the palmitoylation-resistant mutant form β2a(C3,4S) (β2aMT; Fig. 2 A, right). The functional effects of HOOK region mutants were similar to those seen with β2c derivatives (Fig. 1). For example, $Ca_V2.2$ channels coupled with (S), (A), or ΔHOOK derivatives of either WT β2a or β2aMT showed faster inactivation, whereas channels with the (B) derivative showed relatively slower inactivation. In addition, the current was mostly altered in $\tau_{inact,\ fast}$, but not in $\tau_{inact,\ slow}$ (Fig. 2, B and C). Similarly, PIP_2 depletion by Dr-VSP inhibited $Ca_V2.2$ channels with β2a control by 10 ± 1%, which was more than with β2a(B) (4 ± 1%) but less than with β2a(A) (17 ± 1%; Fig. 2, D and E). The effects of β2a(S) and β2aΔHOOK on $Ca_V2.2$ inhibition by PIP_2 depletion were insignificant compared with β2a control (10 ± 1% for β2a(S), 9 ± 1% for β2a ΔHOOK). PIP_2 depletion also inhibited $Ca_V2.2$ channels with β2aMT control by 38 ± 1%, which was less than with β2aMT(A) (56 ± 1%), β2aMT(S) (42 ± 1%), and β2aMT ΔHOOK (42 ± 1%) and more than with β2aMT(B) (9 ± 1%). These results show that the modulatory effects of the HOOK region on $Ca_V2.2$ channel biophysics were almost the same between membrane-tethered β2a and cytosolic β2c, suggesting that the HOOK region independently regulates Ca_V channel gating and that regulation is not affected by N-terminal targeting of the β subunit to the plasma membrane.

Charged amino acids in HOOK region mainly determine PIP_2 sensitivity and inactivation kinetics of $Ca_V2.2$ channels

It has been reported that the basic amino acids of the polybasic linker segment of the β2a subunit are important in slowing the inactivation rate of $Ca_V2.3$ channels (Miranda-Laferte et al., 2012). To further test which amino acids of the basic B domain affect the PIP_2 sensitivity of $Ca_V2.2$ channels, we made several mutant constructs of the B domain and investigated their actions in gating regulation (Fig. 3 A). First, the B domain–deleted mutant β2cΔB or substitution of the basic residues for alanine within the B domain (β2c-B_{Ala}) increased the inactivation and PIP_2 sensitivity of $Ca_V2.2$ channels (Fig. 3, B–E). These phenomena also similarly appeared in channels with the Phe-mutated

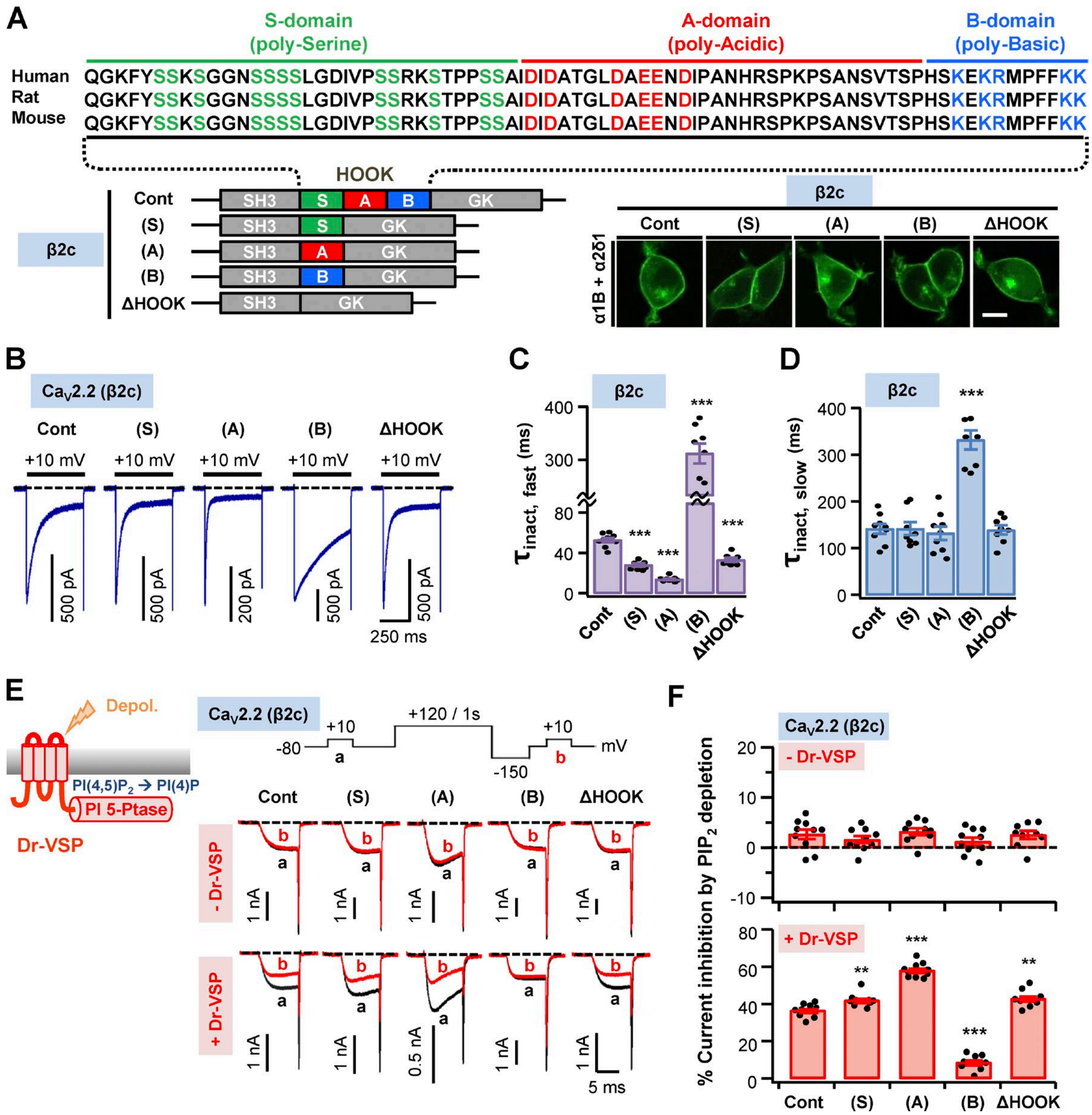

Figure 1. **The HOOK region of β2c subunit plays an important role in determining current inactivation and PIP_2 regulation of $Ca_V2.2$ channels.** (A) Schematic diagram of HOOK region deletion constructs of β2c used in this study. The domain structure of Ca_V β subunit consists of the N terminus, SH3 domain (gray), HOOK region (green, blue, and red), GK domain (gray), and C terminus. Sequence alignment of the HOOK region of β2c from human, rat, and mouse is shown (top). The HOOK region of the β2c subunit was divided into three domains: S (polyserine), A (polyacidic), and B (polybasic). Serine residues within the S domain are indicated in green, acidic residues within the A domain are in blue, and basic residues within the B domain are in red. (inset) Confocal images of tsA-201 cells expressing α1B, α2δ1, and β2c deletion mutants fused to GFP. Bar, 10 µm. (B) Current inactivation of $Ca_V2.2$ channels with β2c mutant derivatives were measured during 500-ms test pulses to 10 mV. (C and D) The current decay of $Ca_V2.2$(β2c) was fitted to a double exponential function. Summary of time constants of fast ($\tau_{inact,\ fast}$; C) and slow ($\tau_{inact,\ slow}$; D) current inactivation. (E) Current inhibition of $Ca_V2.2$ channels with β2c derivatives by Dr-VSP–mediated PIP_2 depletion. Cells received a test pulse (a) and then a depolarization to 120 mV and a hyperpolarization to less than −150 mV, followed by the second test pulse (b). $Ca_V2.2$(β2c) currents before (a) and after (b) the depolarizing pulse are superimposed in control (top) and Dr-VSP–transfected (bottom) cells. (F) Summary of $Ca_V2.2$(β2c) current inhibition (percentage) by PIP_2 depletion in control (top) and Dr-VSP-expressing (bottom) cells. Dots indicate the individual data points for each experiment. **, $P < 0.01$; ***, $P < 0.001$, compared with control. Data are mean ± SEM.

form (β2cPhe$_{Ala}$). The results suggest that both basic amino acids and aromatic Phe residues of the B domain are important in slowing the inactivation of $Ca_V2.2$ channels and decreasing their PIP_2 sensitivity. We also examined the effects of acidic amino acids by constructing A domain mutant forms. The A domain of the HOOK region can be divided further into A-I and A-II domains (Fig. 3 F). The A-I domain possesses all the acidic residues. When the acidic amino acids were replaced with alanine (β2cA$_{Ala}$), the inactivation

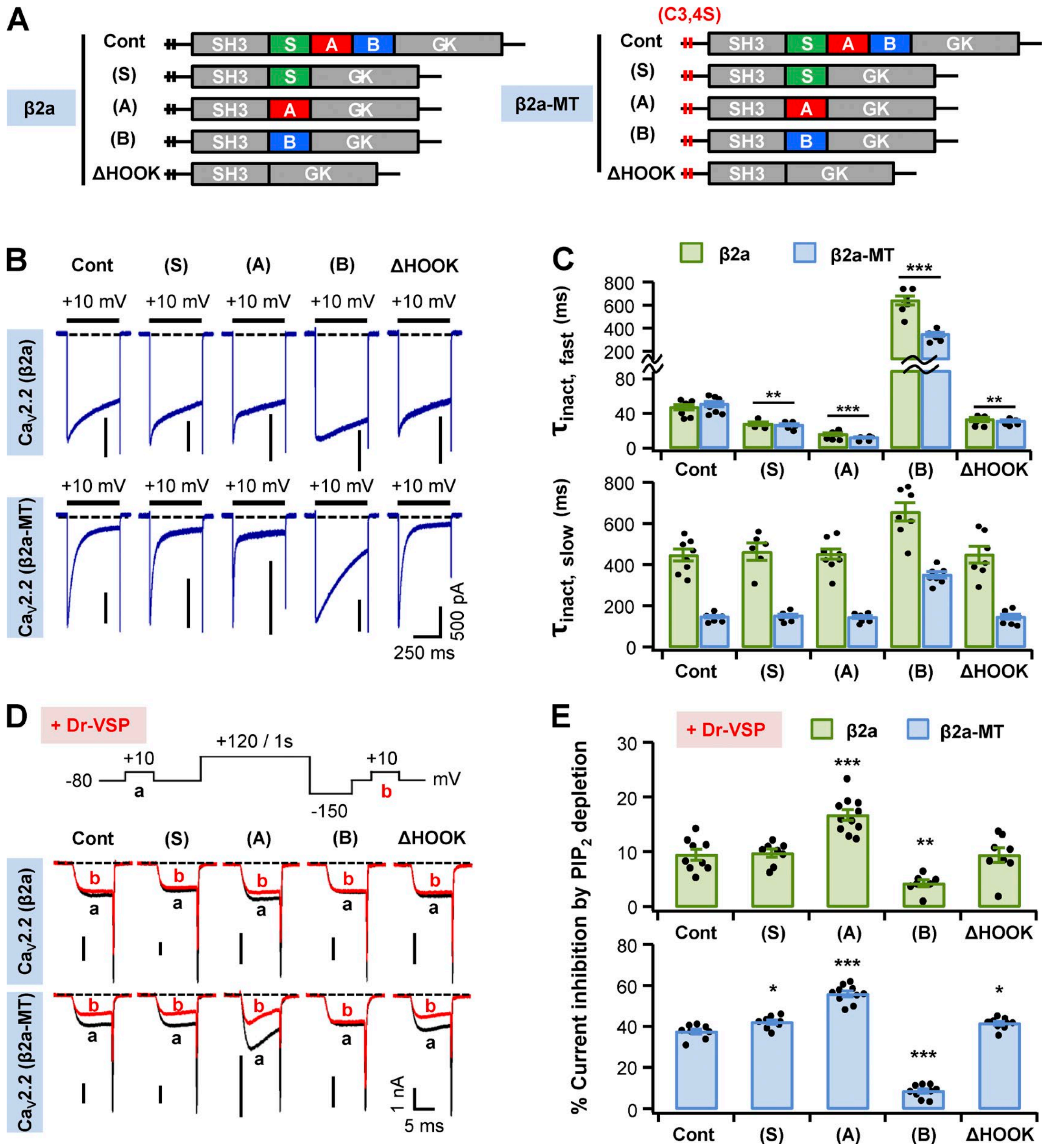

Figure 2. **Both the N terminus and the HOOK region are important in determining the current inactivation and PIP_2 regulation of $Ca_V2.2$ channels with a β2a subunit.** (A) Schematic diagram of HOOK region deletion constructs of β2a (left) and β2aMT (right) used in this study. In the palmitoylation-resistant mutant β2aMT, the palmitoylation residues cysteine 3 and 4 (black) are replaced with serine (red). (B) Current inactivation was measured during 500-ms test pulses to 10 mV in cells expressing $Ca_V2.2$ channels with β2a derivatives (top) or β2aMT derivatives (bottom). (C) The current decay of the $Ca_V2.2$ channels was fitted to a double exponential function. Summary of the time constants for fast ($\tau_{inact,\ fast}$; top) and slow ($\tau_{inact,\ slow}$; bottom) current inactivation. (D) Current trace of $Ca_V2.2$ channels with β2a derivatives (top) or β2aMT derivatives (bottom) before (a) and after (b) depolarizing-pulse in Dr-VSP–expressing cells. (E) Summary of current inhibition (percentage) by PIP_2 depletion in cells with $Ca_V2.2$ channels with β2a derivatives (top) or β2aMT derivatives (bottom). Dots indicate the individual data points for each experiment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with control. Data are mean ± SEM.

of $Ca_V2.2$ channels was slowed similarly to that of the A-I domain–deleted mutant (β2cΔA-I), but they showed slightly faster inactivation than the A domain–deleted mutant (β2cΔA; Fig. 3, G and H). Consistently, β2cΔA, β2cΔA-I, and β2cA_{Ala} showed significantly lower PIP_2 sensitivity than β2c control, and there was no difference among the mutant forms (Fig. 3, I and J). Therefore, our data suggest that the PIP_2 sensitivity and inactivation of Ca_V channels are dependent on the net charges of the HOOK region; the positively charged HOOK region decreases these responses, whereas the negatively changed HOOK region increases them. These results also demonstrate that two hydrophobic Phe residues in the B domain play important roles in decreasing the responses together with the basic amino acid residues.

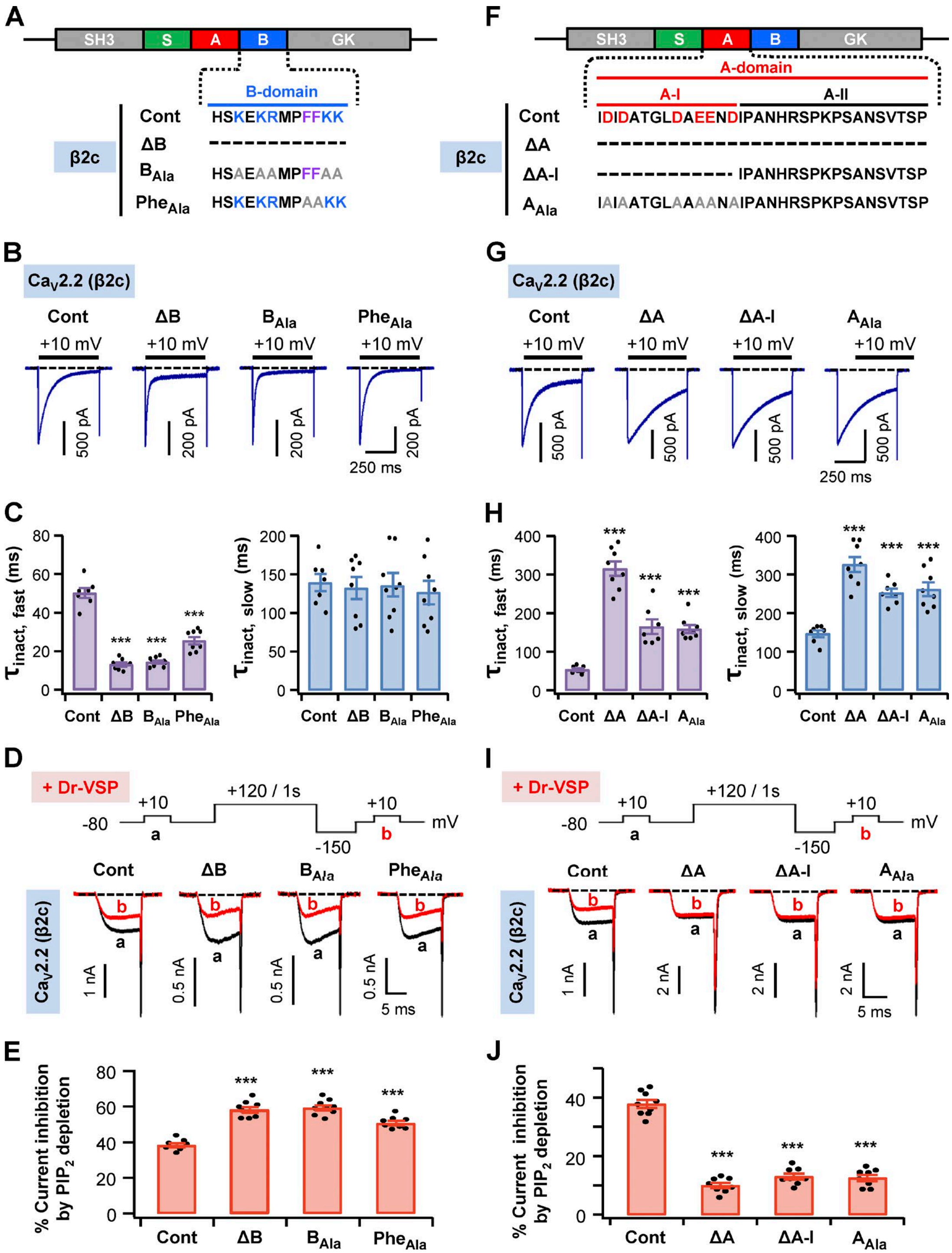

Figure 3. **Charged amino acids in the HOOK region play important roles in determining the current inactivation and PIP_2 sensitivity of $Ca_V2.2$ channels.** (A) Schematic diagram of β2c showing the amino acid sequence of WT B domain (control), B domain–deleted mutant (ΔB), basic residues replaced with alanine within the B domain (B_{Ala}) and two phenylalanines replaced with alanine (Phe_{Ala}). (B) Current inactivation of $Ca_V2.2$ channels was measured during 500-ms test pulses to 10 mV in cells with β2c control, ΔB, B_{Ala}, or Phe_{Ala}. (C) The current decay of the $Ca_V2.2$ channels was fitted to a double exponential function. Summary of time constants for fast ($\tau_{inact,\ fast}$; top) and slow ($\tau_{inact,\ slow}$; bottom) current inactivation. (D) Current inhibition of $Ca_V2.2$ channels with B domain derivatives of β2c subunit by Dr-VSP-mediated PIP_2 depletion. The currents before (a) and after (b) the depolarizing pulse are superimposed. (E) Summary of current inhibition (percentage) by PIP_2 depletion in cells with $Ca_V2.2$ channels with B domain derivatives of β2c. Note that basic residues of the B domain decrease the PIP_2 sensitivity of $Ca_V2.2$ channels. (F) Schematic diagram of β2c showing the amino acid sequence of WT A domain (control), A domain–deleted mutant (ΔA), A-I domain–deleted mutant (ΔA-I), and acidic residues substituted by alanine within the A-I domain (A_{Ala}). (G) Current inactivation of $Ca_V2.2$ channels was measured during 500-ms test pulses to 10 mV in cells with β2c control, ΔA, ΔA-I, or A_{Ala}. (H) Summary of time constants for fast ($\tau_{inact,\ fast}$; top) and slow ($\tau_{inact,\ slow}$; bottom)

Charged amino acids in the HOOK region affect muscarinic modulation of $Ca_V2.2$ channels

We then tested whether the HOOK region of the β2c subunit also affects the G_q-coupled receptor–mediated modulation of $Ca_V2.2$ channels by coexpressing M_1 muscarinic receptors (M_1Rs; Fig. 4). Our data show that M_1R activation with oxotremorine M inhibited $Ca_V2.2$ channels with β2c control by 56 ± 2%, which is less than with β2cΔB (70 ± 3%) and $β2cB_{Ala}$ (68 ± 2%) but more than with β2cΔA (39 ± 2%) and $β2cA_{Ala}$ (42 ± 3%; Fig. 4 F), which are similar to the mode of regulation by Dr-VSP–mediated PIP_2 depletion. However, because M_1 muscarinic modulation of $Ca_V2.2$ channels depends not only on the PIP_2-dependent pathway but also on the Gβγ-dependent pathway (Keum et al., 2014), the regulatory effects of HOOK region derivatives on the muscarinic modulation of the channels appear weak compared with Dr-VSP regulation.

The short form of the HOOK region in the β2c-short subunit shows similar regulatory effects on PIP_2 sensitivity and inactivation of $Ca_V2.2$ channels

The amino acid sequences of β2c and β2c-short are the same except for the HOOK region (Fig. 5 A). The polybasic (Bs) domain of β2c-short possesses half of the basic residues of the B domain of β2c without hydrophobic Phe residues (Fig. 5 A). In addition, there is no A domain in the β2c-short HOOK region. However, we could not detect any significant differences between $Ca_V2.2$ channels with β2c and β2c-short in terms of PIP_2 sensitivity and current inactivation (Fig. 5, B–F). When $Ca_V2.2$ channels were expressed with β2c-short containing only Bs, the current was not much different from that of β2c-short control. However, the channels exhibited faster inactivation and higher PIP_2 sensitivity compared with the channels with β2c containing only the B domain (Fig. 5, B–F). These results suggest that the three basic amino acids without hydrophobic residues in the Bs domain may be insufficient to slow the current inactivation and decrease the PIP_2 sensitivity of $Ca_V2.2$ channels.

The B domain of the HOOK region tethers a β subunit to the plasma membrane via electrostatic and hydrophobic interactions

How does the HOOK region of the β subunit regulate Ca_V channel biophysics? β2c(B) decreased both PIP_2 sensitivity and current inactivation of the Ca_V channel like the membrane-tethering β2a subunit. Therefore, we examined whether the B domain of the HOOK region can directly interact with the plasma membrane. First, we used a peptide-to-liposome FRET assay using synthesized peptides of the B domain of the HOOK region (Fig. 6 A). These peptides were labeled with the green fluorescence dye BODIPY 493/503 and served as the energy donor. Liposomes were labeled with the red fluorescence dye rhodamine and served as the energy acceptor. This FRET assay measures the change in emission intensity of green fluorescence (Fig. 6 A, left). When B domain WT peptides were added to liposomes lacking anionic phospholipids (Fig. 6 A, black traces), the intensity of green fluorescence was insignificantly attenuated, indicating no peptide–liposome interaction (Fig. 6 B). However, when 2% PIP_2 and 10% PS (Fig. 6 B, red traces) were added to the liposomes, there was a noticeable decrease in FRET, indicating the binding of B domain WT peptides to the liposomes (Fig. 6, B and C). It is known that basic amino acids and aromatic residues are major determinants of the membrane incorporation of peripheral proteins (Gelb et al., 1999; Miranda-Laferte et al., 2014; Kim et al., 2015a,b; Kim and Suh, 2016). Thus, we investigated whether mutant forms of B domain peptide (B_{Ala} and Phe_{Ala}) can interact with liposomes containing anionic lipids. When the mutant peptide forms of the B domain were added to the liposomes, FRET signals were not different between liposomes without (black traces) and with (red traces) anionic lipids (Fig. 6, B and C). This suggests that both basic amino acids and aromatic residues are essential for membrane binding of the B domain, which is consistent with the decreasing effects of the residues on inactivation kinetics and PIP sensitivity (Fig. 4). We also examined whether the Bs domain peptide of β2c-short can bind liposomes. No differences in FRET signals of the Bs domain peptide of β2c-short were found between liposomes without (black) and with (red) anionic phospholipids (Fig. 6, B and C). This result indicates that the short Bs domain is not sufficient to make a stable interaction with the membrane, probably because this peptide does not have aromatic residues and has insufficient basic residues.

We then examined which phospholipids are responsible for the interaction with the B domain WT peptide. When either 2% PIP_2 (Fig. 6, D and E, green traces) or 10% PS (blue traces) was applied to the liposomes independently, the intensity of green fluorescence showed a slight attenuation compared with liposomes without both anionic lipids (Fig. 6, D and E, black traces). When

current inactivation. Dots indicate the individual data points for each experiment. *, P < 0.05; ***, P < 0.001 compared with control. (I) Current inhibition of $Ca_V2.2$ channels with A domain derivatives of the β2c subunit by Dr-VSP activation. The currents before (a) and after (b) the depolarizing pulse are superimposed. (J) Summary of current inhibition (%) by PIP_2 depletion in cells with $Ca_V2.2$ channels with A domain derivatives of β2c. Note that acidic residues of the A domain increase PIP_2 sensitivity of $Ca_V2.2$ channels. Dots indicate the individual data points for each experiment. ***, P < 0.001 compared with control. Data are mean ± SEM.

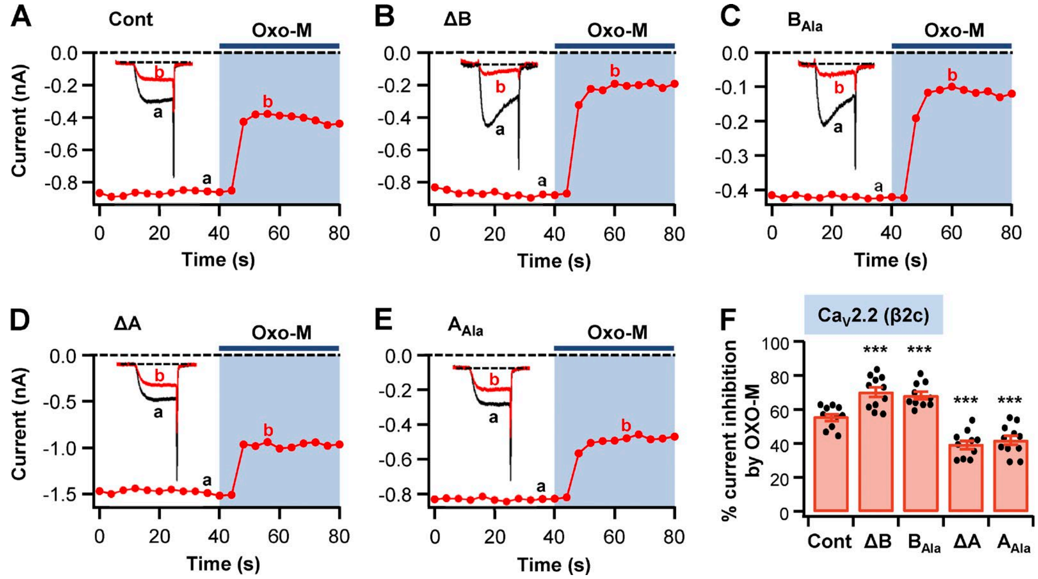

Figure 4. **The HOOK region of the β subunit controls M_1 muscarinic modulation of $Ca_V2.2$ channels.** (A–E) Current inhibition by M_1 muscarinic receptor activation with 10 µM oxotremorine M (Oxo-M) in cells expressing $Ca_V2.2$ with β2c derivatives. The current was measured at +10 mV every 4 s. Insets show that current traces of before (a) and during (b) Oxo-M application. (F) Summary of M_1 muscarinic inhibition of $Ca_V2.2$ currents in cells with β2c derivatives. Dots indicate the individual data points for each experiment. ***, P < 0.001 compared with control. Data are mean ± SEM.

both 2% PIP_2 and 10% PS (Fig. 6, D and E, red traces) were applied to the liposomes, the intensity of the emitted green fluorescence showed a greater reduction. These results indicate that both PIP_2 and PS mediate the interaction of the B domain to the plasma membrane.

The membrane interaction of the β2c HOOK region was examined in intact live cells. In control tsA201 cells, the intact β2c, β2c(A), β2c(B), and β2cΔHOOK were all distributed throughout the cytoplasm in the absence of α1 and α2δ subunits (Fig. 7, A and B). However, when the anionic phosphoinositide PIP_2 was increased at the plasma membrane by overexpressing PIPKIγ, β2c(B) was selectively translocated to the plasma membrane (Fig. 7 B). The mutant β2c(B_{Ala}) and β2c(A+B), which is a β2c subunit of the A domain and B domain in the HOOK region, were still localized in the cytosol, suggesting that basic residues of the B domain are important for the interaction with PIP_2 at the plasma membrane and the interaction can be blocked by adjacent A domain. We then applied the chemically inducible dimerization system to investigate whether the depletion of PIP_2 can trigger the dissociation of β2c(B) from the plasma membrane. This chemically inducible dimerization system is used to irreversibly and rapidly deplete PIP_2 by membrane recruitment of pseudojanin (PJ), which contains both a lipid-4-phosphatase and a lipid-5-phosphatase (Hammond et al., 2012). Our results showed that recruiting PJ to the plasma membrane by rapamycin (Rapa) application leads to the release of plasma membrane β2c(B) to the cytoplasm (Fig. 8, A–D). When we measured the fluorescence intensity of RFP-PJ and β2c(B)-GFP, the plasma membrane distribution of PJ increased upon Rapa application, whereas that of β2c(B) decreased (Fig. 8 B). Consistently, the cytosolic fluorescence intensity of PJ decreased upon Rapa application, whereas that of β2c(B) increased (Fig. 8, C and D). However, recruiting Dead, a mutant form with inactivated lipid-4-phosphatase and lipid-5-phosphatase, to the plasma membrane had no effects on the localization of β2c(B) (Fig. 8 E). Consequently, the intensity of β2c(B) of the plasma membrane and the cytosol remained unchanged after the addition of Rapa (Fig. 8, F–H). These results suggest that the B domain of the HOOK region of the β subunit can directly interact with the plasma membrane in intact cells.

DISCUSSION

In this study, we found that the HOOK region of the β2 subunit regulates the PIP_2 sensitivity and inactivation kinetics of Ca_V channels. (a) When the HOOK region of the β2 subunit was divided into three domains according to amino acid composition (i.e., S, A, and B), the charged amino acids of the A and B domains played important roles in determining the $Ca_V2.2$ channel gating properties. Acidic residues within the A domain increased the inactivation and PIP_2 regulation of $Ca_V2.2$ channels, whereas basic residues within the B domain decreased the responses. (b) The regulatory effect of the HOOK region was commonly detected in channels with either a membrane-tethered β2a subunit or cytoso-

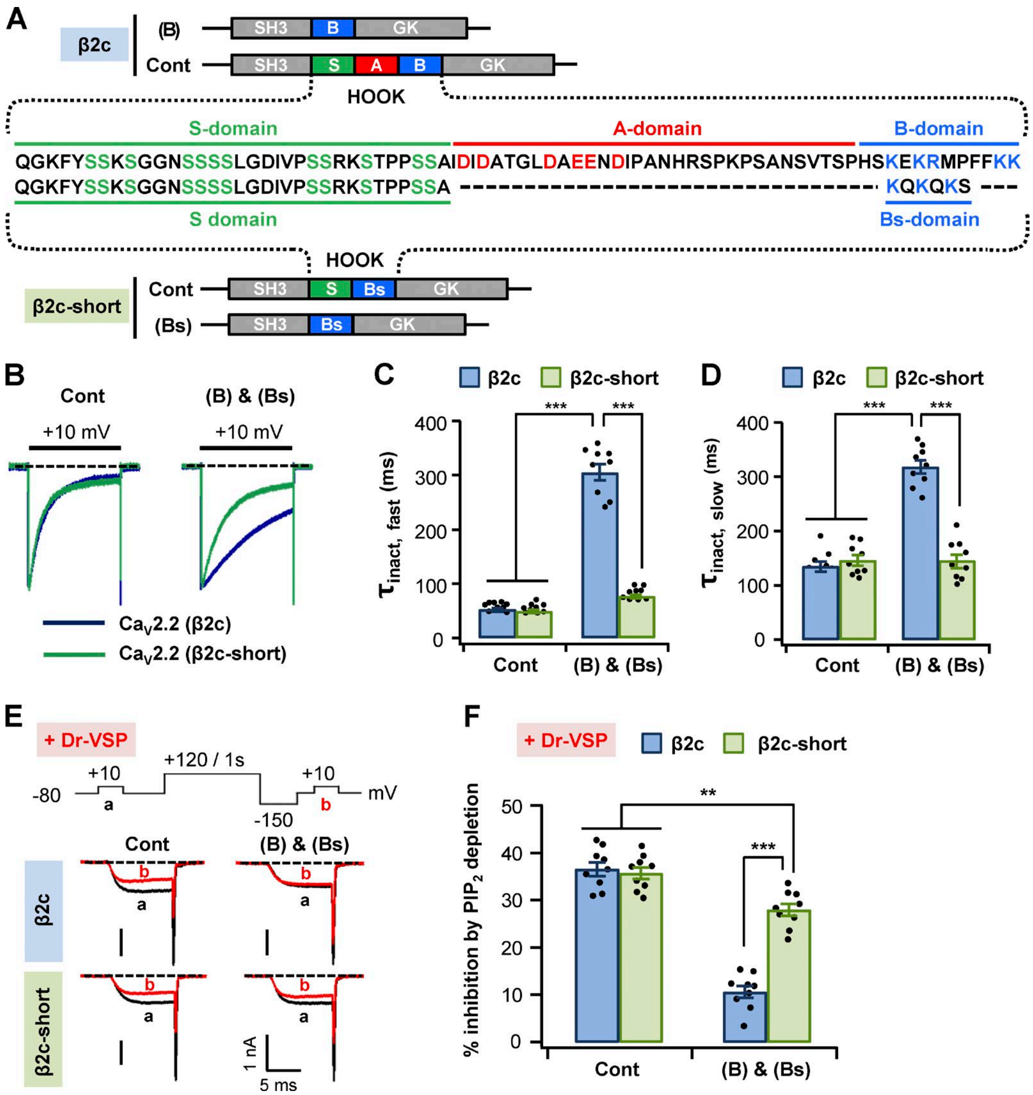

Figure 5. **Current inactivation and PIP_2 sensitivity of $Ca_V2.2$ channels with β2c-short.** (A) Schematic diagram of HOOK region deletion constructs of β2c (top) and β2c-short (bottom) used in this study. Amino acid sequence alignment of the HOOK region between β2c and β2c-short (middle). (B) Current inactivation was recorded during 500-ms test pulses to 10 mV in cells expressing $Ca_V2.2$ channels with β2c derivatives (blue traces) or β2c-short derivatives (green traces). Current traces with β2c-short derivatives (green traces) are scaled to the peak amplitude of current with β2c derivatives (blue traces). (C and D) Summary of time constants for fast ($\tau_{inact,\ fast}$; C) and slow ($\tau_{inact,\ slow}$; D) current inactivation. The decay of $Ca_V2.2$ currents was fitted to a double exponential function. (E) Current inhibition of $Ca_V2.2$ channels with β2c-short derivatives (top) or β2c derivatives (bottom) by Dr-VSP activation. Cells received a test pulse (a) and then were depolarized to 120 mV for 1 s and hyperpolarized to less than −150 mV for 0.4 s, followed by the second test pulse (b). Currents before (a) and after (b) the depolarizing pulse are superimposed. (F) Summary of current inhibition (percentage) by Dr-VSP activation in cells with β2c derivatives (blue bars) or β2c-short derivatives (green bars). Dots indicate the individual data points for each experiment. **, $P < 0.01$; ***, $P < 0.001$ compared with β2c-short derivatives. Data are mean ± SEM.

lic β2c subunit. This suggests that the modulation of Ca_V channel gating by the HOOK region is independent of the regulation by N-terminal–mediated membrane targeting of the β subunit. (c) When the HOOK region–deleted β subunits were expressed, the inactivation and PIP_2 sensitivity of channels were increased compared with channels with a control β subunit and close to those with β2(A). The results suggest that the net charge of the HOOK region in the control β subunit is basic, and thus, the HOOK region is partially interacting with acidic phospholipids in the plasma membrane. Nevertheless, because the targeting of the β subunit to the plasma membrane also required two aromatic Phe residues in the B domain, the HOOK region may bind to the plasma membrane through electrostatic and hydrophobic interaction.

Previous studies have shown that the plasma membrane targeting of the cytosolic β subunit through N-terminal modification reduces both the current inactivation and PIP_2 sensitivity of Ca_V channels (Suh et

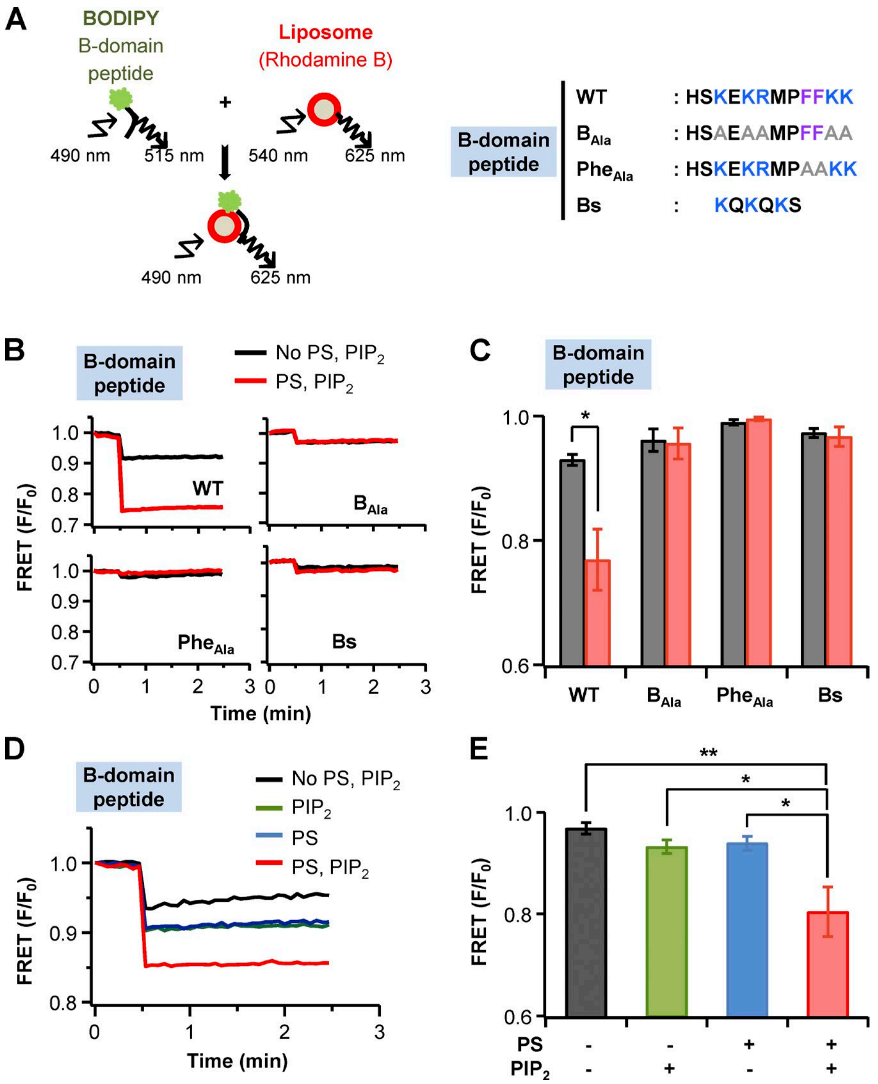

Figure 6. **B domain of HOOK region electrostatically interacts with liposome anionic phospholipids through basic amino acids and two Phe residues.** (A) Schematic diagram of FRET analysis (left) and amino acid sequence of B domain peptides (right). Binding was investigated using FRET between peptides labeled with BODIPY 493/503 (green fluorescence dye; donor) and liposomes labeled with rhodamine (red fluorescence dye; acceptor). Fluorescence emitted from BODIPY 493/503 is quenched by rhodamine, indicating peptides binding to liposomes. (B) Fluorescence intensity of BODIPY B domain peptides without (black traces) or with (red traces) 2% PIP_2 and 10% PS in the liposomes. (C) Summary of FRET changes with different peptides depending on liposomes without (black bars) or with (red bars) 2% PIP_2 and 10% PS (n = 3). *, $P < 0.05$ compared with liposome lacking PIP_2 and PS. (D) Fluorescence of BODIPY without anionic lipids (black trace) or with 2% PIP_2 (green trace), 10% PS (blue trace), or both 2% PIP_2 and 10% PS (red trace) in the liposomes. (E) Summary of FRET changes with different lipid compositions in the liposomes (n = 3–5). *, $P < 0.05$; **, $P < 0.01$, one-way ANOVA followed by Bonferroni post-hoc test. The initial fluorescence of BODIPY 493/503 was determined in the absence of liposomes (F_0) and the subsequent fluorescence was recorded after liposome application (F). FRET is presented as F/F_0. Data are mean ± SEM.

al., 2012; Keum et al., 2014). In this work, we showed that the HOOK region of the β2 subunit also serves similar functions in regulating Ca_V channel gating. According to our findings, the mechanism of Ca_V channel regulation by the HOOK region is similar to the channel regulation by the N-terminal–dependent subcellular localization of the β subunit. β2c(B), the β2c subunit with only the B domain in the HOOK region, binds to the plasma membrane in the presence of high PIP_2 and exhibits slower inactivation and lower PIP_2 sensitivity on $Ca_V2.2$ channels, acting like the palmitoylation-mediated membrane-targeted β2a subunit. Actually, our data show that the synthetic peptide of the B domain can directly bind to the liposome-containing anionic lipids. However, the confocal data indicate that β2c(B) by itself does not interact with the plasma membrane in intact cells, but high PIP_2 levels are required. This may suggest that the β2c(B) subunit is too big to interact with the plasma membrane through only electrostatic interaction, whereas B domain peptides are small, and therefore, the presence of several acidic amino acids and two aromatic residues is sufficient for the peptides to interact with liposomes. We also found that β2c(B) is localized at the plasma membrane when the concentration of PIP_2 is increased over 10-fold at the plasma membrane by overexpressing PIPKIγ (Suh and Hille, 2007). This suggests that although the B domain alone is not sufficient to target the whole β2C protein to the plasma membrane in normal cells, β2c(B) may have a chance to interact with the plasma membrane when β2c(B) is coexpressed with the α1B subunit and thus localized closely to the plasma membrane by binding to the I-II linker of the α1B subunit (Fig. 9). It has been reported that both basic and aromatic residues of the N terminus play essential roles in the electrostatic interaction of the Ca_V β2e subunit with the plasma membrane (Miranda-Laferte et al., 2014; Kim et al., 2015a,b; Kim and Suh, 2016). Here, we also found that the interaction of the HOOK region with the plasma membrane depends on both polybasic and hydrophobic residues within the B domain. Based on these results, the mechanism by which the HOOK region regulates the inactivation ki-

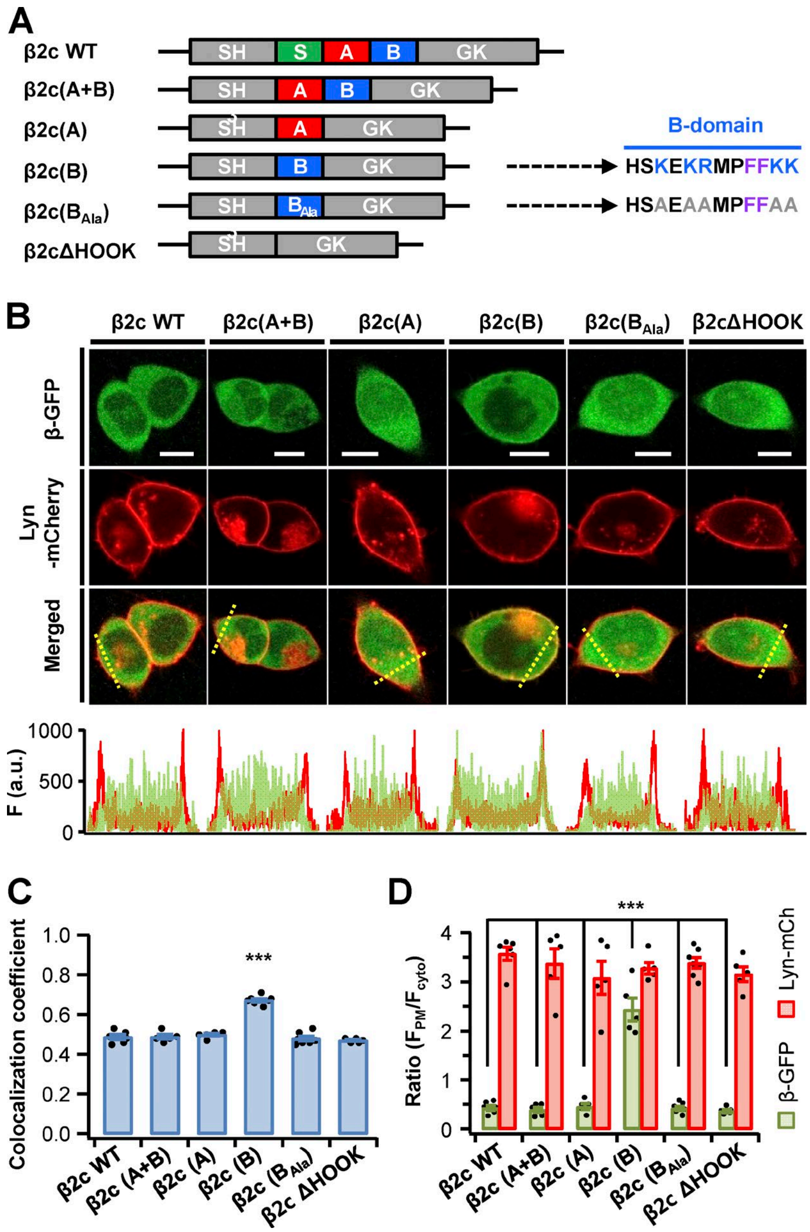

Figure 7. **The B domain in the HOOK region of the β2c subunit directly interacts with the plasma membrane in intact cells.** (A) Schematic diagram of HOOK region deletion constructs of β2c used in this study. (B) Confocal images showing subcellular distribution of GFP-tagged β2c derivatives and Lyn-mCherry (the plasma membrane marker) in the presence of PIPKIγ (top), with line scans crossing the cytosol and plasma membrane (bottom). a.u., arbitrary units. Bars, 10 µm. Note that GFP-tagged β2c(A+B) are distributed through both the cytosol and the plasma membrane. (C) The colocalization coefficients were obtained from merged images of cells expressing GFP-labeled β2c derivatives and Lyn-mCherry. (D) Quantification of fluorescence intensity ratio of plasma membrane and cytosol (F_{PM}/F_{cyto}) was calculated from line intensity histograms of cells expressing β2c derivatives-GFP and Lyn-mCherry in the presence of PIPKIγ. Dots indicate the individual data points for each experiment. ***, $P < 0.001$, one-way (C) or two-way (D) ANOVA followed by Bonferroni post-hoc test. Data are mean ± SEM.

netics and PIP_2 sensitivity of Ca_V channels is caused by the direct interaction of the HOOK region with the plasma membrane.

Our data show that the PIP_2 sensitivity and inactivation kinetics of $Ca_V2.2$ channels are not significantly different in channels with β2c or β2c-short, although the β2c-short subunit has half of the basic residues compared with β2c without the Phe residues in the B domain and no A domain. If the A and B domains in the HOOK region of the β2c subunit bind each other through charge–charge interaction, the total net charge of the HOOK region of the β2c subunit may not be different from that of β2c-short (Fig. 9). Our data suggest that the net charge of the HOOK region of both β2c and β2c-short is weak basic because deletion of the whole HOOK region triggered the β subunit to be located in the cytosol and the $Ca_V2.2$ channels with β2cΔHOOK to present faster inactivation and higher PIP_2 sensitivity. These results suggest that even though the WT β2c and β2c-short are located in the cytosol in the absence of α1 subunit, when bound to the α1 through α1-binding pocket, they are able to slightly interact with the plasma membrane via the basic amino acids of the HOOK region. Previous studies showed many of membrane-interacting proteins have a very low affinity to phospholipids to direct membrane targeting on their own but cooperate with other domains in the same protein or other membrane proteins to drive multivalent membrane binding through multidomain complex formation (Lemmon, 2008). For example, some proteins cooperate with tyrosine-phosphorylated membrane receptors through the phosphotyrosine-binding

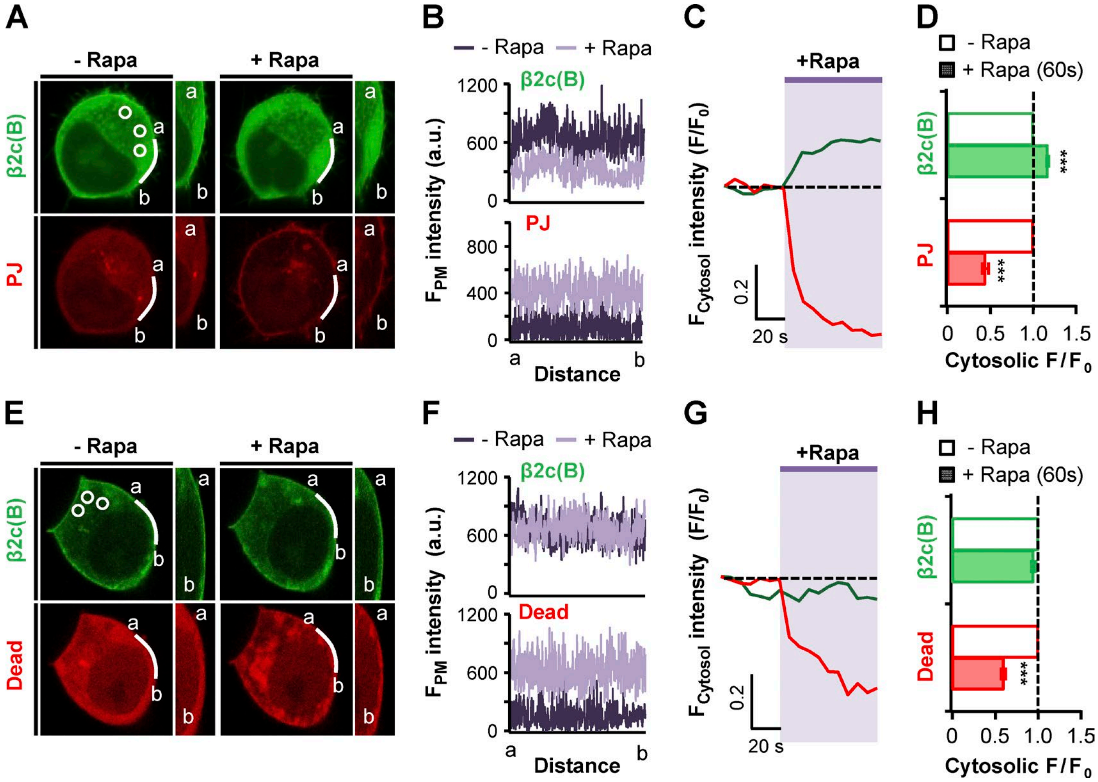

Figure 8. **PIP_2 depletion induces the dissociation of β2c(B) from the plasma membrane.** (A and E) Time-series confocal images of cells expressing β2c(B)-GFP, LDR and either RFP-PJ (A) or RFP-Dead (E). Images before and after 1 µM rapamycin (Rapa) addition for 60 s. Bar, 5 µm. (B and F) Analysis of the plasma membrane fluorescence intensity of β2c(B)-GFP and either RFP-PJ (B) or RFP-Dead (F) before (black) and after (purple) rapamycin application. a.u., arbitrary units. (C and G) Cytosolic fluorescence intensities of GFP (green) and RFP (red) for the cells shown in A (C) or E (G). Normalized fluorescence intensity is shown as a function of time. $\Delta F/F_0$, fluorescence alteration divided by initial fluorescence. (D and H) Summary of normalized fluorescence intensity of GFP (green) and RFP (red) in the cytosol for the cells shown in A or E before (white bars) and after (colored bars) 60-s rapamycin addition. ***, $P < 0.001$. Data are mean ± SEM.

domain, such as SH2, to recruit a phospholipid-binding domain to the plasma membrane containing specific phospholipids (Schlessinger, 2000).

In this work, we used artificially modified β2c constructs after deletion or point mutation of the HOOK region. Is it possible to alter the net charge of the HOOK region of the β2 subunit in vivo? We envisage that the net charge of the HOOK region can be changed by phosphorylation of the S domain. As mentioned previously, the S domain retains a serine-rich sequence, and it is possible that the serine residues are phosphorylated by protein kinases (Brunet et al., 2015). So far, the crystal structure of the HOOK region is unresolved, but it is highly flexible (Chen et al., 2004; Opatowsky et al., 2004; Van Petegem et al., 2004; Buraei and Yang, 2010). Therefore, the structure and the net charge of the HOOK region could be dynamically changed by the degree of phosphorylation in the S domain. In addition, depending on the domains exposed by the phosphorylation of the S domain, the net charge of the HOOK region will be dynamically changed. In resting state, the net charge of HOOK region may be weak positive, because the A domain and B domain of the HOOK region can partially mask each other. Our data suggest that when the net charge of the HOOK region is changed to basic because of B domain exposure, the β subunit will move toward the plasma membrane and thereby decrease the current inactivation and PIP_2 sensitivity of Ca_V channels (Fig. 9). When the β subunit, meanwhile, moves toward the cytosol because of the exposure of acidic residues, it will increase both responses. Recent studies have reported evidence supporting our hypothesis. Those studies identified that two conserved sites of the S domain of the HOOK region can be phosphorylated in vivo and that the phosphomimetic mutations of these sites slowed $Ca_V1.2$ channel inactivation (Brunet et al., 2015). In addition, it has been recently reported that HOOK region mutation of the β2 subunit might be involved in autism spectrum disorders (Breitenkamp et al., 2014). Those studies showed that rare missense mutations of the β2d subunit that altered $Ca_V1.2$ channel gating were discovered in autism spectrum disorder–affected families. In one of these mutations, serine was replaced by phenylalanine within the S

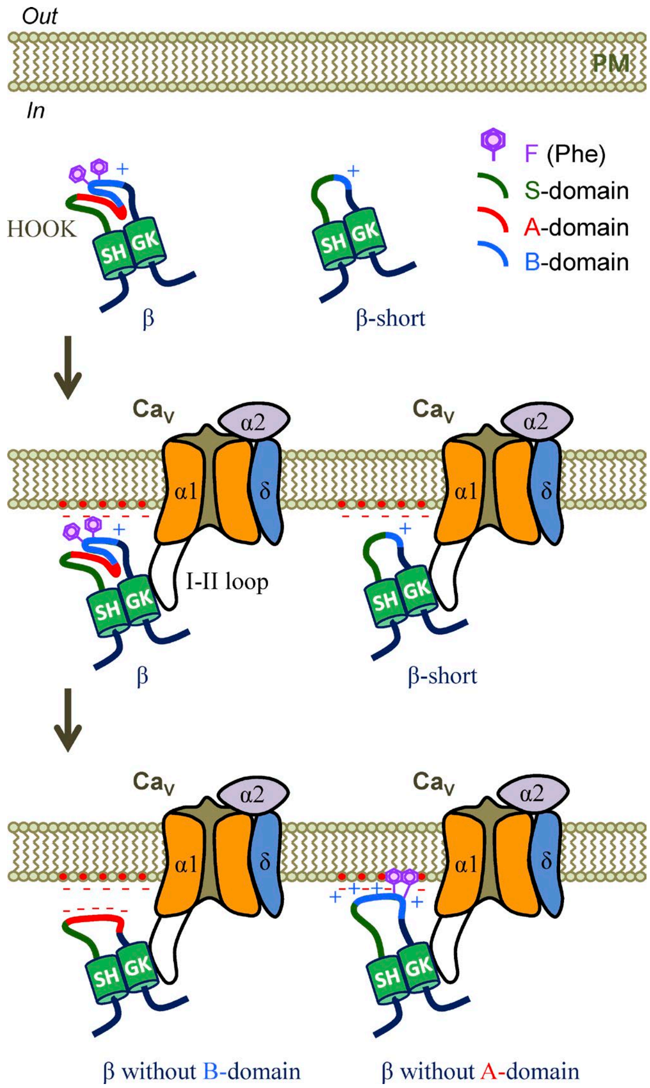

Figure 9. **Schematic model of β subunit HOOK region–mediated regulation of Ca$_V$ channels.** In the absence of the α1 subunit, the β or β-short forms bind membrane phospholipids too weakly to direct stable membrane association on their own (top). They are localized in the cytosol in cells without an α1 subunit. In the presence of the α1 subunit (middle), they can be recruited to the plasma membrane through the cooperative interaction between GK domain and I-II loop, and the polyacidic (A) and polybasic (B) domains of the HOOK region may partially associate each other; thus, the HOOK region of the β subunit looks charged as weak positive with two hydrophobic Phe residues. Therefore, the HOOK region may interact with the anionic membrane phospholipids through electrostatic forces. The HOOK region of the β-short subunit does not have an A domain but contains three basic amino acids without Phe residues. The HOOK region of the β-short subunit is also charged as positive and can interact with the plasma membrane. When the B domain was deleted from β subunit (bottom), HOOK becomes acidic and cannot interact with the anionic membrane phospholipids. The dissociation of the β subunit HOOK region from the plasma membrane promotes the inactivation of Ca$_V$ currents and augments the PIP_2 sensitivity of Ca$_V$ channels like the cytosolic β subunits. In contrast, when the A domain was deleted from the β subunit, the HOOK region becomes more positive, can directly associate with the plasma membrane, and enhances the inactivation kinetics and PIP_2 sensitivity of Ca$_V$ channels like the membrane-tethered β subunits. In this configuration, both the net charges and hydrophobicity of the HOOK region are important for deciding the interaction affinity of β subunits to the plasma membrane. All HOOK region deletion derivatives still remain stably bound to the I-II loop of the α1 subunit.

domain (S197F). β2d S197F exhibited slow inactivation of the Ca$_V$1.2 channel compared with WT S197F. The HOOK region of this mutant may have a greater chance of interacting with the plasma membrane because of the mutation to the aromatic residue. Still, further studies should investigate which factor changes the net charge of the HOOK region in physiological conditions.

It seems clear that the plasma membrane PIP_2 regulates Ca$_V$ channel gating through both direct and indirect pathways. Recently, Kaur et al. (2015) reported that several basic amino acids in the C terminus of the I–II link are involved in the PIP_2 binding to the Ca$_V$ α1C subunit and mediate the direct phospholipid regulation of Ca$_V$ channel inactivation. In contrast, our recent studies with the β2e subunit showed that PIP_2 indirectly regulates the channel gating through the interaction with the N terminus of β2e, where the PIP_2 in the plasma membrane acts as an anchoring site for the β2e subunit and thus slows the inactivation of Ca$_V$ channels with β2e. Here, we provide another case showing that the PIP_2 indirectly regulates Ca$_V$ channels through the coupling with the HOOK region of the β subunit. The last pathway is relatively weak and needs the help of the α1 subunit to hold the β subunit adjacent to the plasma membrane.

In conclusion, our findings offer new insight into the mechanism by which the HOOK region regulates Ca$_V$ channels. It has been reported that the HOOK region may directly associate with the α1 subunit to regulate the current inactivation of Ca$_V$ channels (He et al., 2007; Richards et al., 2007), but the HOOK region flexible interaction site in the α1 subunit has not been determined. Here, we speculate that the HOOK region interacts with the plasma membrane through electrostatic and hydrophobic interactions independently from the isotypes of the β subunit. Future work should reveal this dynamic regulatory mode of the HOOK region in native neurons and investigate the physiological consequences of the flexible HOOK regulation of neuronal Ca$_V$ channels.

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