Molecular basis of the PIP$_2$-dependent regulation of Ca$_V$2.2 channel and its modulation by Ca$_V$ β subunits

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Abstract

High-voltage-activated Ca$^{2+}$ (Ca$_V$) channels that adjust Ca$^{2+}$ influx upon membrane depolarization are differentially regulated by phosphatidylinositol 4,5-bisphosphate (PIP$_2$) in an auxiliary Ca$_V$ β subunit-dependent manner. However, the molecular mechanism by which the β subunits control the PIP$_2$ sensitivity of Ca$_V$ channels remains unclear. By engineering various α1B and β constructs in tsA-201 cells, we reported that at least two PIP$_2$-binding sites, including the polybasic residues at the C-terminal end of I–II loop and the binding pocket in S4II domain, exist in the Ca$_V$2.2 channels. Moreover, they were distinctly engaged in the regulation of channel gating depending on the coupled Ca$_V$ β2 subunits. The membrane-anchored β subunit abolished the PIP$_2$ interaction of the phospholipid-binding site in the I–II loop, leading to lower PIP$_2$ sensitivity of Ca$_V$2.2 channels. By contrast, PIP$_2$ interacted with the basic residues in the S4II domain of Ca$_V$2.2 channels regardless of β2 isotype. Our data demonstrated that the anchoring properties of Ca$_V$β2 subunits to the plasma membrane determine the biophysical states of Ca$_V$2.2 channels by regulating PIP$_2$ coupling to the nonspecific phospholipid-binding site in the I–II loop.

Editor's evaluation

This manuscript describes experiments using heterologous expression to achieve molecular dissection of the effects of PIP2 and CaVβ2 auxiliary subunits on CaV2.1 (P/Q-type) calcium channels. The experiments also probe interplay between lipid effects and other modulatory pathways. Understanding the functional regulation of this channel is important because CaV2.1 channels play significant roles in neuronal plasticity.

Introduction

Voltage-gated Ca$^{2+}$ (Ca$_V$) channels that mediate Ca$^{2+}$ influx upon membrane depolarization contribute to various physiological events, including synaptic transmission, hormone secretion, excitation–contraction coupling, and gene transcription (Berridge et al., 2000; Catterall, 2011; Clapham, 2007; Li et al., 2016). Ca$_V$ channels can be divided into high-voltage-activated (HVA) and low-voltage-activated (LVA) channels based on their activation voltage threshold. The HVA Ca$^{2+}$ channels, which consist of the Ca$_V$1 and Ca$_V$2 families, are multiprotein complexes with a pore-forming α1 subunit and auxiliary α2δ and β subunits. Diverse cellular factors regulate Ca$_V$ channel activity (Felix, 2005; Huang and Zamponi, 2017).

Among the various intracellular regulatory signals of Ca$_V$ channels, we focus on the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP$_2$). Previous studies have shown that PIP$_2$ activates several types of HVA Ca$_V$ channels in recombinant systems and native tissue cells (Hille et al., 2015; Rodriguez-Menchaca et al., 2012; Suh and Hille, 2008; Wu et al., 2002; Xie et al., 2016).
Dr-VSP, a voltage-sensing lipid phosphatase from zebrafish, can be used to examine the effects of PI₂P on Caᵥ channels without the involvement of other downstream second messengers generated from Gₐ-coupled receptors (Murata et al., 2005; Okamura et al., 2009; Suh et al., 2010). In vitro experiments using Dr-VSP have shown that most HVA Ca²⁺ channels are suppressed by membrane PI₂P depletion without influencing LVA Ca²⁺ channels (Jeong et al., 2016; Suh et al., 2010). PI₂P induces two distinct and opposing regulatory effects on Caᵥ2.1 channels (Wu et al., 2002). Thus, the Caᵥ2.1 channel was suggested to contain two distinct PI₂P-interaction sites with different binding affinity (Wu et al., 2002). A more recent study showed that four arginine residues within the C-terminal end of the I–II loop of L-type Caᵥ1.2 channels are involved in nonspecific phospholipid interactions; therefore, the substitution of these basic residues for alanine decreases current inhibition via PI₂P breakdown and increases the open probability of Caᵥ1.2 channels (Kaur et al., 2015). The precise PI₂P-binding sites have not been fully determined in Caᵥ channels yet.

Among the auxiliary subunits, Caᵥ β subunits directly bind to an α-interacting domain (AID) within the N-terminal region of the I–II loop. They play key roles in regulating membrane trafficking and fine-tuning the gating of Caᵥ channels (Buraei and Yang, 2010; Buraei and Yang, 2013). A single β subunit can be divided into five distinct regions: conserved src homology-3 (SH3) and guanylate kinase (GK) domains, a flexible HOOK region connecting the two domains, and variable N- and C-terminus. The GK domain contains an α-binding pocket (ABP), which is a site for interaction with the AID of the I–II loop (Buraei and Yang, 2010; Buraei and Yang, 2013; Chen et al., 2004; Opatowsky et al., 2004; Van Petegem et al., 2004). Additionally, the HOOK region, a flexible linker composed of around 70 amino acids, is important in determining the inactivation kinetics, current density, and PI₂P sensitivity (Kim et al., 2015a; Kim et al., 2015b; Kim et al., 2016; Suh et al., 2012). However, the underlying mechanisms for the differential regulation of Caᵥ2.2 channel gating depending on the subcellular localization of Caᵥ β subunits has not been clearly resolved.

Previous studies have proposed a bidentate model where two palmitoyl chains of the Caᵥ β2a subunit compete with the interaction of the two fatty acyl chains of PI₂P. Subsequently, this dislodges the PI₂P molecule from its binding site on the N-type Caᵥ2.2 channels, decreasing the requirement for PI₂P (Hennehan et al., 2009; Hille et al., 2015; Mitra-Ganguli et al., 2009; Roberts-Crowley and Rittenhouse, 2009). Using cryo-electron microscopy (cryo-EM), Dong et al., 2021 and Gao et al., 2021 have recently shown that human Caᵥ2.2 channels possess a PI₂P-binding pocket within the S₄ᵣᵣ domain of α1B subunit. PI₂P interaction to this site is required for a minor shift of the S₄ᵣᵣ domain to the I–II loop. The functional role of the PI₂P-binding site in Caᵥ2.2 channel gating and the modulatory effects of Caᵥ β subunits on the PI₂P interaction are yet to be defined. In this study, we developed diverse engineered α1B and β constructs and found that the Caᵥ2.2 channels were regulated by PI₂P through at least two distinct interacting sites, including a nonspecific phospholipid-binding motif in the distal I–II loop and the binding pocket in the S₄ᵣᵣ domain. Our results revealed that the PM-anchored β2a subunit selectively disrupted PI₂P interaction with the phospholipid-binding site in the I–II loop, leading to a channel state less sensitive to Dr-VSP-induced PI₂P depletion. However, the S₄ᵣᵣ-binding pocket of Caᵥ2.2 channels interacted with PI₂P regardless of the coupled β2 isotype. The present study provides new insights into the reciprocal roles of the Caᵥ β subunits and membrane PI₂P in HVA Caᵥ channel regulation.
Results

N-terminal length of PM-tethering CaV β subunit is important in determining current inactivation and PIP2 sensitivity of CaV2.2 channels

We have previously reported that subcellular localization of the CaV β subunit plays an important role in determining the inactivation kinetics and PIP2 sensitivity of CaV2.2 channels (Keum et al., 2014; Kim et al., 2015a, Kim et al., 2016). By manipulating the β2 constructs, we further examined how CaV β subunits determine the gating properties of the CaV2.2 channel depending on their subcellular localization. First, we used a palmitoylation-resistant cytosolic mutant form of β2a, β2a(C3,4S), where two palmitoylation sites (C3 and C4) in the N-terminus of the β2a subunit were mutated to serine residues (Chien et al., 1996; Hurley et al., 2000; Olcese et al., 1994; Qin et al., 1998; Figure 1A and Figure 1—figure supplement 1A). Additionally, we constructed two more membrane-recruited β2c analogs by adding membrane-targeting Lyn11 (N-terminal G2-myristoylation and C3-palmitoylation modification sequence from Lyn kinase; Resh, 1994) or Lyn11 plus a flexible 48 amino acid linker (Lyn-48aa) to the N-terminus of β2c. When these CaV β constructs were expressed in cells without the pore-forming α1B, β2a(C3,4S) was distributed through the cytosol similar to β2c. By contrast, the engineered Lyn-β2c and Lyn-48aa-β2c were localized at the PM like the membrane-anchored β2a subunit (Figure 1B, C). However, in the presence of α1B and α2δ1, all the β2 constructs were mainly distributed at the PM, probably via binding to α1B subunits (Figure 1—figure supplement 1B, C). This suggested that amino acid mutation or chimeric modification of the β2 subunit does not affect the formation of the CaV2.2 channel multicomplex. Next, we tested the effects of the β2 constructs on current inactivation and PIP2 sensitivity of the CaV2.2 channels. PIP2 regulation of CaV2.2 channel gating was measured as the difference before and after a + 120 mV depolarizing pulse using Dr-VSP (see Figure 1—figure supplement 2A). Coexpression of β2a(C3,4S) accelerated current inactivation and increased the PIP2 sensitivity of CaV2.2 channels, such as those with the cytosolic β2c subunit. Expression of the chimeric Lyn-β2c slowed down the inactivation rate and decreased PIP2 sensitivity, like the channels with the PM-anchored β2a subunit (Figure 1D, E). Interestingly, cells co-transfected with the PM-tethered chimeric Lyn-48aa-β2c showed faster current inactivation and higher PIP2 sensitivity in CaV2.2 channels, which were similar to the responses of channels with the cytosolic β2c subunit. In control experiments without Dr-VSP, we confirmed that the current amplitudes of CaV2.2 channels with the developed β2 constructs were not significantly different before and after the depolarizing pulse (Figure 1—figure supplement 2A, B). Additionally, we verified that the effects of Dr-VSP were not due to relieving the Gβγ-mediated tonic inhibition from the CaV2.2 channels. As shown in Figure 1—figure supplement 2C, prepulse depolarization did not change the current amplitudes in cells intracellularly perfused with 1 mM of the G protein inhibitor GDP-β-S instead of GTP in the absence of Dr-VSP. Moreover, the CaV2.2 channels with GDP-β-S showed very similar PI(4,5)P2 sensitivities to those in experiments with GTP in cells expressing Dr-VSP (Figure 1—figure supplement 2D). This suggested that 0.1 mM GTP concentration in the pipette solution was not sufficient to trigger spontaneous G protein activation or suppress CaV2.2 channels through Gβγ binding.

We further examined the effects of the length of the flexible linker between Lyn and the β2c subunit on the inactivation kinetics and PIP2 sensitivity of CaV2.2 channels. As shown in Figure 1—figure supplement 3, when the inserted linkers were longer than 24 aa, current inactivation was faster and current inhibition by PIP2 depletion was stronger. Together, these data suggest that the N-terminal length of the PM-tethering CaV β subunit is critical in determining the inactivation kinetics and PIP2 sensitivity of CaV2.2 channels.

Proximal interaction of the fatty acyl chains with channel complex underlies the β subunit-dependent regulation of CaV2.2 channel gating

It has been previously reported that disruption of the SH3–GK interaction in the membrane-anchored β2a subunit accelerates the channel inactivation of CaV2.1 channels (Chen et al., 2009). The GK domain of the CaV β subunit interacts directly with the AID domain in the I–II loop of CaV α1 subunits (Buraei and Yang, 2010; Buraei and Yang, 2013; Chen et al., 2004; Opatowsky et al., 2004; Van Petegem et al., 2004); therefore, disruption of the SH3–GK interaction in the CaV β subunit may increase the length between the N-terminus and the GK–AID complex through the flexible HOOK region. To test the possible effects of increased N-terminal length from the AID–GK complex on CaV...
Figure 1. Current inactivation and PIP₂ sensitivity in N-type Caᵥ2.2 channels with different subtypes of the β2 subunit. (A) Schematic diagram of high-voltage-activated (HVA) calcium channel complex viewed from the intracellular side (left). Caᵥ β subunit is located beside the domain II of α1B in the cytosolic side while Caᵥ α2δ subunit is mostly localized at the extracellular surface of the channel protein (Gao et al., 2021). Schematic model of Caᵥ2.2 channels with plasma membrane (PM)-anchored β2a, cytosolic β2a(C3,4S) and β2c, or N-terminus engineered PM-recruited β2c (right). (B) Figure 1 continued on next page
channel gating, we constructed mutant β2a subunits in which the SH3–GK intramolecular interaction was disrupted by mutating seven amino acids in the SH3 and GK domains to alanine residues (Figure 2A). Additionally, the N-terminus was deleted to abolish membrane targeting of the β2a subunit by itself, and Lyn11 was inserted to the N-terminus for membrane recruitment. Without α1B and α2S1, both N-terminus-deleted (ΔN)β2 WT and (ΔN)β2 Mut, in which the SH3–GK interaction was disrupted, were expressed in the cytosol. Conversely, Lyn-(ΔN)β2 WT and Lyn-(ΔN)β2 Mut constructs were localized to the PM (Figure 2A, inset images). In CaV2.2 channels with the N-terminus-deleted mutant (ΔN)β2 WT, the current exhibited fast inactivation and high PIP2 sensitivity (Figure 2B–D). These phenomena similarly appeared in channels with the (ΔN)β2 Mut. In contrast, CaV2.2 channels with Lyn-(ΔN)β2 WT exhibited slow inactivation and weak PIP2 sensitivity. However, the channels with Lyn-(ΔN)β2 Mut exhibited fast inactivation and strong PIP2 sensitivity, like channels with cytosolic (ΔN)β2 WT and (ΔN)β2 Mut (Figure 2B–D). We also confirmed that disruption of the SH3–GK interaction did not shift the current–voltage (I–V) curve of CaV2.2 currents (Figure 2—figure supplement 1). These data suggested that the length from the N-terminal lipid anchor to the GK domain of β subunit is crucial in determining the inactivation rate and PIP2 sensitivity of CaV2.2 channels.

To further examine the functional role of length between lipid anchor and GK domain on CaV channel gating in live cells, we developed new chimeric β2 constructs by applying the rapamycin-induced dimerizing system FK506-binding protein (FKBP) and FKBP–rapamycin-binding (FRB) protein (Banaszynski et al., 2005; Inoue et al., 2005; Suh et al., 2006). As shown in Figure 3A, FKBP and FRB proteins irreversibly assembled to form a ternary complex upon application of rapamycin, which led to shortening of the length between the lipid anchor Lyn11 and GK–AID domains. We fused a Förster resonance energy transfer (FRET) probe YFP to the C-terminus of all β2 constructs by applying the rapamycin-induced dimerizing system FK506-binding protein (FKBP) and FKBP–rapamycin-binding (FRB) protein (Banaszynski et al., 2005; Inoue et al., 2005; Suh et al., 2006). As shown in Figure 3A, FKBP and FRB proteins irreversibly assembled to form a ternary complex upon application of rapamycin, which led to shortening of the length between the lipid anchor Lyn11 and GK–AID domains. We fused a Förster resonance energy transfer (FRET) probe YFP to the C-terminus of all β2 constructs by applying the rapamycin-induced dimerizing system FK506-binding protein (FKBP) and FKBP–rapamycin-binding (FRB) protein (Banaszynski et al., 2005; Inoue et al., 2005; Suh et al., 2006). As shown in Figure 3A, FKBP and FRB proteins irreversibly assembled to form a ternary complex upon application of rapamycin, which led to shortening of the length between the lipid anchor Lyn11 and GK–AID domains. We fused a Förster resonance energy transfer (FRET) probe YFP to the C-terminus of all β2 constructs by applying the rapamycin-induced dimerizing system FK506-binding protein (FKBP) and FKBP–rapamycin-binding (FRB) protein (Banaszynski et al., 2005; Inoue et al., 2005; Suh et al., 2006). As shown in Figure 3A, FKBP and FRB proteins irreversibly assembled to form a ternary complex upon application of rapamycin, which led to shortening of the length between the lipid anchor Lyn11 and GK–AID domains. We fused a Förster resonance energy transfer (FRET) probe YFP to the C-terminus of all β2 constructs by applying the rapamycin-induced dimerizing system FK506-binding protein (FKBP) and FKBP–rapamycin-binding (FRB) protein (Banaszynski et al., 2005; Inoue et al., 2005; Suh et al., 2006). As shown in Figure 3A, FKBP and FRB proteins irreversibly assembled to form a ternary complex upon application of rapamycin, which led to shortening of the length between the lipid anchor Lyn11 and GK–AID domains. We fused a Förster resonance energy transfer (FRET) probe YFP to the C-terminus of all β2 constructs by applying the rapamycin-induced dimerizing system FK506-binding protein (FKBP) and FKBP–rapamycin-binding (FRB) protein (Banaszynski et al., 2005; Inoue et al., 2005; Suh et al., 2006). As shown in Figure 3A, FKBP and FRB proteins irreversibly assembled to form a ternary complex upon application of rapamycin, which led to shortening of the length between the lipid anchor Lyn11 and GK–AID domains. We fused a Förster resonance energy transfer (FRET) probe YFP to the C-terminus of all β2 constructs by applying the rapamycin-induced dimerizing system FK506-binding protein (FKBP) and FKBP–rapamycin-binding (FRB) protein (Banaszynski et al., 2005; Inoue et al., 2005; Suh et al., 2006). As shown in Figure 3A, FKBP and FRB proteins irreversibly assembled to form a ternary complex upon application of rapamycin, which led to shortening of the length between the lipid anchor Lyn11 and GK–AID domains. We fused a Förster resonance energy transfer (FRET) probe YFP to the C-terminus of all β2 constructs by applying the rapamycin-induced dimerizing system FK506-binding protein (FKBP) and FKBP–rapamycin-binding (FRB) protein (Banaszynski et al., 2005; Inoue et al., 2005; Suh et al., 2006). As shown in Figure 3A, FKBP and FRB proteins irreversibly assembled to form a ternary complex upon application of rapamycin, which led to shortening of the length between the lipid anchor Lyn11 and GK–AID domains. We fused a Förster resonance energy transfer (FRET) probe YFP to the C-terminus of all β2 constructs by applying the rapamycin-induced dimerizing system FK506-binding protein (FKBP) and FKBP–rapamycin-binding (FRB) protein (Banaszynski et al., 2005; Inoue et al., 2005; Suh et al., 2006).
Lyn-FRBS-HOOK-GK-Linker-FKBP (RCF), where a 194-aa linker was inserted between GK and FKBP, rapamycin enhanced the FRET signal without causing significant changes in the current amplitude (Figure 3B, right and Figure 3—figure supplement 1). The effects of rapamycin on inactivation kinetics and PIP₂ sensitivity were much weaker in Ca V2.2 channels with RCF when compared with those in channels with RF (Figure 3C–F). This suggested that rapamycin-induced dimerization may be insufficient to shorten the length between the lipid anchor and isolated GK domain of β subunit in channels with RCF.

Next, we measured the effects of the N-terminal length of PM-tethered β subunit on Ca V2.2 channel activity by inserting flexible linkers of various lengths between Lyn11 and the GK domain of β₂.

Figure 2. Disruption of SH3–GK interaction in the plasma membrane (PM)-recruited Ca₃.2 β₂ subunit leads to an increase in both current inactivation and PIP₂ sensitivity of CaV₂.2 channels. (A) Left, a diagram showing how the SH3–GK intramolecular interaction is disrupted in β₂ constructs (top). Phenylnalanine 92, histidine 94, arginine 107, and valine 109 residues in the SH3 domain and tyrosine 406, lysine 408, and threonine 410 residues in the GK domain are replaced with alanine. Schematic model of CaV₂.2 channels with engineered β₂ constructs in which the SH3–GK intramolecular interaction is disrupted. Lyn-(ΔN)β₂: Lyn-labeled N-terminus-deleted β₂ construct. Lyn-(ΔN)β₂ Mut: Lyn-(ΔN)β₂ construct with a disrupted SH3–GK intramolecular interaction. Inset: confocal images of tsA-201 cells expressing engineered β₂ constructs labeled with mCherry without α₁B and α₁δ₁ subunits. Scale bar, 5 μm. (B) Representative currents of CaV₂.2 channels with engineered β₂ constructs. The currents were measured during 500-ms test pulses to +10 mV (top). Current traces before (a) and after (b) a +120-mV depolarizing pulse in cells expressing CaV₂.2 channels with engineered β₂ constructs and Dr-VSP (bottom). Peak tail current is indicated by arrowheads (trace a, black head; trace b, red head). (C) Summary of CaV₂.2 current inactivation (n = 9–12). r₁₀₀ indicates the fraction of current remaining after 100-ms depolarization to +10 mV. (D) Summary of CaV₂.2 current inhibitions (%) by PIP₂ depletion in Dr-VSP-expressing cells (n = 9–11). Dots indicate the individual data points for each cell. Data are mean ± standard error of the mean (SEM). **p < 0.01, ***p < 0.001, using one-way analysis of variance (ANOVA) followed by Tukey post hoc test.

The online version of this article includes the following source data and figure supplement(s) for figure 2:

Source data 1. Current inactivation (r₁₀₀) and current inhibition (%) by PIP₂ depletion in N-type CaV₂.2 channels with the engineered β₂ construct.

Figure supplement 1. Disruption of the SH3–GK intramolecular interaction of β₂ subunit does not shift current–voltage (I–V) curve of CaV₂.2 current.

Figure supplement 1—source data 1. Current–voltage (I–V) curve of CaV₂.2 current.
Figure 3. Effects of the real-time translocation of the GK domain to the plasma membrane (PM) on CaV2.2 channel gating. (A) Left, a schematic diagram showing rapamycin-induced translocatable β2 chimeric constructs. Translocatable β2 chimeric constructs were invented by fusing FRB or FKBP to the N- and C-termini of the GK domain, respectively. The new constructs were tagged with Lyn11 (RF or Lyn-FRB-Hook-GK-FKBP) to be tethered to the PM. Rapamycin (Rapa) addition triggers the formation of a tripartite FRB–rapamycin–FKBP complex, resulting in the movement of the FKBP domain to the PM (right). For Förster resonance energy transfer (FRET) imaging, chimeric β constructs labeled with YFP in the C-terminus and PM-targeting Lyn-CFP were coexpressed. Right, schematic model of CaV2.2 channels with RF before and after rapamycin application. Rapamycin induces the formation of the tripartite complex, resulting in a shift of the FKBP domain to the PM and an enhanced FRET signal. (B) Time courses of CaV2.2 currents (blue traces) and FRET ratio (green traces) were measured simultaneously in single cells expressing CaV2.2 channels with Cont (left), RF (middle), or RCF (right) and the membrane marker Lyn-CFP. (C) Current inactivation of CaV2.2 channels with Cont (left), RF (middle), and RCF (right) was measured during 500-ms test pulses to +10 mV before (black traces) and after (red traces) rapamycin addition. (D) Summary of inactivation of CaV2.2 currents before (black bars) and after (red bars) rapamycin application (n = 7–9). The fraction of the current remaining after 100-ms depolarization (r_{100}) to +10 mV. (E) Current inhibition of Dr-VSP-mediated PIP2 depletion on CaV2.2 channels with Cont (left), RF (middle), and RCF (right) before and after rapamycin addition. The traces before (a) and after (b) the depolarizing pulse to +120 mV were superimposed. Peak tail current is indicated by arrowheads (trace a, black head; trace b, red head). (F) Summary of Dr-VSP-induced CaV2.2 current inhibition before (black bars) and after (red bars) rapamycin addition (n = 7–9). Dots indicate the individual data points for each cell. Data are mean ± standard error of the mean (SEM). **p < 0.01, ***p < 0.001, using two-way analysis of variance (ANOVA) followed by Sidak post hoc test.

The online version of this article includes the following source data and figure supplement(s) for figure 3:

Source data 1. Time courses of CaV2.2 currents and Förster resonance energy transfer (FRET) ratio.

Figure 3 continued on next page
The real-time translocation of the GK domain to the plasma membrane increased the current amplitude of CaV2.2 channels. (Figure 4A). The inserted linkers were unstructured flexible peptides (see Figure 4—figure supplement 1); therefore, the length of the linkers was calculated using the worm-like chain (WLC) model (see Methods). Our results showed that both the current inactivation and PIP2 sensitivity of CaV2.2 channels became gradually stronger as the inserted flexible linkers became longer (Figure 4B–D). Consistently, the current activation was gradually accelerated by the increase in linker length (Figure 4—figure supplement 2). However, no additional difference was detected in channels with the membrane-tethered Lyn-43aa-GK subunit when compared with the cytosolic GK subunit. This indicated that the GK domain with the length of the inserted 43-aa linker is sufficient to act like the cytosolic CaV β subunit (Figure 4B–D). Interestingly, the PIP2 sensitivity and inactivation kinetics of CaV channels were differentially regulated by the length between the lipid anchor and the GK domain: the channels with Lyn-43aa-GK showed faster inactivation than the channels with Lyn-22aa-GK, whereas the PIP2 sensitivity of the two channels was not significantly different (Figure 4B–E). Additionally, our data analysis indicated that the biophysical gating properties of CaV2.2 channels with a membrane-anchored β2a subunit were similar to those of channels with Lyn-9aa-GK. Furthermore, the gating properties of CaV2.2 channels coupled with cytosolic β2c were similar to those of channels with Lyn-20aa-GK (Figure 4E).

Previous studies have reported that subcellular localization of the CaV β subunit is important in determining the current density of CaV channels, where CaV channels with the membrane-anchored β subunit show relatively higher current density than channels with the cytosolic β subunit (Suh et al., 2012). In line with this, we found that the current density of CaV2.2 channels with β2a was significantly higher than that of channels with β2c (Figure 4—figure supplement 3A–C). Therefore, we tested whether the current density of CaV channels was dependent on the N-terminal length. CaV2.2 channels showed slightly decreased current density that was dependent on the expansion of the flexible linker length between Lyn and the GK domain alone (Figure 4—figure supplement 4A, C). This phenomenon was observed in channels with the whole β2c subunit with Lyn (Figure 4—figure supplement 3D–F). We tested whether the length between N-terminal lipid anchor and GK domain affected the voltage-dependent gating of CaV channels. Voltage-dependent activation of CaV2.2 channels with Lyn-linker-GK derivatives showed a greater shift to positive voltage as the inserted flexible linkers increased in length (Figure 4—figure supplement 4B, D). This suggested that incremental increases in linker length lead to a decreased voltage sensitivity. There was no difference in the current density and voltage-dependent activation between CaV channels with the Lyn-43aa-GK and GK subunit. Together, these results suggested that differential regulation of CaV2.2 channel gating by β subunits is mainly determined by the anchoring properties of the β subunits to PM.

**Polybasic motif at the C-terminal end of I–II loop plays an important role in the PIP2 regulation of CaV2.2 channels**

How does the N-terminal length of the PM-tethering CaV β subunit regulate CaV channel gating? Recently, Kaur et al., 2015 have reported that a polybasic motif consisting of four basic amino acids within the C-terminal end of L-type CaV1.2 channels interacts with membrane phospholipids, including PIP2. Additionally, the putative PIP2-binding site is conserved in the I–II loop of N-type CaV2.2 channels (Figure 5—figure supplement 1). We examined whether the polybasic motif affects the PIP2 sensitivity of CaV2.2 channels. First, we eliminated the potential phospholipid-binding motif from the CaV2.2 channel I–II loop by mutating the four polybasic residues to alanine (4A α1B) (Figure 5A). In CaV2.2 channels with the β2a subunit, the inactivation kinetics of the current did not differ between WT α1B and 4A α1B (Figure 5B, C, left). However, in CaV2.2 with β2c, the inactivation rate was slower in 4A α1B channels (Figure 5B, C, right). The effects of PIP2 depletion on current amplitude were also measured in these channels. In control experiments without Dr-VSP, the current of WT or 4A-mutant CaV2.2 channels did not significantly differ before and after a + 120-mV depolarizing
Figure 4. Flexible linker length between Lyn and the GK domain of the β subunit performs a key role in determining both the current inactivation and the PIP2 sensitivity of CaV2.2 channels. (A) Schematic diagram of diverse flexible linkers (ΔN) inserted between Lyn and GK (G) domain. The length of each linker is calculated by the worm-like chain (WLC) model (see Methods). Amino acid sequences of Lyn (Lyn11 plus 12 aa linker) and the additional linkers are listed. (B) Current inactivation of CaV2.2 channels with diverse CaVβ-GK derivatives was measured during 500 ms test pulses to +10 mV. (C) Effects of Dr-VSP-mediated PIP2 depletion on CaV2.2 channels with GK domain derivatives. Peak tail current is indicated by arrowheads (trace a, black head; trace b, red head). (D) Summary of current inactivation (blue bars; n = 9–12) and inhibition (%) by PIP2 depletion (red bars; n = 8–10) in CaV2.2 channels with CaVβ GK derivatives. Data are mean ± standard error of the mean (SEM). Dots indicate the individual data points for each cell. (E) Normalized mean current inactivation and mean current inhibition by PIP2 depletion versus additional linker length (aa) of CaVβ GK derivatives measured in CaV2.2 channels. The normalized current regulation in cells expressing CaV2.2 with β2a and β2c is indicated with dashed arrows. The online version of this article includes the following source data and figure supplement(s) for figure 4:

Source data 1. Current inactivation (r100) and current inhibition (%) by PIP2 depletion in CaV2.2 channels with the engineered β2 GK derivatives.

Figure supplement 1. IUPRED web-server result of inserted linker.

Figure supplement 2. Summary of time constants for CaV2.2 current activation.

Figure supplement 2—source data 1. Time constants of current activation in CaV2.2 channels with diverse CaVβ-GK derivatives.

Figure supplement 3. Current density in N-type CaV2.2 channels with β2 variants.

Figure supplement 3—source data 1. Population current density versus voltage relations for CaV2.2 channels with β2 variants.

Figure supplement 4. Flexible linker length between Lyn and GK domain of β subunit is important in determining the current density and the voltage-dependent gating of CaV2.2 channels.

Figure 4 continued on next page
pulse in cells with either β2a or β2c subunits (Figure 5D). By contrast, PIP2 depletion by Dr-VSP activation similarly inhibited the CaV current by approximately 5% in cells expressing either WT or 4A CaV2.2 channels with a PM-anchored β2a subunit (Figure 5E, left). This indicated the presence of another PIP2-binding site in the α1B subunit other than this polybasic motif in I–II loop. On the other hand, the PIP2 sensitivity in channels with β2c was dramatically reduced in 4A channels, indicating that the polybasic motif in the I–II loop plays a key role in PIP2 regulation of CaV2.2 channels with the cytosolic β subunit (Figure 5D, E). However, in cells expressing 4A CaV2.2 channels with β2c, we observed another ~5% current inhibition by PIP2 depletion. This was similar to the CaV2.2 channels with β2a.

Next, we investigated whether the polybasic motif affects the PIP2 sensitivity of CaV2.2 channels with Lyn-β2c and Lyn-48aa-β2c (Figure 5—figure supplement 2). Similar to β2a, we did not detect any significant differences in current inactivation and PIP2 sensitivity between WT and 4A mutant CaV2.2 with Lyn-β2c (Figure 5—figure supplement 2). Conversely, WT CaV2.2 channels with Lyn-48aa-β2c exhibited faster inactivation and higher PIP2 sensitivity, which was similar to the responses of CaV2.2 channels with cytosolic β2c. However, in cells expressing 4A mutant CaV2.2 channels with Lyn-48aa-β2c, the current inactivation was slowed and the PIP2 sensitivity was decreased to ~5% (Figure 5—figure supplement 2). The PIP2 sensitivities of 4A CaV2.2 channels with Lyn-β2c and Lyn-48aa-β2c did not significantly differ and were similar to that of WT channels with Lyn-β2c. Consistent with the data in Figure 5, these results suggested that the polybasic motif within the I–II loop interacts with membrane PIP2 in CaV2.2 channels with β2c-like Lyn-48aa-β2c, but not with β2a-like Lyn-β2c subunits. On the other hand, in channels with the β2a subunit, there was no significant difference in the voltage-dependent activation between WT α1B and 4A α1B (Figure 5F, G). However, the activation of 4A α1B with the β2c subunit was significantly shifted toward the hyperpolarization direction when compared with WT α1B channels with β2c (Figure 5F, G). In addition, the activation curve of 4A α1B with β2c was similar to the curves of WT and 4A α1B with β2a (Figure 5F, G). Together, our data suggested that two different PIP2-interacting sites with differential PIP2 sensitivities exist in CaV2.2 channels. More importantly, our data indicate that PIP2 interacts with the polybasic motif when CaV2.2 is expressed with cytosolic β subunits but not when expressed with lipidated membrane-anchored β subunit.

Finally, we determined whether other arginine residues in the distal region of polybasic motif also affected the PIP2 sensitivity of CaV2.2 channels (Figure 5—figure supplement 3). For this, two arginine residues (R476 and R477) near the polybasic motif were replaced with alanine (α1B R476,477A) (Figure 5—figure supplement 3A). We also constructed a α1B R465,466A by mutating only two arginine residues (R465 and R466) in the polybasic motif (R465, R466, R469, and R472) (Figure 5—figure supplement 3A). In CaV2.2 channels with the β2a subunit, we did not detect any significant differences in current inactivation and PIP2 sensitivity among WT α1B, α1B R465,466A, and α1B R476,477A (Figure 5—figure supplement 3B–E). However, in CaV2.2 with β2c, the inactivation rate was slower and the PIP2 sensitivity was weaker in both α1B R465,466A and α1B R476,477A compared to WT α1B (Figure 5—figure supplement 3B–E).

**Differential modulation of CaV2.2 channels by muscarinic receptor stimulation in cells expressing PM-anchored or cytosolic β subunit**

To examine whether the polybasic motif influenced the Gq-coupled modulation of CaV2.2 channels, we applied the muscarinic acetylcholine receptor agonist, oxotremorine-M (Oxo-M), to cells co-transfected with the M1 muscarinic receptor (M1R) (Figure 6). Since the M1R stimulation suppressed CaV2.2 channels through both Gβγ binding to channels and PIP2 depletion (Keum et al., 2014), we then used a Gβγ-insensitive chimeric CaV2.2 channel construct, α1C-1B, to examine the effect of PIP2 depletion alone on channel regulation (Figure 6). In this chimera construct, the N-terminus of CaV2.2 (α1B), which contains one of the Gβγ interaction sites, is replaced by the N-terminus of CaV1.2 (α1C) (Agler et al., 2005). M1R activation inhibited the current by approximately 5% in cells expressing either α1C-1B WT or 4A channels with β2a subunit, which were similar to the responses of regulation by Dr-VSP-mediated PIP2 depletion in those channels (Figure 6B, C). However, consistent with the results
Figure 5. Polybasic motif at the C-terminal end of the I–II loop influences determination of steady-state activation, current inactivation, and PIP$_2$ sensitivity of Ca$_{v}$2.2 channels. (A) Schematic diagram of phospholipid-binding residue-neutralizing mutations within the C-terminal end of the I–II loop in the α$_{1}$B subunit. The phospholipid-binding residues (R465, R466, K469, and R472) highlighted in blue were mutated to alanine (4A). (B) Current inactivation was measured during 500-ms test pulses to +10 mV in cells expressing α$_{1}$B WT (black traces) and 4A mutants (green traces) with β2a (left) or β2c (right).
for Dr-VSP-induced channel modulation, current suppression was much weaker in α1C-1B 4A channels with β2c than in α1C-1B WT with β2c (Figure 6B, C). We confirmed that the current suppression by M1R activation were not recovered in both α1C-1B WT and α1C-1B 4A channels by a prepulse regardless of the coupled β2 isotypes (Figure 6D, E). We additionally used G1-coupled M2 muscarinic receptor (M1R) to further examine whether the polybasic motif in I–II loop affects the Gβγ-mediated modulation of CaV2.2 currents (Figure 6—figure supplement 1). M1R activation inhibited the currents evoked by a + 10-mV test pulse without significant difference between WT and 4A α1B with β2a or β2c (Figure 6—figure supplement 1D). We have previously reported that subcellular localization of the CaV β subunit plays important roles in determining the Gβγ-dependent inhibition of CaV2.2 channels; membrane-anchored β2a subunit changes CaV2.2 channels are more sensitive to Gβγ-mediated voltage-dependent inhibition, whereas cytosolic β2b and β3 subunit changes channels are less sensitive to Gβγ-mediated voltage-dependent inhibition (Keum et al., 2014). In CaV2.2 channels with the β2a subunit, the recoveries from Gβγ-mediated inhibition did not significantly differ between WT α1B and 4A α1B (Figure 6—figure supplement 1E, F). However, in CaV2.2 with the β2c subunit, there was less recovery from Gβγ-mediated inhibition in α1B WT than in α1B 4A (Figure 6—figure supplement 1E, F). Recovery from M1R-mediated inhibition in 4A α1B with β2c was similar to the values of WT and 4A α1B with β2a (Figure 6—figure supplement 1E, F).

**PIP2-binding site in S4b domain is important in maintaining the CaV2.2 channel activity regardless of the coupled β2 isotype**

Recently, the cryo-electron microscopic structure of human CaV2.2 complex composed of α1B, α2δ1, and β3 subunits was revealed at a resolution of 3.0 Å (Dong et al., 2021; Gao et al., 2021). These studies have shown that the 5-phosphate group of membrane PIP2 interacts with two basic residues (R584 and K587) within S4b domain of α1B. We examined whether the two basic residues affect the PIP2 sensitivity of CaV2.2 channels. First, we constructed neutralized mutant α1B subunits in which the two basic residues in S4b were replaced by alanine residues (α1B RA/KA) (Figure 7A). In CaV2.2 channels with β2a, the inactivation kinetics of the current were not changed in α1B and α1B RA/KA, regardless of the 4A mutation (Figure 7B, C). In CaV2.2 with β2c, WT α1B RA/KA showed faster inactivation than WT α1B, whereas 4A α1B RA/KA showed much slower but similar inactivation to those of α1B and α1B RA/KA with β2a (Figure 7B, C). Additionally, the effects of PIP2 depletion on current amplitude were
measured in these mutant channels. Mutation of the two basic residues in S4, completely abolished the Dr-VSP-mediated current inhibition in cells expressing WT α1B RA/KA or 4A α1B RA/KA with the β2a subunit, while there was approximately 5% inhibition in cells expressing WT and 4A α1B with β2a (Figure 7D, E). Importantly, PIP2 depletion significantly inhibited the currents in cells expressing WT
Figure 7. PIP_2-binding residues within the S4_1 domain play an important role in determining steady-state activation and PIP_2 sensitivity of Ca_2.2 channels. (A) Distance analysis of PIP_2-binding site in the S4_1 domain of α_1B subunit. Two amino acids (R584 and K587) interacting with the 5-phosphate of PIP_2 were neutralized to alanine residues (RA/KA). (B) Current inactivation was measured during 500-ms test pulses to +10 mV in cells expressing WT α_1B (black traces), WT α_1B RA/KA (red traces), 4A α_1B (green traces), and 4A α_1B RA/KA (orange traces) with β_2a (upper) or β_2c (bottom). Gray traces

Figure 7 continued on next page
Discussion

This study has expanded our understanding of the inter-regulatory actions of the Ca_{\beta} subunit and membrane PIP_{2} on Ca_{\alpha} channel gating properties, including inactivation kinetics, current density, and voltage dependency. Our data predict that Ca_{2.2} channels complexed with any \beta isotype can interact with membrane PIP_{2} through the binding pocket in the S4_{\alpha} domain (Figure 8). However, in Ca_{2.2} channels with cytotoxic \beta_{2c}, there seems to be another interaction with PIP_{2} through the nonspecific phospholipid-binding site at the distal end of the \alpha_{1} I–II loop. This leads to the channel becoming highly sensitive to Dr-VSP-mediated PIP_{2} depletion (Figure 8, lower panel). In channels with \beta_{2a}, the membrane anchoring of the subunit may interfere with the interaction between the phospholipid-binding site and PIP_{2}. This converts the channels to a less PIP_{2}-sensitive state (Figure 8, upper panel). Additionally, the neutralization of polybasic residues in the I–II loop to alanine abolished PIP_{2} binding on the phospholipid-binding site regardless of \beta isotype, which led to the less PIP_{2}-sensitive state (Figure 8, 4A \alpha_{1}B). By contrast, the neutralization of two basic residues in the S4_{\beta}-binding pocket slightly reduced PIP_{2} sensitivity in channels with cytotoxic \beta_{2c} subunits and completely abolished the response in channels with a \beta_{2a} subunit (Figure 8, \alpha_{1}B RA/KA). Taken together, these data showed that when PIP_{2} molecules were depleted at the VSD_{\alpha}, PIP_{2} and polybasic phospholipid-binding sites or both sites were mutated to neutralized amino acid residues, the channels move to a nonconducting state (Figure 8, 4A \alpha_{1}B RA/KA).
CaV \( \beta \) subunits regulate bidentate \( \text{PIP}_2 \) binding to CaV2.2 channels

Previous studies have proposed a bidentate model for the \( \text{PIP}_2 \) modulation of N-type CaV2.2 channel regulation (Heneghan et al., 2009; Hille et al., 2015; Mitra-Ganguli et al., 2009; Roberts-Crowley and Rittenhouse, 2009). In this model, lipidation on the N-terminus of CaV \( \beta \) subunits disrupts the hydrophobic interaction between the two fatty acyl chains of \( \text{PIP}_2 \) and CaV2.2 channels, and thus reduces current inhibition by \( \text{PIP}_2 \) depletion. For example, \( \beta 2 \)a subunits interact with the PM through two palmitoyl fatty acyl chains in the N-terminus, leading to competition in binding to CaV channels with the fatty side chains of \( \text{PIP}_2 \). This competition removes \( \text{PIP}_2 \) from the channel-binding site. Thus, CaV channels with \( \beta 2 \)a are uncoupled from the membrane \( \text{PIP}_2 \) and show lower \( \text{PIP}_2 \) sensitivity to \( \text{PIP}_2 \) depletion. By contrast, non-lipidated \( \beta 3 \) subunits expressed in the cytosol do not interrupt the interaction between the fatty acyl chains of \( \text{PIP}_2 \) and CaV2.2 channels, and show high \( \text{PIP}_2 \) sensitivity of channels (Heneghan et al., 2009; Hille et al., 2015; Suh et al., 2012). Consistently, we found that when the \( \beta 3 \) subunits were anchored to the PM by adding the lipidation signal of Lyn to the N-terminus, the engineered Lyn-\( \beta 3 \) construct decreased the \( \text{PIP}_2 \) sensitivity of CaV2.2 channels, similar to \( \beta 2 \)a. The Lyn \( \eta \) domain incorporates into the PM through the G2-myristoylated and C3-palmitoylated lipid anchors;
therefore, Lyn-β3 mimics β2a in competing with PIP₂ for the hydrophobic Ca₂⁺,2.2 channel interaction. Conversely, the lipid anchor of Lyn-48aa-β2c may be localized far from the channel complex because of its long N-terminal flexible linker, suggesting that these mutant subunits cannot disrupt the hydrophobic interaction between PIP₂ and channels.

Our results provide advance information about the bidentate model. First, we confirmed that two distinct PIP₂-interacting sites were preserved in the Ca₂⁺,2.2 channel: the binding pocket in VSD₂ and phospholipid-binding site in the I–II loop. Our data are consistent with that the 5-phosphate group of membrane PIP₂ interacts with the two basic residues within the S₄ᵢ domain of Ca₂⁺,2.2 channels regardless of β₂ isotype. The additional interaction of PIP₂ with the nonspecific phospholipid binding site in the distal I–II loop of Caᵢ channels was mainly observed in Ca₂⁺,2.2 channels with the cytosolic Caᵢ β₂c subunit. Our data indicate that PIP₂-binding to the I–II loop phospholipid-binding site is selectively disrupted by the lipid anchor of membrane-anchored β2a. The hydrophobic interaction of the palmitoyl or myristoyl groups of Caᵢ β2a or Lyn-β constructs with channel complex may be the cause of PIP₂ release from the lower-affinity I–II loop phospholipid-binding site (Roberts-Crowley and Rittenhouse, 2009). When PIP₂ interacts with the VSDᵢ PIP₂-binding site of Ca₂⁺,2.2 channels complexed with β2a, the PIP₂ sensitivity of the channels dramatically decreased to approximately 5%. Our results suggested that this minimal PIP₂ sensitivity specifically caused by PIP₂ degradation on VSDᵢ-binding pocket by Dr-VSP activation.

This work suggests that the PIP₂ sensitivity of the Ca₂⁺,2.2 channel is mainly affected by the length between the lipid anchor and GK domain of the Caᵢ β subunit. Although both Lyn-β2c and Lyn-48aa-β2c are localized at the PM, the PIP₂ sensitivity and inactivation kinetics of Ca₂⁺,2.2 channels are significantly different from each other: Ca₂⁺,2.2 channels with Lyn-β2c subunits exhibited relatively slower inactivation kinetics and lower PIP₂ sensitivity, similar to channels with the membrane-anchored β2a subunit. By contrast, Ca₂⁺,2.2 channels with Lyn-48aa-β2c subunits exhibited faster inactivation kinetics and higher PIP₂ sensitivity, similar to channels with the cytosolic β2c subunit. Similarly, disruption of the SH3-GK interaction in the membrane-anchored β2a subunit accelerated current inactivation and increased the current inhibition by PIP₂ depletion. Moreover, real-time translocation of the lipid anchor, Lyn₁₁₁, to the channel complex by rapamycin-inducible dimerization systems slowed the inactivation and decreased the PIP₂ sensitivity of Ca₂⁺,2.2 channels. Inversely, incremental increases in flexible linker length between the lipid anchor and GK domain of Caᵢ β₂ subunits gradually accelerated the inactivation kinetics and increased the PIP₂ sensitivity of Ca₂⁺,2.2 channels. However, the mechanism by which the physical distance from the PM lipid to GK domain of the Caᵢ β subunit affects the PIP₂ sensitivity of the Ca₂⁺,2.2 channel is not fully understood yet. Another possibility is that torsional rigidity of the linker domain may be different depending on the length and thus differently restrict the cytoplasmic movement of Caᵢ β subunit as well as the gating of Ca₂⁺,2.2 channels.

Colecraft et al. have reported that chemically induced anchoring of intracellular loops of the channels to the PM can modulate the gating of the HVA Ca<sup>2+</sup> channel (Subramanyam and Colecraft, 2015; Yang et al., 2013). They have shown that PdBu-induced translocation to the PM of chimeric β3-C₁PKCc, which is assembled by fusing the C₁ domain of PKCc to the C-terminus of the β3 subunit, leads to the inhibition of the Ca₂⁺,2.2 current. Conversely, the C₁PKCc β3 subunit, which is assembled by adding C₁PKCc to the N-terminus of the β3 subunit, has no effect on the current (Yang et al., 2013). These studies suggest that the polarity of the PM-targeting domain may play an important role in determining the Ca₂⁺,2.2 channel gating; however, the molecular basis of the differential regulation mechanism remain unclear. On the basis of our results, we speculate that the C₁PKCc β3 form may be insufficient to disrupt the interaction with between phospholipid-binding site and PIP₂ in Ca₂⁺,2.2 channels because the length from the C₁PKCc and the GK domain of the β3 subunit is 175 aa. This could be too long to interfere the interaction between PIP₂ and Ca₂⁺,2.2 channels.

Recently, Gao et al., 2021 have shown that two basic gating charge residues (R584 and K587) within the S₄ᵢ domain of human Caᵢ,2.2 channel interact with the 5-phosphate group of membrane PIP₂. In our present work, we found that mutation of the two residues (RA/KA) in the S₄ᵢ domain completely blocked the Dr-VSP-induced current suppression in channels with β2a and shifted the voltage-dependent activation curve toward the depolarization direction regardless of Caᵢ β2 isotype. The cryo-EM structure does not show the nonspecific PIP₂-binding site in the channels probably because it is located in the flexible I–II loop. We hypothesize that the polybasic residues in the I–II loop tether to the anionic phospholipids through the electrostatic interaction and this dipole–dipole
interaction may contribute to the low-affinity phospholipid-binding site (Yeon et al., 2018). In contrast, the VSDα PIP2-binding site forms a pocket-like structure inside the S4 domain and covered by the AID domain in the cytosolic side (Dong et al., 2021; Gao et al., 2021), which could stabilize the domain in a high-affinity PIP2 interacting site. Thus, it is possible that the PIP2 molecule inside the VSDα PIP2-binding pocket is relatively less accessible to the degradation by phospholipase C or Dr-VSP, leading to the lower PIP2 sensitivity in CaV2.2 channels.

In conclusion, our findings provide new insights on the regulatory mechanism of CaV2.2 channel gating by CaV β subunits. Our recent study has reported that when intracellular Ca2⁺ is increased by depolarizing the cells or activating Gq-coupled receptors, the high intracellular Ca2⁺ concentration induces a dissociation of the N-terminus of the CaV β2e subunit from the PM. This increases both the inactivation kinetics and PIP2 sensitivity of CaV2.2 channels (Kim et al., 2016). The N-terminus of the β2e subunit is anchored to the PM via electrostatic interaction with the anionic phospholipids of these PM. These studies suggest that dissociation of the β2e subunit from the membrane leads to an interaction between the I–II loop phospholipid-binding site and PIP2, which changes the gating properties of CaV channels in physiological conditions. The interaction of CaV α1B with β subunits can be dynamically exchanged by other free β isoforms in intact cells (Yeon et al., 2018); therefore, the displacement of cytosolic β subunits by membrane-tethered β subunits on CaV channels will abolish the interaction with between PIP2, and the I–II loop phospholipid-binding site via lipid anchor of membrane-tethered β subunits, which alters the CaV channel gating properties. Further studies are needed to investigate whether the conformational shift of the I–II loop to the membrane or cytosolic face by endogenous β subunit combinations determines CaV channel gating in neurons and other excitable cells.

Materials and methods

Cell culture and transfection

Human embryonic kidney tsA-201 cells (large T-antigen transformed HEK293 cells; RRID:CVCL_2737) were a kind gift from Dr Bertil Hille at University of Washington. The identity of this cell line has been authenticated by STR analysis and has recurrently tested negative for mycoplasma contamination using PCR (Cosmogenetech, Daejeon, South Korea). Cells were maintained in Dulbecco modified Eagle medium (Invitrogen, CA) supplemented with 10% fetal bovine serum (Invitrogen, CA) and 0.2% penicillin/streptomycin (Invitrogen, CA) in 100 mm culture dishes at 37°C with 5% CO2. The cells were segregated into 6-well plates and used between passages 1–5. The experiments were repeated three times. Human embryonic kidney tsA-201 cells (large T-antigen transformed HEK293 cells; RRID:CVCL_2737) were a kind gift from Dr Bertil Hille at University of Washington. The identity of this cell line has been authenticated by STR analysis and has recurrently tested negative for mycoplasma contamination using PCR (Cosmogenetech, Daejeon, South Korea). Cells were maintained in Dulbecco modified Eagle medium (Invitrogen, CA) supplemented with 10% fetal bovine serum (Invitrogen, CA) and 0.2% penicillin/streptomycin (Invitrogen, CA) in 100 mm culture dishes at 37°C with 5% CO2. The cells were transiently transfected with Lipofectamine 2000 (Invitrogen, CA) when the confluency of the cells reached 50–70%. For assessment of CaV channel expression, the cells were co-transfected with α1 of CaV, α2δ1, and various β2 chimera constructs in a 1:1:1 molar ratio. The transfected cells were plated onto a coverslip chip coated with poly-L-lysine (0.1 mg/ml, Sigma-Aldrich, MO) 24–36 hr after transfection. plated cells were used for electrophysiological and confocal experiment within 24 hr after plating, as described previously (Park et al., 2017).

Plasmids

The following plasmids were used: The calcium channel subunits α1B of rat CaV2.2e[37b] (GenBank Sequence accession number AF055477) and rat α2δ1 (AF286488) were from Diane Lipscombe, Brown University, Providence, RI. Chimeric α1C-1B was generously donated by David T. Yue, Johns Hopkins University, Baltimore, MD. Mouse cDNAs of β2a and β2c were generously donated by Veit Flockerzi, Saarland University, Homburg, Germany. The Dr-VSP (AB308476) was obtained from Yasushi Okamura, Osaka University, Osaka, Japan.

Molecular cloning

Cloning of β2a-GFP, β2a(C3,4S)-GFP, and β2c-GFP was performed as previously described (Park et al., 2017). For the generation of various β2 chimera constructs, we used the one-step sequence- and ligation-independent cloning (SLIC) as a time-saving and cost-effective cloning strategy (Jeong et al., 2012). First, pEGFP-N1, pEYFP-N1, and mCherry-N1 vectors (Clontech) were linearized by KpnI restriction enzyme digestion. The cDNAs encoding β2a, β2c, Lyn, FRB, or FKBP were amplified by PCR using primers with an 18-bp homologous sequence attached to each end of the linearized vector. Primers used for β2 chimera constructs are listed in Supplementary file 1. Second, the linearized vector and PCR fragments were blended and incubated at room temperature for 2.5 min with T4

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DNA polymerase (NEB, The Netherlands). Third, the DNA mixture was kept on ice for 10 min, after which competent Escherichia coli cells were transformed directly. For the deletion and point mutation of GK-SH3 interaction sites of the β2 subunit and the potential PIP2-interaction sites of α1B, first, the α1B or β2 subunits were amplified by inverse PCR using nPfu-special DNA polymerase (Enzymomics, Daejeon, South Korea). Second, the PCR product was 5'-phosphorylated by T4 polynucleotide kinase (Enzymomics, Daejeon, South Korea) and plasmid DNA was digested by Dpn I (Agilent Technologies, Santa Clara, CA). Finally, the PCR product was ligated by T4 DNA ligase (NEB, The Netherlands). The primers used for mutagenesis are listed in Supplementary file 2. All the chimera and mutant constructs were verified by DNA sequencing (Macrogen, South Korea).

Electrophysiology

The whole-cell configuration of the patch-clamp technique was used to record Ba^{2+} currents using HEKA EPC-10 patch-clamp amplifier with pulse software (HEKA Elektronik). 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%. For all recordings, cells were maintained at −80 mV. The external solution 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 and an osmolarity of 321–350 mOsm. The internal solution of the pipette consisted of 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\(_2\)ATP, and 0.1 mM Na\(_3\)GTP, adjusted to pH 7.4 with CsOH and an osmolarity of 321–350 mOsm.

Confocal imaging

All imaging examinations were performed with an LSM 700 confocal microscope (Carl Zeiss AG) at room temperature (22–25°C). The external 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 and an osmolarity of 321–350 mOsm. For live-cell imaging, images were obtained by scanning cells with a ×40 (water) apochromatic objective lens at 1024 × 1024 pixels using digital zoom. Analysis of line scanning of fluorescence images was performed using the ‘profile’ tool in Zen 2012 lite imaging software (Carl Zeiss Microimaging). To analyze colocalization, we performed quantitative colocalization analysis using Fiji software with the Colocalization Threshold plugin to determine the Pearson’s correlation coefficient (R). Pixel intensities were presented as 2D intensity histograms with a linear regression line and as bar graphs with mean R values. All images were transferred from LSM4 to JPEG format.

Förster resonance energy transfer

FRET experiments were performed using a monochromator (Polychrome V; TILL Photonics) with a ×40, NA 0.95 dry immersion objective lens (Olympus). Regular pulses of indigo light (438 ± 12 nm) excited the fluorescent proteins. Emission was separated into short (460–500 nm) and long (520–550 nm) wavelengths by appropriate filters and then acquired by two photomultipliers. Donor and acceptor signals obtained by photometry (TILL Photonics) were transferred to the data acquisition board (PCI-6221; National Instruments). Signal acquisition and real-time calculation of the FRET ratio were conducted by a custom program. The FRET ratio was calculated as follows:

\[ \text{FRET}_{r} = \frac{(\text{YFP}_{C} - c\text{Factor} \times \text{CFP}_{C})}{\text{CFP}_{C}} \]

CFP\(_{C}\) is the CFP emission detected by the short-wavelength photomultiplier, and YFP\(_{C}\) is the YFP emission detected by the long-wavelength photomultiplier, as described previously (Keum et al., 2014).

Calculation of distance with a WLC model

The Lyn-Linker-(additional Link) structure was suggested as an unstructured structure from the IUPRed Web-server (http://iupred.elte.hu/) (Dosztányi et al., 2005) to predict disorder tendency. To calculate the distance between the GK domain and the inner surface of the PM, the WLC model was used. This model is usually used to describe the behavior of polymers that are semi-flexible: quite stiff with successive segments pointing in roughly the same direction, and with persistence length within a few orders of magnitude of the polymer length. This model is also used to describe unstructured proteins.
like this linker structure (Zhou, 2001). In the WLC, the mean square end-to-end distance $<R^2>$ is written as:

$$<R^2> = 2P\lambda_0 \left[1 - \frac{P}{\lambda_0} \exp\left(-\frac{L_0}{P}\right)\right]$$

where $P$ is the polymer’s characteristic persistence length and $\lambda$ is the maximum length. We used $P = 0.6$ and $\lambda = (N - 1)^3 \times 3.8$, where $N$ is number of amino acids in the unstructured protein (Lapidus et al., 2002). We then removed three amino acids in Lyn(MGC), which is directly connected to the membrane via palmitoylation and myristoylation. The root mean square end-to-end distance $\sqrt{<R^2>}$, which can be suggested as the average distance, was calculated. $\sqrt{<R^2>}$ was 32.7 Å for six additional linkers, 36.0 Å for 11 aa, 38.4 Å for 15 aa, 42.4 Å for 22 aa, 52.5 Å for 43 aa, and 28.2 Å for no additional linker.

### Statistical analysis

Patch clamp data acquisition and analysis used Pulse/Pulse Fit 8.11 software with the EPC-10 patch clamp amplifier (HEKA Elektronik). Further data processing was performed with Igor Pro 6.2 (WaveMetrics, Inc), Excel office 365 (Microsoft), and GraphPad Prism 7.0 (GraphPad Software, Inc). All quantitative data were presented as mean ± standard error of the mean values. Comparisons between groups were analyzed by Student’s two-tailed unpaired t-test. Comparisons among more than two groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Comparisons among more than two groups with two independent variables were analyzed using two-way ANOVA followed by Sidak post hoc test. Differences were considered significant at the *p < 0.05, **p < 0.01, and ***p < 0.001, as appropriate.

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Cheon-Gyu Park, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Visualization, Methodology, Writing - original draft; Wookyung Yu, Formal analysis; Byung-Chang Suh, Conceptualization, Resources, Supervision, Visualization, Project administration, Writing - review and editing

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Additional files

Supplementary files
- Supplementary file 1. Primers for β2 chimera constructs.
- Supplementary file 2. Primers for deletion or mutagenesis of CaV α1B and β2 constructs.
- Transparent reporting form

Data availability
All data generated or analyzed during this study are included in the manuscript and supporting files.

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