

Dynamic phospholipid interaction of $\beta 2e$ subunit regulates the gating of voltage-gated Ca^{2+} channels

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High voltage-activated Ca^{2+} (Ca_V) channels are protein complexes containing pore-forming $\alpha 1$ and auxiliary β and $\alpha 2\delta$ subunits. The subcellular localization and membrane interactions of the β subunits play a crucial role in regulating Ca_V channel inactivation and its lipid sensitivity. Here, we investigated the effects of membrane phosphoinositide (PI) turnover on $\text{Ca}_V 2.2$ channel function. The $\beta 2$ isoform $\beta 2e$ associates with the membrane through electrostatic and hydrophobic interactions. Using chimeric β subunits and liposome-binding assays, we determined that interaction between the N-terminal 23 amino acids of $\beta 2e$ and anionic phospholipids was sufficient for $\beta 2e$ membrane targeting. Binding of the $\beta 2e$ subunit N terminus to liposomes was significantly increased by inclusion of 1% phosphatidylinositol 4,5-bisphosphate (PIP_2) in the liposomes, suggesting that, in addition to phosphatidylserine, PIs are responsible for $\beta 2e$ targeting to the plasma membrane. Membrane binding of the $\beta 2e$ subunit slowed $\text{Ca}_V 2.2$ current inactivation. When membrane phosphatidylinositol 4-phosphate and PIP_2 were depleted by rapamycin-induced translocation of pseudojanin to the membrane, however, channel opening was decreased and fast inactivation of $\text{Ca}_V 2.2(\beta 2e)$ currents was enhanced. Activation of the M_1 muscarinic receptor elicited transient and reversible translocation of $\beta 2e$ subunits from membrane to cytosol, but not that of $\beta 2a$ or $\beta 3$, resulting in fast inactivation of $\text{Ca}_V 2.2$ channels with $\beta 2e$. These results suggest that membrane targeting of the $\beta 2e$ subunit, which is mediated by nonspecific electrostatic insertion, is dynamically regulated by receptor stimulation, and that the reversible association of $\beta 2e$ with membrane PIs results in functional changes in Ca_V channel gating. The phospholipid–protein interaction observed here provides structural insight into mechanisms of membrane–protein association and the role of phospholipids in ion channel regulation.

INTRODUCTION

This paper concerns the localization and actions of a regulatory subunit of voltage-gated Ca^{2+} (Ca_V) channels. The intracellular Ca^{2+} ion is a potent second messenger, important for diverse biological processes such as neurotransmitter release, hormone secretion, excitation–contraction coupling, and gene expression. Ca_V channels, which are multi-protein complexes made up of at least three types of subunit named α , β , and $\alpha 2\delta$, are major machinery for Ca^{2+} influx (Catterall, 2000; Ertel et al., 2000). Thus, regulation of Ca_V channels is critical for maintaining a variety of cellular signaling. The β subunits are intracellular proteins directly associated with the large pore-forming $\alpha 1$ subunits. They tune the electrophysiological and kinetic properties of the channel (Buraei and Yang, 2010; Waithe et al., 2011). Equally

importantly, they act as chaperones to traffic $\alpha 1$ subunits to the plasma membrane and confer functional complexity on Ca_V channels (Bichet et al., 2000; Altier et al., 2011). Unlike the other cytosolic β subunits, the $\beta 2a$ subunit is known to be posttranslationally palmitoylated at two cysteines of the N terminus and therefore is localized on the plasma membrane. It slows the inactivation of Ca_V channels (Chien et al., 1996; Qin et al., 1998; Hurley et al., 2000). With other cytosolic (nonlipidated) subunits, Ca_V channels can be inhibited by phosphatidylinositol 4,5-bisphosphate (PIP_2) depletion, but with the lipidated $\beta 2a$ subunit, channels are much less sensitive to PIP_2 depletion (Heneghan et al., 2009; Roberts-Crowley et al., 2009; Suh et al., 2012). Similar to the $\beta 2a$ subunit, the $\beta 2e$ subunit is localized at the plasma membrane even in the absence of $\alpha 1$ subunit, and it slows the inactivation of Ca_V channels (Takahashi et al., 2003; Link et al., 2009). Recently, Miranda-Laferte

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Abbreviations used in this paper: 2BP, 2-bromopalmitate; 2-HM, 2-hydroxymyristate; Ca_V , voltage-gated Ca^{2+} ; FRET, fluorescence resonance energy transfer; G_q PCR, G_q protein-coupled receptor; M_1 R, M_1 muscarinic receptor; N-del, N terminus-deleted; Oxo-M, oxotremorine-M; PI, phosphoinositide; $\text{PI}(4)\text{P}$, phosphatidylinositol 4-phosphate; PIP_2 , phosphatidylinositol 4,5-bisphosphate; PJ, pseudojanin; PS, phosphatidylserine; qRT-PCR, quantitative real-time PCR.

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et al. (2014) have reported that with protein–liposome cosedimentation assays, this membrane targeting of the $\beta 2e$ subunit depends on the interaction of its N terminus, which consists of positively charged residues, with plasma membrane phosphatidylserine (PS) (Miranda-Laferte et al., 2014). Thus, the membrane localization of β subunits by lipidation contributes unique properties to Ca_v channels. However, the functional roles of β subunits in the modulation of Ca_v channels by G_q protein–coupled receptors (G_q PCRs) remain to be further explained.

The subcellular distribution of peripheral membrane proteins is regulated in part by unique interactions between the protein and the cytoplasmic lipid leaflet of the membrane (Cho and Stahelin, 2005; Rosenhouse-Dantsker and Logothetis, 2007; van Meer et al., 2008). Indeed, for the plasma membrane, the lipid composition plays an important role in many cellular processes involving signal transduction, protein trafficking, cytoskeleton organization, and vesicular transport (McLaughlin and Murray, 2005; Balla, 2013). In particular, anionic plasma membrane lipids including phosphatidic acid, PS, and phosphoinositides (PIs) not only recruit peripheral proteins to the plasma membrane but also allow them to be involved in a wide variety of signaling pathways in a spatio-temporally organized manner. Especially, the levels of phospholipids are intensely regulated by receptor signal transductions and intracellular energy metabolism. Thus, the dynamics in the interaction of peripheral proteins to the plasma membrane would be very important for the functional regulation of membrane molecules (DiNitto et al., 2003).

Here, we investigated the effect of reversible interaction between the $\beta 2e$ subunit and the plasma membrane PI phospholipids on regulation of Ca_v channels. The $\beta 2e$ subunit is a splice variant of a single $\beta 2$ gene and is expressed broadly in excitable tissues, such as brain, heart, lung, and kidney, in rodents and human (Massa et al., 1995; Colecraft et al., 2002; Link et al., 2009). Using a series of experimental approaches including *in vitro* binding assays between peptides and liposomes and rapamycin-inducible dimerization, we found that $\beta 2e$ subunits are membrane localized through interactions between the $\beta 2e$ N terminus and PI phospholipids, and that the depletion of membrane PIs accelerates the inactivation of $Ca_v 2.2$ channels. In addition, G_q -coupled muscarinic receptor stimulation triggers translocation of the $\beta 2e$ subunit to the cytosol and enhances the current inactivation. Our results suggest that $\beta 2e$ regulates Ca_v channels through a reversible interaction with membrane poly-PIs, a different membrane-targeting mechanism from that of the $\beta 2a$ subunit. These findings provide better understanding of the role of PIs in direct interaction between plasma membrane and peripheral proteins as well as in ion channel regulation by membrane phospholipids.

MATERIALS AND METHODS

cDNAs

The following calcium channel subunits were used: rat $\beta 2a$ (M80545; provided by W.A. Catterall, University of Washington, Seattle, WA); rat $\beta 2a$ (C3,4S; provided by J. Hurley, Indiana University, Bloomington IN); $\alpha 1B$ (GenBank accession no. AF055477), rat $\beta 3$ (RefSeq accession no. NM_012828), and rat $\alpha 2\delta-1$ (AF286488; provided by D. Lipscombe, Brown University, RI); Lyn- $\beta 3$ -YFP, RFP-Dead, RFP-Sac1, RFP-INPP5E, and RFP-PJ (provided by B. Hille, University of Washington); M_1 muscarinic receptor (M_1R ; provided by N.N. Nathanson, University of Washington); and M_2 muscarinic receptor (Guthrie Resource Center, Rolla, MO).

Mouse $\beta 2e$ cloning

Mouse brain cDNA library was isolated from 2-mo-old C57 male mice. This PCR product of brain cDNA library was amplified by PCR using the primers of $\beta 2b$ (GenBank accession no. AF423193) and $\beta 2e$ (RefSeq accession no. XM_006497319) subunits: $\beta 2b$ forward primer: 5'-CGCTAGCAGCATCTCGACCCCTGTTG-3', reverse primer: 5'-CGGATCCCCCTTGGCGGATGTATACATCCCTG-3'; $\beta 2e$ forward primer: 5'-CGCTAGCATGAAGGCCACCTGGATCAGGCTT-3', reverse primer: 5'-CGGATCCCCCTTGGCGGATGCGGATCCCCCTTGGCGGATGTATACATCCCT-3'. The cDNAs encoding the mouse $\beta 2$ subunits were TA cloned into T-easy vector (Promega) and cloned in pCDNA3.1, pEGFP-N1 (Takara Bio Inc.), and mCherry-N1 (Takara Bio Inc.). For the $\beta 2e/3$ chimeric construct, the N-terminal region (69 base pairs) of $\beta 2e$ subunit was amplified by PCR using mouse $\beta 2e$ subunit and the following primers: forward primer: 5'-CCGGTACCATGAAGGCCACCTGGAGGCTT-3', reverse primer: 5'-CCGGTACCACAGATGTCCGAACCTCTTCAGC-3'. For insertion of N terminus of $\beta 2e$ in $\beta 3$, Kpn1 site was generated in the N-terminal region of $\beta 3$ using point mutagenesis using the following primer: sense: 5'-GGTACCGTCGACTGCAGAATTGCAAGCTTG-3', antisense: 5'-TAGGAGTCGGCGGTACCCGCTCCGAGTC-3'. Constructs were subsequently inserted into the N-terminal region of $\beta 3$ for expression. An N-terminal region–deleted construct of $\beta 2e$ subunit was amplified by PCR using the following primers: forward primer: 5'-GCCATGGTTGGTTTTTCGGCAGACTCCTACACCAG-3', reverse primer: 5'-CGGATCCCCCTTGGCGGATGTATACATCCCC-3'.

RNA isolation

For analysis of mRNA expression profile, cortex, cerebellum, hippocampus, and hypothalamus were removed from 2-mo-old C57 male mice, following national and international standards of animal care and welfare. Total RNA from each brain tissue was extracted from homogenized samples using RNeasy total RNA isolation system (Promega). The total RNA of brain tissues was used to generate double-strand cDNA using SuperScript III reverse transcription (Invitrogen).

Quantitative SYBR Green real-time RT-PCR assay

The relative abundance of $\beta 2a$ and $\beta 2e$ subunits was determined using CFX96 Touch Real-Time PCR machine (Bio-Rad Laboratories), using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories). The following primers were used for quantitative real-time PCR (qRT-PCR) of fragments of β subunits and β actin using cDNA from various mouse brain tissues: $\beta 2a$ forward primer: 5'-ATGCAGTGTGCGGGCTGGTACAT-3', reverse primer: 5'-CATGCCAGGCACCGGAACGTCAT-3'; $\beta 2a$ forward primer: 5'-TGAAGGCCACCTGGATCAGGCTTC-3'; and as a control, β -actin forward primer: 5'-TGTTTGAGACCTTCAACACC-3', reverse primer: 5'-TAGGAGCCAGAGCAGTAATC-3'. The qRT-PCR was set up in a 20- μ l reaction volume containing 10 μ l of 2 $\times$ SsoAdvanced Universal SYBR Green Supermix, 1.5 μ l cDNA (100 ng), 1 μ l (10 μ M)

of each primer, and 5.5 μ l of nuclease-free water. The optimized cycling conditions were as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, primer annealing at 60°C for 30 s, and extension at 72°C for 20 s. A melt curve analysis was performed after amplification to verify the specificity of the amplified products. Melting curve analysis consisted of 65°C for 15 s, followed by temperature from 55 to 96°C in increments of 0.5°C with continuous reading of fluorescence. Each sample was quantified by determining the cycle threshold, and triplicate qRT-PCR reactions were done for β 2a and β 2e subunits. To evaluate concentrations of β 2a, β 2e, and β actin in brain tissues, Gene Expression Module of CFX manager software (Bio-Rad Laboratories) was used.

Cell culture and transfection

TsA201 cells were maintained in DMEM (HyClone; Thermo Fisher Scientific) containing 10% fetal bovine serum and 0.2% penicillin/streptomycin at 37°C with 5% CO₂. For transfection, cells were plated in 3.5-cm culture dishes at 50–80% confluency. For Cav channel expression, cells were transiently transfected using Lipofectamine 2000 (Invitrogen). The transfected DNA mixture consisted of plasmids encoding α 1, β , and α 2 δ -1 at a 1:1:1 molar ratio. When needed, enhanced GFP was also included in DNA mixtures. Cells were plated on poly-L-lysine-coated chips the day after transfection. Currents were recorded within 2 d after transfection.

Preparation of liposomes

All lipids were purchased from Avanti Polar Lipids, Inc., except *N*-[5-(dimethylamino) naphthalene-1-sulfonyl]-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (dansyl-PE), from Invitrogen. Liposomes consist of PC (1- α -phosphatidylcholine), PE (1- α -phosphatidylethanolamine), PS (1- α -phosphatidylserine), cholesterol, dansyl-PE, and PIP₂, and (44:10:15:25:5:1 mol percent). In case of no PS or PIP₂, the PC content was adjusted accordingly. In brief, the lipid mixture was dried under a gentle stream of nitrogen in the hood, thereby generating a lipid film. The film was then dissolved with 100 μ l of buffer containing 150 mM KCl, 20 mM HEPES/KOH, pH 7.4, and 5% sodium cholate. A size-exclusion column was applied to remove detergent (Sephadex G50 in 150 mM KCl and 20 mM HEPES, pH 7.4). Liposomes were collected as eluted ($\sim$ 400 μ l); note that liposomes were easily detected by UV because of dansyl-PE.

Assay for peptide–liposome binding

Binding of peptide to liposomes was monitored using fluorescence resonance energy transfer (FRET) measurements in which dansyl-PE incorporated in liposomes quenches the fluorescence of tryptophan in the peptide (Nalefski and Falke, 2002). All measurements were performed in a Fluoromax (HORIBA Scientific) and performed at 37°C in 1 ml of buffer containing 150 mM KCl and 20 mM HEPES-KOH, pH 7.4. The peptide (750 nM) contains one tryptophan residue. Tryptophan was excited at 280 nm (slit width of 5 nm), and emission spectra were recorded from 320 to 420 nm (slit width of 5 nm) with the peak at 355 nm. FRET was normalized as F_0/F , where F_0 and F represent the fluorescence intensity at 355 nm before and after liposome addition, respectively. Peptide–liposome interaction increases FRET (F_0/F) as a result of tryptophan quenching.

Patch-clamp recording

Whole-cell Ba²⁺ currents or Ca²⁺ currents were recorded at room temperature (20–24°C) using an EPC-10 amplifier with pulse software (HEKA). Electrodes were pulled from glass micropipette capillary (Sutter Instrument) to yield pipettes with resistances of 2–2.5 M Ω . Series-resistance errors were compensated at >60%, and fast and slow capacitance were compensated before the applied test-pulse sequences. Voltage-clamp records were acquired

at 10 kHz and filtered at 3 kHz. For all recordings, cells were held at -80 mV. All data presented here are leak and capacitance subtracted before analysis. The external Ringer's solution contained 150 mM NaCl, 10 mM BaCl₂ or CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 8 mM glucose; pH was adjusted to 7.4 with NaOH. The internal solution of the pipette consisted of 175 mM CsCl, 5 mM MgCl₂, 5 mM HEPES, 0.1 mM 1,2-bis(2-aminophenoxy) ethane *N,N,N',N'*-tetraacetic acid (BAPTA), 3 mM Na₂ATP, and 0.1 mM Na₃GTP; the pH was adjusted to 7.5 with CsOH. Reagents were obtained as follows: CsOH, BAPTA, Na₂ATP, and Na₃GTP (Sigma-Aldrich), and other chemicals (MERCK).

Curve fitting

Voltage dependence of steady-state inactivation was fitted by the following equation:

$$I/I_{\max} = R + (1 - R) / (1 + \exp((V - V_{50,\text{inact}})/K)),$$

where I is the Cav2.2 current amplitude measured during the test pulse at 10 mV (at -10 mV for Cav1.3 channel) for conditioning depolarizations varying from -70 to 70 mV, $I_{\max}$ is the maximal current, V is the conditioning depolarization, $V_{50,\text{inact}}$ is the half-maximal voltage for current inactivation, R is the proportion of non-inactivating current, and K is a slope factor.

Confocal imaging

TsA201 cells were imaged 24–48 h after transfection on poly-L-lysine-coated coverslips with a confocal microscope (LSM 700; Carl Zeiss). Cell images were scanned by using a 40 $\times$ (water) apochromatic objective lens at 1,024 $\times$ 1,024 pixels using digital zoom. For time courses, 524 $\times$ 524 pixels were used. During time course experiments, images were taken every 5 s in imaging software (ZEN; Carl Zeiss). Analysis of line scanning and quantitative analysis of the cytosolic or the plasma membrane fluorescence of target proteins were performed using the “profile” and the “measure” tools, respectively, in ZEN 2012 lite imaging software for region of interest (Carl Zeiss). All images were stored in LSM4 format, which is like JPEG, and raw data from time course was processed with Excel 2012 (Microsoft) and summarized in Igor Pro (WaveMetrics). For β -subunit localization analysis, the colocalization coefficient module in ZEN software was used for quantifying the colocalization between the various β subunits labeled with GFP and the PIP₂ probe PH-PLC δ -RFP (PH-RFP) as a marker of the plasma membrane. Threshold intensities for each fluorescent protein were determined using the plasma membrane region of the images, and the overlap coefficient in the ZEN software was used as an indicator of colocalization coefficient.

Chemicals

2-Bromopalmitic acid and 2-hydroxymyristic acid were prepared as 100-mM stock solutions in DMSO and stored at -20°C . To make working concentrations of 100 μ M, working solutions were prepared by diluting the stock at 1:1,000 with Ringer's solution. Oxo-tremorine-M (Oxo-M) was dissolved in sterile water to make a 10-mM stock solution and was diluted further at 1:1,000 in Ringer's solution for a working solution. Rapamycin was dissolved in DMSO to make a 1-mM stock solution and was diluted at 1:1,000 in Ringer's solution. All chemicals were purchased from Sigma-Aldrich.

RESULTS

The β 2e subunit is localized on the plasma membrane and is widely expressed

Without α 1 subunits to bind to, most β subunits are cytosolic proteins, whereas because of palmitoylation of

two cysteines, the $\beta 2a$ subunit expresses at the plasma membrane (Fig. 1, A and B). Similarly, as shown in Fig. 1 (B and C), the $\beta 2e$ subunit shows a membrane distribution and slows inactivation of $\text{Ca}_v2.2$ channels whether with 10 mM Ba^{2+} or 10 mM Ca^{2+} in the Ringer's solution. In the Ca^{2+} Ringer's solution, inactivation of $\text{Ca}_v2.2$ current is faster with all types of β subunits than in Ba^{2+} (Fig. 1 B, bottom), probably through Ca^{2+} -dependent inactivation via interaction of its C terminus with calmodulin (Liang et al., 2003; Wykes et al., 2007). Even in the presence of Ca^{2+} ions, a slower inactivation is detected in channels with $\beta 2a$ and $\beta 2e$ subunits (Fig. 1 C, bottom). We then investigated relative mRNA expression profiles of $\beta 2e$ subunits in different mouse brain tissues. Previous studies showed that all β -subunit isoforms are expressed in the central nervous system (Ludwig et al., 1997; Dolphin, 2003; Schlick et al., 2010). Using qRT-PCR, we observed that like the $\beta 2a$ subunit, the $\beta 2e$ subunit is expressed in cortex, cerebellum, hippocampus, and hypothalamus (Fig. 1 D). Notably, in cerebellum, the $\beta 2e$ subunit was expressed at a fourfold higher level compared with the $\beta 2a$ subunit, whereas $\beta 2a$ is highly expressed in the rest of brain tissues (Fig. 1 E). The results

indicate that the $\beta 2e$ subunit is localized at the plasma membrane in the presence or absence of $\alpha 1$ subunits and is expressed widely in the brain.

Membrane targeting of $\beta 2e$ subunit is independent from lipid modification

Several previous studies showed that lipidation of the $\beta 2a$ subunit can be blocked by inhibitors such as 2-bromopalmitate (2BP) and tunicamycin, leading to a cytosolic distribution of the protein and restoring fast inactivation to the channels (Hurley et al., 2000; Webb et al., 2000). In addition to palmitoylation, myristoylation can target peripheral proteins to membranes. Accordingly, we began by testing whether lipidation accounts for membrane localization of the $\beta 2e$ subunit using two types of inhibitors: 2BP for palmitoylation and 2-hydroxymyristate (2-HM) for myristoylation. As a positive control, we used a chimeric Lyn- $\beta 3$ -YFP construct known to be localized on the plasma membrane through both palmitoylation and myristoylation (Inoue et al., 2005). The confocal images for this Lyn control show that in the absence of $\alpha 1$, treatment with either lipidation inhibitor or both inhibitors together induces a cytosolic distribution

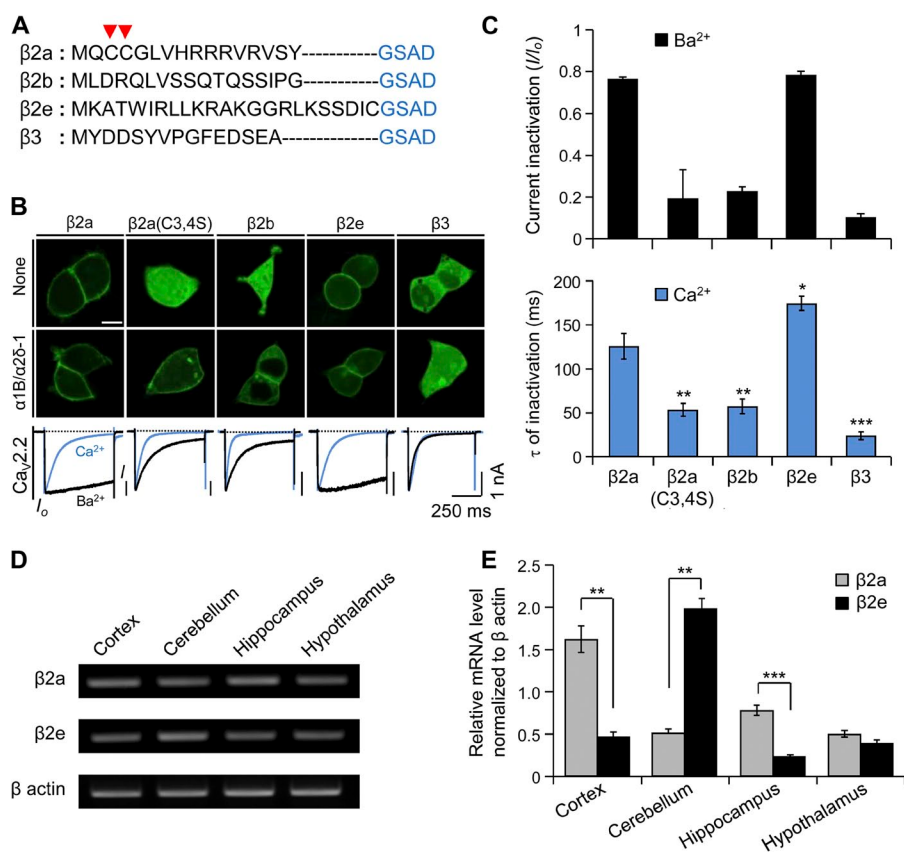


Figure 1. Subcellular localization of different β subunits in tsA201 cells and mRNA expression of $\beta 2a$ and $\beta 2e$ subunits in brain tissues. (A) Amino acid multi-alignment of the N-terminal region of various β subunits. Arrowheads indicate two cysteine residues responsible for the palmitoylation and membrane binding of the $\beta 2a$ subunit. (B) Confocal images showing subcellular distribution of β subunits in the absence (top) or presence (middle) of $\alpha 1B$ and $\alpha 2\delta 1$ subunits in tsA 201 cells (middle). Current inactivation of $\text{Ca}_v2.2$ channels with different β subunits (bottom) was recorded during a 500-ms test pulse at 10 mV. Blue and black curves indicate Ca^{2+} currents and Ba^{2+} currents, respectively. Scale bar, 10 μm . (C) Summary of current inactivation of Ba^{2+} currents (top) and time constant (τ) for current inactivation of Ca^{2+} currents (bottom). Current inactivation (I/I_0) was calculated as current amplitude (I) divided by initial peak amplitude (I_0). For Ba^{2+} currents, $\beta 2a$ ($n = 5$), $\beta 2a(\text{C3,4S})$ ($n = 5$), $\beta 2b$ ($n = 5$), $\beta 2e$ ($n = 6$), or $\beta 3$ ($n = 6$); for Ca^{2+} currents, $\beta 2a$ ($n = 4$), $\beta 2a(\text{C3,4S})$ ($n = 6$), $\beta 2b$ ($n = 5$), $\beta 2e$ ($n = 5$), or $\beta 3$ ($n = 5$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with $\beta 2a$. (D) mRNA expression of $\beta 2e$ subunit

in cortex, cerebellum, hippocampus, and hypothalamus. mRNA expression of β actin was used as a positive control. (E) qRT-PCR mRNA expression profile for $\beta 2e$ and $\beta 2e$ subunits in cortex ($n = 3$), cerebellum ($n = 3$), hippocampus ($n = 3$), and hypothalamus ($n = 3$). mRNA expression levels of $\beta 2a$ and $\beta 2e$ subunits were normalized to the mRNA level of β actin. $n = 3$. **, $P < 0.01$; ***, $P < 0.001$, compared with $\beta 2a$. Data are mean $\pm$ SEM.

of Lyn- β 3-YFP, as predicted (Fig. 2 A). To quantify the localization of β subunits in the plasma membrane, we sampled cells expressing Lyn- β 3-YFP and the membrane PIP₂ probe PH-RFP in a blinded manner and calculated colocalization coefficients. The analysis shows that colocalization of two proteins decreases slightly in cells treated with 2BP or 2-HM and more in cells treated with both inhibitors (Fig. 2 C), confirming that Lyn targeting to the plasma membrane uses both myristoylation and palmitoylation. The β 2a subunit was localized to the cytoplasm only when 2BP was added (Fig. 2, B and C). Additionally, inactivation of Ca_v channels was dramatically accelerated in cells treated with 2BP or both inhibitors (Fig. 2, D and E, top panels). On the other hand, with the β 2e subunit, no combination of inhibitors had any effect on the subcellular distribution or the current inactivation (Fig. 2, B–E, bottom panels of D and E). These results suggest that unlike the membrane localization of the β 2a subunit, membrane targeting of the β 2e subunit does not depend on lipid modification.

The N-terminal 23 amino acids determine the plasma membrane localization of the β 2e subunit

As the β 2 subunit splice variants differ only in their N-terminal region (Fig. 1 A), we examined whether the N terminus of the β 2e subunit is a major determinant for

membrane localization. We engineered a chimeric construct. The 23 N-terminal residues (MKATWIRLLKRAKG-GRLLKSSDIC) of the β 2e subunit replaced the N terminus of the cytosolic β 3 subunit (β 2e/3). In the absence of α 1, the chimeric constructs now expressed, in part, on the plasma membrane. However, they were also present in the cytoplasm and nucleus (Fig. 3 A). This incomplete targeting led us to consider that other domains of β 2e also contribute to the membrane localization. Localization analysis indicated that the colocalization coefficient of β 2e/3 subunit is intermediate between that of β 3 and β 2e subunits and significantly different from either (Fig. 3 B). Furthermore, the chimeric β 2e/3 subunit attenuated inactivation of Ca_v2.2 current about fourfold compared with the cytosolic β 3 subunit (Fig. 3, C and D). It has been reported previously that membrane anchoring of β subunits shifts the voltage dependence of inactivation to more depolarized voltages, whereas cytosolic β subunits shift to more hyperpolarized voltages (Buraei and Yang, 2010). Indeed, our results show that the midpoints ($V_{50,inact}$) of steady-state inactivation with β 3 and β 2e subunits are -15.9 ± 0.5 mV and 5.1 ± 0.5 mV, respectively, and $V_{50,inact}$ of chimeric β 2e/3 subunit is -7.7 ± 0.6 mV (Fig. 3 E).

In addition to the inactivation, β subunits regulate the PIP₂ sensitivity of Ca_v channels (Suh et al., 2012).

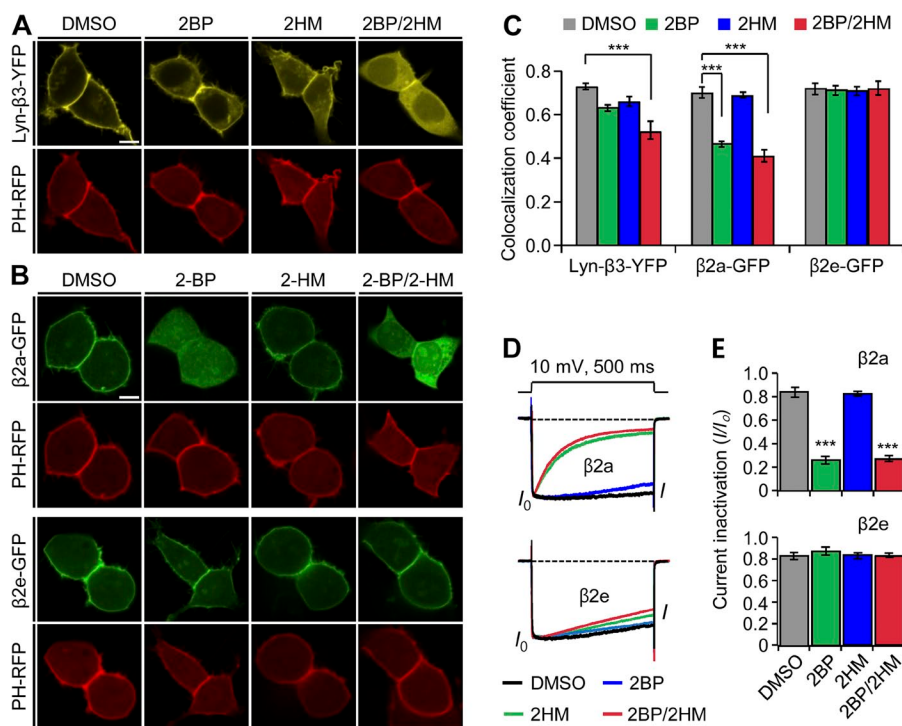


Figure 2. The N-terminal 23 amino acids determine the plasma membrane localization of the β 2e subunit. (A) Confocal images of Lyn- β 3-YFP-expressed cells. The PIP₂ probe PH-PLC δ -RFP (PH-RFP) was cotransfected as a marker of the plasma membrane. Lyn- β 3-YFP-expressed cells were preincubated with inhibitors for 12 h after transfection. Confocal images were taken by confocal microscope in the presence of vehicle (0.2% DMSO); a palmitoylation inhibitor, 2-BH (100 μ M); a myristoylation inhibitor, 2-HM (100 μ M); or both 2-BH and 2-HM. Bar, 5 μ m. (B) Effect of lipidation inhibitors on subcellular localization of β 2a (top) and β 2e subunits (bottom). Cells expressing β 2e subunit or β 2e subunit were preincubated in the absence or presence of 100 μ M 2-BH, 100 μ M 2-HM, or both and were imaged by confocal microscope 24 h after transfection. Note that the β 2e location was not affected by the inhibitors. Bar, 5 μ m. (C) Quantification of membrane localization of Lyn- β 3 ($n = 8$), β 2a ($n = 10$), and β 2e ($n = 10$) subunits compared with

PH-RFP. The colocalization coefficients were obtained from merged images of Lyn- β 3 and PH-RFP, β 2a and PH-RFP, or β 2e and PH-RFP, as shown in A and B. ***, $P < 0.001$, compared with DMSO. (D) Effect of lipidation inhibitors on current inactivation of Ca_v2.2 channels with β 2a (top) or β 2e subunits (bottom). Currents were measured during 500-ms test pulses to 10 mV. (E) Summary of current inactivation with β 2a (top) and β 2e (bottom) subunits. $n = 4$ –6 for β 2a; $n = 4$ –5 for β 2e. ***, $P < 0.001$, compared with DMSO. Data are mean $\pm$ SEM.

To investigate lipid modulation, we depleted PIP_2 using the voltage-sensitive lipid phosphatase Dr-VSP, which can convert PIP_2 to phosphatidylinositol 4-phosphate (PI(4)P) during depolarizing voltage pulses (Yeung et al., 2008; Suh et al., 2010). We asked whether the membrane-targeted chimeric $\beta 2\text{e}/3$ subunit reduces the sensitivity to membrane PIP_2 depletion. With Dr-VSP, PIP_2 depletion by a 120-mV depolarizing pulse inhibited $\text{Ca}_v2.2$ current with the chimeric $\beta 2\text{e}/3$ subunit by $24 \pm 3\%$, less than with the cytosolic $\beta 3$ subunit ($43 \pm 3\%$), but more than with $\beta 2\text{e}$ subunit ($10 \pm 1\%$; Fig. 3, F and G). This result suggests that the N-terminal region of $\beta 2\text{e}$ determines not only membrane targeting and slow inactivation but also low PIP_2 sensitivity. Such data are consistent with our previous finding that localization of the β subunit is a key determinant both for the inactivation kinetics of Ca_v channels and for regulation by PIP_2 depletion (Chan et al., 2007).

Phospholipids determine the binding affinity of peptides to liposomes

Neutralization of positive residues in the N terminus by mutagenesis relocated $\beta 2\text{e}$ subunits to the cytoplasm. To further investigate the mechanism for binding to the plasma membrane, we used a peptide-to-liposome

FRET assay. This FRET approach measures the change of the peptide Trp fluorescence associated with proximity to liposome membranes. In the experiment, a 23-residue peptide of the N terminus of the $\beta 2\text{e}$ subunit (MKATWIRLLKRAKGGRLKSSDIC) was synthesized. Trp (W) served as the energy donor, and dansyl-phosphatidylethanolamine (dansyl-PE) was the energy acceptor. Quenching of Trp fluorescence was monitored using excitation (λ_{ex}) at 284 nm and emission (λ_{em}) at 355 nm (Fig. 4 A). We first measured FRET with liposomes lacking anionic phospholipids (no PIP_2). When liposomes were added to the peptide, the intensity of Trp fluorescence showed only a minor attenuation, indicating at best a weak hydrophobic peptide–liposome interaction (Fig. 4 B). However, when 1% PIP_2 was present in the liposome membranes, there was a marked decrease of Trp fluorescence, indicating significant binding of N-terminal peptides to these liposomes. When 1% PIP_2 and 15% PS were present in the liposome membranes, there was an additional reduction of Trp fluorescence, confirming that anionic phospholipids increase the binding of N-terminal peptides to liposome membranes. (Fig. 4, B and C). These results suggest that PIP_2 as well as PS mediate the membrane binding of the $\beta 2\text{e}$ subunit to the plasma membrane.

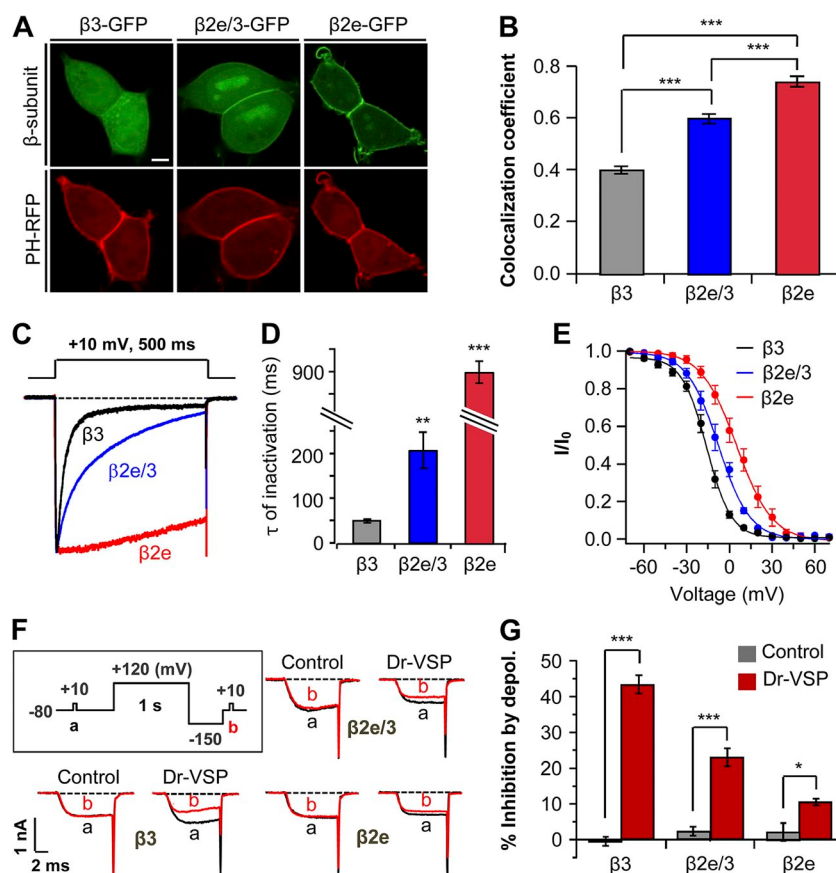


Figure 3. Subcellular localization of β subunits affects inactivation and PIP_2 sensitivity of Ca_v channels. (A) Confocal images of cells expressing $\beta 3$ -GFP, chimeric $\beta 2\text{e}/3$ -GFP ($\beta 3$ subunit containing $\beta 2\text{e}$ N-terminal 23 amino acids), and $\beta 2\text{e}$ -GFP with membrane PIP_2 probe PH-PLC δ -RFP (PH-RFP). Bar, 5 μm . (B) Quantification of plasma membrane localization of $\beta 3$ -GFP, chimeric $\beta 2\text{e}/3$ -GFP, and $\beta 2\text{e}$ -GFP subunits. ***, $P < 0.001$. (C) Effect of membrane-anchored $\beta 2\text{e}/3$ -GFP on the inactivation of $\text{Ca}_v2.2$ currents. Currents were measured during 500-ms test pulses to 10 mV in cells expressing $\text{Ca}_v2.2$ channels with $\beta 3$, $\beta 2\text{e}/3$ -GFP, or $\beta 2\text{e}$ subunits. (D) Summary of time constant (τ) for $\text{Ca}_v2.2$ current inactivation with $\beta 3$ ($n = 4$), $\beta 2\text{e}/3$ ($n = 4$), and $\beta 2\text{e}$ ($n = 5$) subunits. **, $P < 0.01$; ***, $P < 0.001$, compared with $\beta 3$. (E) Voltage dependence of normalized steady-state inactivation for $\text{Ca}_v2.2$ channels with $\beta 3$ ($n = 5$), chimeric $\beta 2\text{e}/3$ ($n = 6$), and $\beta 2\text{e}$ ($n = 6$) subunits. Normalized data were plotted against the conditioning potential. (F) Test protocol for depolarizing pulse and current inhibition of $\text{Ca}_v2.2$ channels with $\beta 3$, $\beta 2\text{e}/3$ and $\beta 2\text{e}$ subunits by Dr-VSP. The currents before (a) and after (b) a depolarizing pulse to 120 mV were superimposed in control and Dr-VSP transfected cells. (G) Summary of $\text{Ca}_v2.2$ current inhibition by membrane depolarization in control and Dr-VSP-expressing cells. For control, $\beta 3$ ($n = 5$), $\beta 2\text{e}/3$ ($n = 4$) or $\beta 2\text{e}$ ($n = 5$); for Dr-VSP, $\beta 3$ ($n = 4$), $\beta 2\text{e}/3$ ($n = 4$) or $\beta 2\text{e}$ ($n = 5$). * $P < 0.05$, *** $P < 0.001$, compared with control. Data are mean $\pm$ SEM.

Depletion of poly-PIs induces cytosolic translocation of $\beta 2e$ subunits and fast channel inactivation

In recent studies with rapamycin-translocatable enzyme systems, it was reported that membrane recruitment of a lipid 4-phosphatase (Sac), a lipid 5-phosphatase (INPP5E), or pseudojanin (PJ; which has both Sac and INPP5E) can trigger the dissociation of peripheral membrane proteins from the plasma membrane inner surface (Hammond et al., 2012). Thus, PI(4)P and/or PIP₂ function as anchors recruiting proteins to the membrane. We applied these tools to observe whether a direct depletion of poly-PIs can cause the dissociation of the $\beta 2e$ subunit from the cell membrane. For convenience, we will call these translocatable phosphatases RF-Sac, RF-INPP5E, and RF-PJ. We first verified the PI specificity of each translocatable enzyme and lipid probe (Fig. 5 A). As expected, recruitment of RF-Sac and RF-INPP5E to the plasma membrane by rapamycin evoked dissociation of the PI(4)P probe Osh1-GFP and of the PIP₂ probe PH-PLC δ -GFP from the membrane, respectively (Fig. 5 B, top). It was proposed previously that Osh1 can also bind to PIP₂ as well as to PI(4)P (Roy and Levine, 2004). Indeed, membrane recruitment of RF-INPP5E also caused a slight movement of Osh1-GFP to the cytosol, whereas Sac recruitment did not affect the PH-PLC δ -GFP (Fig. 5 B, bottom). Using these constructs, we examined whether lipid depletion can change the binding properties of $\beta 2e$ to the membrane in the absence of $\alpha 1$ subunits. Fig. 5 C shows confocal images of $\beta 2e$ -GFP and RF-enzymes before and after a 2-min application of 1 μ M rapamycin. The results show that membrane translocation of either RF-INPP5E or RF-Sac alone is not enough to trigger the dissociation of $\beta 2e$ subunits from the plasma membrane (Fig. 5 C, top). However, when the RF-PJ was recruited to the membrane, it evoked a strong movement of $\beta 2e$ to the cytosol (Fig. 5, C, bottom, and D), suggesting that $\beta 2e$ subunits use both PI(4)P and PIP₂ as plasma membrane anchors.

In addition to measuring the cytosolic fluorescence intensity of $\beta 2e$ subunits, we also analyzed the plasma membrane distribution of $\beta 2e$ before and after rapamycin application. Fig. 6 A shows that recruiting PJ to the plasma membrane by rapamycin application simultaneously induces a robust increase of fluorescence intensity of RFP-PJ at the plasma membrane and a decrease of fluorescence of $\beta 2e$ -GFP. Indeed, only the PJ but not the other translocatable enzymes decreased the presence of $\beta 2e$ at the plasma membrane (Fig. 6 B). Next, we tested the effects of poly-PI depletion on regulation of Ca_v2.2 channels coexpressed with the $\beta 2e$ subunit. As shown in Fig. 6 C is that depletion of PI(4)P or PIP₂ by RF-INPP5E and RF-Sac systems had no significant effect on Ca_v2.2 current inactivation. In contrast, depletion of both lipids by the translocatable PJ system resulted in a stronger and faster current inactivation (Fig. 6, C and D).

With the inactive enzyme PJ-Dead, $V_{50,inact}$ of steady-state inactivation of Ca_v2.2 channel was 7.1 ± 0.4 mV. Depletion

of both PI(4)P and PIP₂ by PJ shifted the voltage dependence of inactivation to more negative voltages ($V_{50,inact} = -7.8 \pm 0.3$ mV), and depletion of PI(4)P or PIP₂ shifted somewhat less ($V_{50,inact} = 1.8 \pm 0.3$ mV and -0.5 ± 0.4 mV, respectively; Fig. 6 E). We also tested the effects of depleting poly-PI on gating of Ca_v1.3 channels. Only PJ accelerated the inactivation of Ca_v1.3 channel significantly (Fig. 6, F and G). Like the voltage dependence of inactivation for the Ca_v2.2 channel, depletion of both PI(4)P and PIP₂ caused the voltage dependence of inactivation to be more negative ($V_{50,inact} = -14.1 \pm 0.7$ mV) compared with that of Dead ($V_{50,inact} = -4.8 \pm 0.5$ mV). The depletion of PI(4)P ($V_{50,inact} = -8.7 \pm 0.9$ mV) or PIP₂ ($V_{50,inact} = -7.7 \pm 0.7$ mV) was intermediate (Fig. 6 H). These results suggest that PI(4)P and PIP₂ together determine current inactivation of Ca_v2.2 and Ca_v1.3 channels with the $\beta 2e$ subunit.

M₁ receptor activation results in fast inactivation of Ca_v current by eliciting transient and reversible translocation of $\beta 2e$ subunit from membrane to cytosol
Activation of G_qPCRs including M₁Rs or AT1a angiotensin receptors depletes both PI(4)P and PIP₂ in the

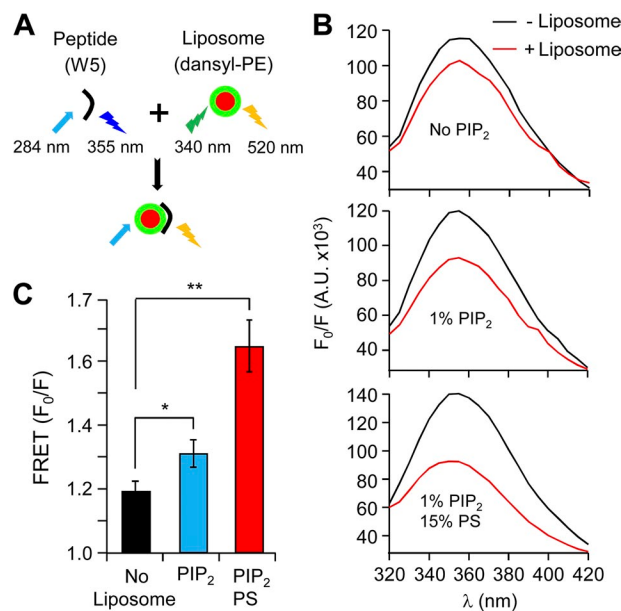


Figure 4. Binding of the N-terminal peptide of $\beta 2e$ to liposomes is strengthened in the presence of anionic poly-PIs. (A) Schematic diagram of FRET analysis. Binding was examined using FRET between peptide containing Trp (W) (donor) and liposomes labeled with dansyl-PE (acceptor). Fluorescence emitted from Trp is quenched by dansyl-PE, suggesting peptide binding to liposomes. The initial spectrum of Trp was determined in the absence of liposomes (F_0), and the subsequent spectrum was recorded after liposome addition (F). (B) FRET signals in the absence (black trace) or presence (red traces) of liposomes with no-liposome, 1% PIP₂, or both 1% PIP₂ and 15% PS. FRET is presented as F_0/F at 355 nm. A.U., absorbance units. (C) Summary of FRET changes with different lipid compositions on the liposomes. For no-liposome, 1% PIP₂, and both 1% PIP₂ and 15% PS, $n = 3$; *, $P < 0.05$; **, $P < 0.01$, compared with no liposome. Data are mean $\pm$ SEM.

plasma membrane via the activation of PLC β (Horowitz et al., 2005; Balla et al., 2008). Based on Fig. 4, we examined whether M₁R activation has any effect on localization of the β 2e subunit in the absence of α 1 subunits. When

the M₁R is activated by Oxo-M, the PH-PLC δ -RFP probe but not the lipidated β 2a subunit migrated to the cytosol (Fig. 7 A). Hence, even when PIP₂ and PI(4)P are depleted by M₁R, the β 2a subunit stays in the membrane

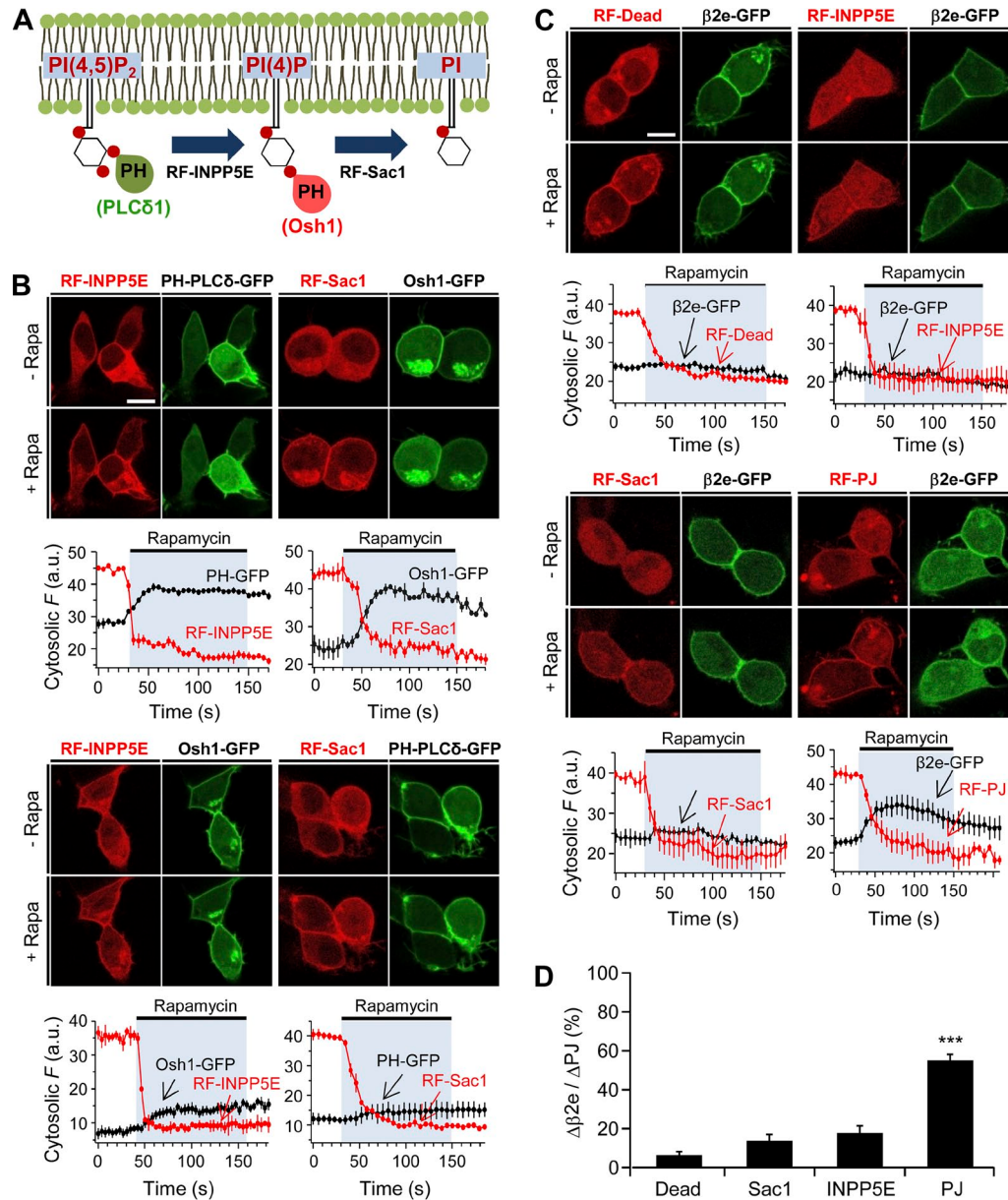
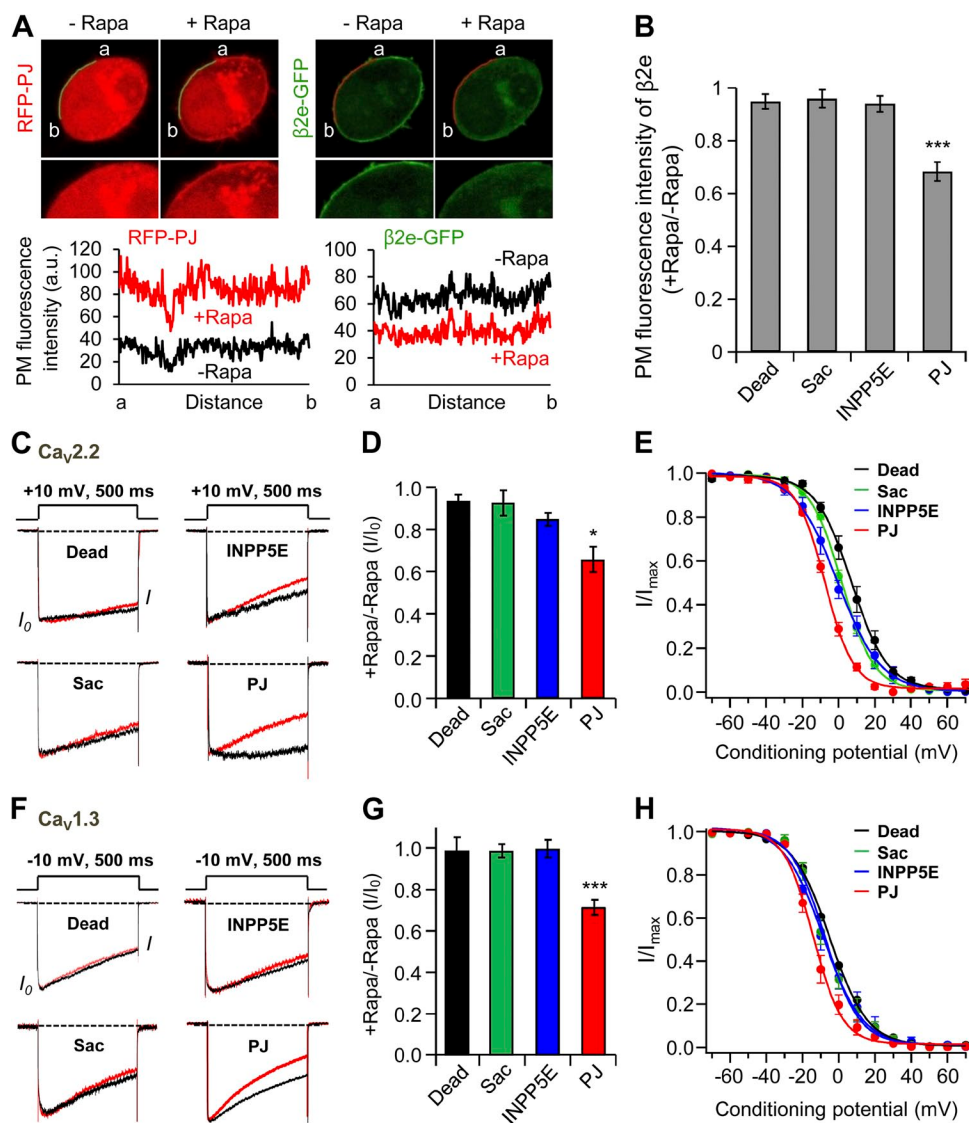


Figure 5. Depletion of PI(4)P and PIP₂ induces the dissociation of β 2e subunit from the plasma membrane. (A) Schematic diagram of a depletion of PI(4)P or PIP₂ by 4- or 5-phosphatases. (B) Confocal images and full time courses of cytosolic fluorescence change in cells transfected with LDR, PH-PLC δ -GFP, Osh1-GFP, RF-INPP5E, and RF-Sac. Translocation of RF-INPP5E or RF-Sac to the plasma membrane by rapamycin depletes PH-PLC δ -GFP (PIP₂ probe) or Osh1-GFP (PI(4)P probe), respectively. For INPP5E, $n = 3$; for Sac, $n = 3$. Bottom images show that plasma membrane translocation of RF-INPP5E or RF-Sac by rapamycin has no effect on depletion of Osh1-GFP or PH-PLC δ -GFP probes, respectively. For INPP5E, $n = 3$; for Sac, $n = 3$. Time courses were taken every 5 s by confocal microscope. Bar, 10 μ m. (C) Confocal images and full time course of cytosolic fluorescence change in cells transfected with LDR, β 2e-GFP, and RF-Dead (RFP-FKBP-dead form of PJ), RF-INPP5E, RF-Sac, and RF-PJ. Confocal images show the subcellular distribution of β 2e-GFP and translocatable RF-phosphatases before and after rapamycin (1 μ M) application for 2 min. For Dead, $n = 4$; for INPP5E, $n = 3$; for Sac, $n = 3$; for PJ, $n = 4$. Bar, 10 μ m. (D) Change in cytosolic intensity of β 2e subunit ($\Delta\beta$ 2e) to cytosolic intensity of each translocatable enzyme (Δ PJs) before and after rapamycin application and expressed as percentage with Dead ($n = 5$), Sac ($n = 5$), INPP5E ($n = 5$), and PJ ($n = 6$). ***, $P < 0.001$, compared with Dead. Data are mean $\pm$ SEM.

through its palmitoylation. However, in $\beta 2e$ -transfected cells, M_1R activation induced strong translocation of both PH-PLC δ -RFP and $\beta 2e$ -GFP simultaneously (Fig. 7 B). We also tested the specificity of G protein-coupled receptors using M_2 -muscarinic receptors (M_2Rs), which are $G_{i/o}$ protein coupled (Fig. 7 C). Activation of M_2R by Oxo-M had no effect on translocation of the $\beta 2a$ subunit. Collectively, these results suggest that membrane binding of the $\beta 2e$ subunit relies on poly-PIs and PS in the plasma membrane, and that PI depletion by activation of M_1R suffices for reversible translocation of the $\beta 2e$ subunit.

Next, we investigated the effect of the $\beta 2e$ subunit on the modulation of $Ca_v2.2$ channel gating by M_1R . Previous

studies showed that muscarinic stimulation inhibits current of $Ca_v2.2$ channels in a β subunit-dependent manner (Heneghan et al., 2009; Suh et al., 2012; Keum et al., 2014). As shown in Fig. 8 A, membrane-anchored $\beta 2a$ and $\beta 2e$ subunits show similar weak muscarinic suppression of the currents ($34 \pm 2\%$ vs. $32 \pm 4\%$ for $\beta 2a$ and $\beta 2e$, respectively), whereas two cytosolic $\beta 3$ and N terminus-deleted (N-del) subunits show a stronger muscarinic suppression ($69 \pm 4\%$ vs. $59 \pm 3\%$ for $\beta 3$ and N-del, respectively; Fig. 8, A and C). Finally, we tested the effect of M_1R on inactivation of Ca_v channels. Oxo-M treatment potentiated inactivation of $Ca_v2.2$ current in cells with the $\beta 2e$ subunit somewhat, whereas Oxo-M treatment had no significant



($n = 4-5$). *, $P < 0.05$, compared with Dead. (E) Voltage dependence of normalized steady-state inactivation for $Ca_v2.2$ channels with Dead ($n = 4$), Sac ($n = 4$), INPP5E ($n = 5$), or PJ ($n = 5$) after rapamycin addition. (F) Effect of various translocatable systems on inactivation of $Ca_v1.3$ channels. $Ca_v1.3$ currents were measured during a 500-ms test pulse to -10 mV. (G) Summary of inactivation of $Ca_v1.3$ currents before and after rapamycin addition in cells expressing different translocatable dimerization constructs ($n = 4-5$). ***, $P < 0.001$, compared with Dead. (H) Voltage dependence of normalized steady-state inactivation for $Ca_v1.3$ channels with Dead ($n = 4$), Sac ($n = 5$), INPP5E ($n = 5$), or PJ ($n = 5$) after rapamycin addition. Data are mean $\pm$ SEM.

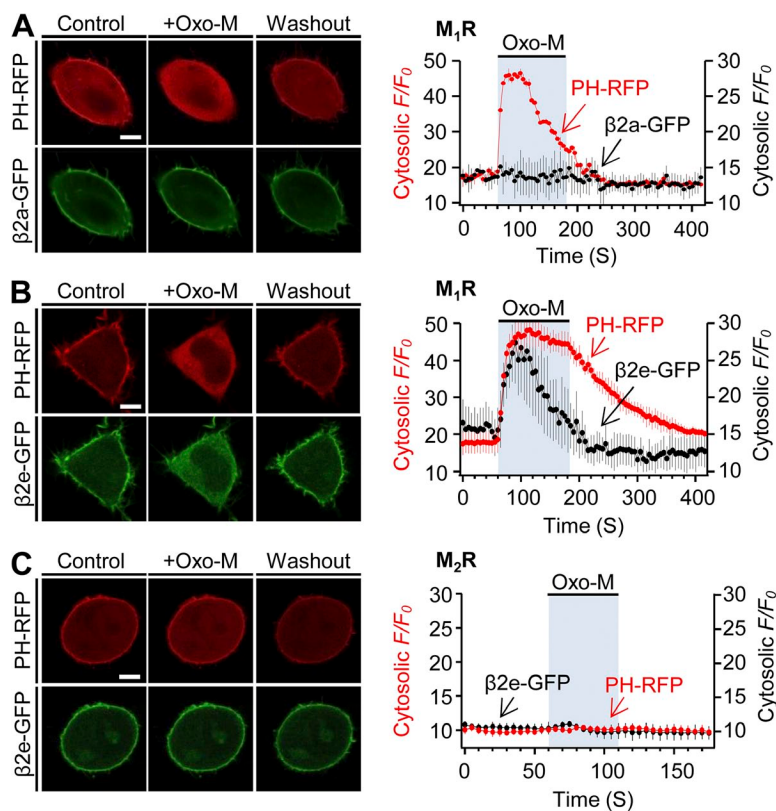


Figure 7. Activation of M₁R induces reversible translocation of the $\beta 2e$ subunit. Confocal images and full time courses of PH-PLC δ -RFP (PH-RFP) and $\beta 2a$ -GFP (A) or PH-RFP and $\beta 2e$ -GFP (B) before, during, and after Oxo-M (10 μ M) application in M₁R-expressing cells. Time courses were taken every 5 s by confocal microscope. (C) Subcellular distributions and a full time course of PH-RFP and $\beta 2e$ -GFP in M₂R-expressing cells. For analysis of time course, $n = 4$ –5. Scale bars, 10 μ m.

effect on current inactivation in $\beta 2a$, $\beta 3$, and N-del subunits expressing cells (Fig. 8 B), consistent with an M₁R-induced translocation of $\beta 2e$ to the cytosol (Fig. 8,

B and D). Collectively, these results suggest that the subcellular redistribution of $\beta 2e$ subunits when G_qPCRs deplete PI(4)P and PIP₂ changes the gating of Ca_v channels.

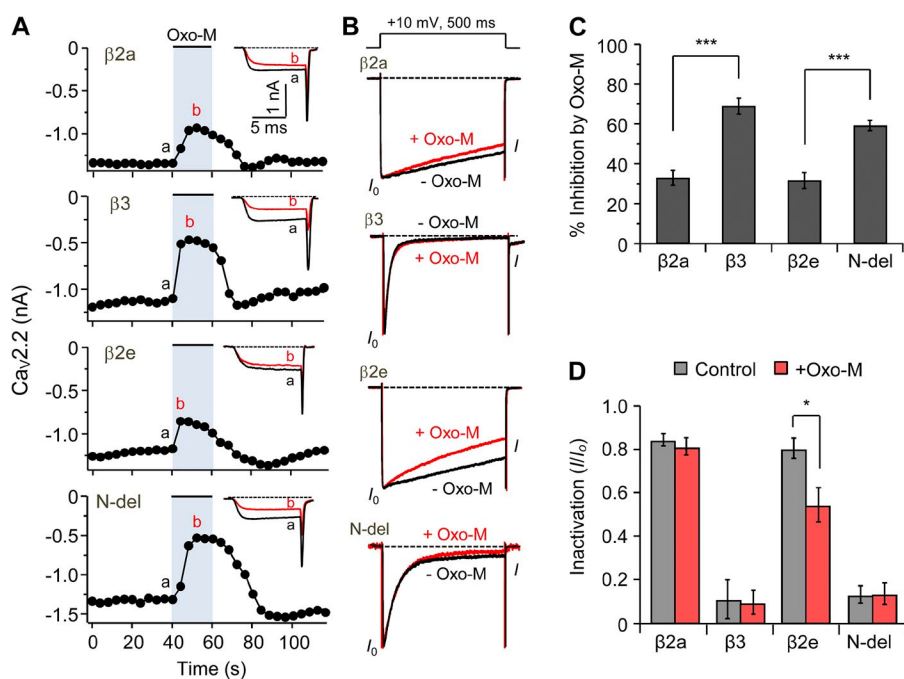


Figure 8. M₁ muscarinic stimulation induces fast inactivation of Ca_v2.2 current by eliciting transient and reversible translocation of the $\beta 2e$ subunit from membrane to cytosol. (A) Current inhibition of Ca_v2.2 channels by M₁R activation with Oxo-M and current inactivation of Ca_v2.2 channels before Oxo-M and during Oxo-M addition in cells expressing $\beta 2a$, $\beta 3$, $\beta 2e$, or N-del. Current traces before (a) and during (b) Oxo-M addition were superimposed (inset). (B) Current inactivation with various β subunits was recorded during a 500-ms test pulse at 10 mV. Current traces before (black trace) and during (red trace) Oxo-M applications were scaled to the peak current amplitude (I_0) and were superimposed. Dashed line indicates zero currents. (C) Summary of Ca_v2.2 current inhibition by M₁R stimulation in cells expressing $\beta 2a$ ($n = 4$), $\beta 3$ ($n = 6$), $\beta 2e$ ($n = 8$), or N-del ($n = 9$). ***, $P < 0.001$, compared with $\beta 2a$ and $\beta 2e$, respectively. (D) Summary of current inactivation before and during Oxo-M application in cells expressing $\beta 2a$ ($n = 6$), $\beta 3$ ($n = 5$), $\beta 2e$ ($n = 5$), or N-del ($n = 5$). *, $P < 0.05$, compared with control. Data are mean $\pm$ SEM.

DISCUSSION

In 1996, the palmitoylation and plasma membrane localization of the $\beta 2a$ splice variant of $\beta 2$ subunits were discovered (Chien et al., 1996). Those properties have functional consequences for Ca_v channels, including slowing inactivation kinetics and decreasing regulation by membrane phospholipids (Chien et al., 1998; Qin et al., 1998; Hurley et al., 2000; Heneghan et al., 2009; Suh et al., 2012). Like $\beta 2a$, $\beta 2e$ was found to locate to the plasma membrane and to slow inactivation of Ca_v channels in HEK293 cells (Takahashi et al., 2003). Recently, interaction of the N-terminal basic residues of $\beta 2e$ with membrane PS was reported to play a role in tethering of the $\beta 2e$ subunit (Miranda-Laferte et al., 2014). Those studies suggested electrostatic and hydrophobic interactions with the membrane. We anticipated that the interactions between the $\beta 2e$ subunit and the membrane PI phospholipids might also be responsible for the dynamic regulation of Ca_v channels by G protein-coupled receptors.

Our study focused on the interaction between the $\beta 2e$ subunit and membrane poly-PIs and how this affects Ca_v channel gating and modulation in living cells. We took advantage of three rapamycin-inducible dimerization tools containing lipid phosphatases (Hammond et al., 2012). Binding of the $\beta 2e$ subunit to the membrane could be reversed by depleting poly-PI with the lipid phosphatases or with direct G_q PCR activation. Recruiting either the 4- or 5-phosphatase alone to the membrane had little effect on $\beta 2e$ localization, but recruiting PJ, which combines both phosphatases, released the $\beta 2e$ subunit from the plasma membrane. Thus, we infer that the $\beta 2e$ subunit binds PI(4)P and PIP₂, and that depleting both lipids allows dissociation of the $\beta 2e$ subunit from the membrane. These results are reminiscent of findings that PI(4)P and PIP₂ activate TRPV1 channels with similar potency (Hammond et al., 2012; Lukacs et al., 2013). Membrane PI(4)P can be involved in scaffolding, recruiting peripheral proteins to the membrane, activation of ion channels, and as a precursor for PIP₂ synthesis (Suh and Hille, 2008; Korzeniowski et al., 2009; Balla, 2013). Although membrane PI(4)P works together with PIP₂ to recruit the intact $\beta 2e$ subunit to the plasma membrane, our FRET analysis using only the N-terminal peptide and model liposomes shows that including sufficient PIP₂ suffices for significant interaction between N-terminal peptide and the liposome. Additionally, Miranda-Laferte et al. (2014) showed that including PS augments $\beta 2e$ binding to liposomes compared with neutral (phosphatidylcholine) liposomes. It seems that PI(4)P and PIP₂ play a major role in membrane targeting of the $\beta 2e$ subunit (see also Cho and Stahelin, 2005; Mulgrew-Nesbitt et al., 2006), but other anionic phospholipids can contribute as well—this might be called a nonspecific interaction.

In addition to the rapamycin-translocatable enzymes, we used activation of M₁R, whose well-characterized

pathway results in depletion of PI(4)P and PIP₂ (Horowitz et al., 2005; Balla et al., 2008). The results showed a striking dynamic action on the $\beta 2e$ subunit. As shown in Fig. 7 with M₁R, we found that receptor activation induces translocation of the $\beta 2e$ subunit, thus altering Ca_v channel gating dynamically. Again, this shows that poly-PIs are essential to guarantee membrane localization in the cell. Because we cannot deplete PS acutely, we cannot rule out that PS also contributes an important component of the interaction energy.

From the standpoint of the neuron, all β subunits are expressed in the central and peripheral nervous systems (Vacher et al., 2008; Obermair et al., 2010; Schlick et al., 2010; Frank, 2014) and thus can play pivotal roles in homeostatic synaptic plasticity via regulation of Ca_v channel complexes (Catterall and Few, 2008; Simms and Zamponi, 2014). Although all β subunits associate with $\alpha 1$ subunits and share common features such as membrane trafficking of $\alpha 1$ and regulation of gating properties, the roles of β subunits in synaptic transmission depend on cellular context. Xie et al. (2007) reported a functional difference for β subunits in hippocampal neurons. They showed that $\beta 2a$ and $\beta 4b$ trigger synaptic vesicle depletion (depression) and paired-pulse facilitation, respectively. Because they slow inactivation, these β subunits enhance Ca²⁺ influx into the presynaptic nerve terminal, ultimately leading to change of transmitter release and synaptic plasticity. Ca_v1 channels are predominantly expressed in postsynaptic membranes (Lipscombe et al., 2004) and Ca_v2 channels in presynaptic terminals (Reid et al., 2003). In parallel qRT-PCR analysis with the $\beta 2a$ subunit, the $\beta 2e$ mRNA is expressed in various brain tissues such as cortex, cerebellum, hippocampus, and hypothalamus. Our finding is that depletion of both PI(4)P and PIP₂ by M₁R activation gives rise to reversible translocation of the $\beta 2e$ subunit, leading to fast inactivation. Therefore, given the abundance of expression of the $\beta 2e$ subunit in brain region, dynamic regulation of $\beta 2e$ location and differential regulation of current inactivation in Ca_v channels with $\beta 2a$ and $\beta 2e$ subunits by G_q PCR-mediated PIP₂ depletion ought to contribute a novel regulation of the synapse and of synaptic plasticity.

In conclusion, our findings suggest that plasma membrane poly-PIs contribute to membrane targeting of the $\beta 2e$ subunit through an electrostatic mechanism. The $\beta 2e$ subunit slows inactivation of Ca_v channels, but when poly-PIs are depleted by M₁R activation, the $\beta 2e$ subunit shifts to the cytosol and channels show more inactivation. Such reversible membrane interaction unique to the $\beta 2e$ subunit invites speculation that dynamic regulation of the $\beta 2e$ subunit by depletion of membrane lipids is another mechanism for regulating function of Ca_v channels. Future work should reveal this regulatory mode in native neurons and investigate the physiological consequence of neuronal Ca_v channels in synaptic plasticity.

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Author contributions: B.-C. Suh designed the research; D.-I. Kim, Y. Park, and D.-J. Jang performed biochemical and electrophysiological research; D.-I. Kim and B.-C. Suh wrote the paper.

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