Dual Regulation of R-Type CaV2.3 Channels by M1 Muscarinic Receptors

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Voltage-gated Ca\(^{2+}\) (Ca\(_v\)) channels are dynamically modulated by G protein-coupled receptors (GPCRs). The M1 muscarinic receptor stimulation is known to enhance CaV2.3 channel gating through the activation of protein kinase C (PKC). Here, we found that M1 receptors also inhibit CaV2.3 currents when the channels are fully activated by PKC. In whole-cell configuration, the application of phorbol 12-myristate 13-acetate (PMA), a PKC activator, potentiated CaV2.3 currents by ~two-fold. After the PMA-induced potentiation, stimulation of M1 receptors decreased the CaV2.3 currents by 52 ± 8%. We examined whether the depletion of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P\(_2\)) is responsible for the muscarinic suppression of CaV2.3 currents by using two methods: the Danio rerio voltage-sensing phosphatase (Dr-VSP) system and the rapamycin-induced translocatable pseudojanin (PJ) system. First, dephosphorylation of PI(4,5)P\(_2\) to phosphatidylinositol 4-phosphate (PI(4)P) by Dr-VSP significantly suppressed CaV2.3 currents, by 53 ± 3%. Next, dephosphorylation of both PI(4)P and PI(4,5)P\(_2\) to PI by PJ translocation further decreased the current by up to 66 ± 3%. The results suggest that CaV2.3 currents are modulated by the M1 receptor in a dual mode—that is, potentiation through the activation of PKC and suppression by the depletion of membrane PI(4,5)P\(_2\). Our results also suggest that there is rapid turnover between PI(4)P and PI(4,5)P\(_2\) in the plasma membrane.

INTRODUCTION

Voltage-gated calcium (Ca\(_v\)) channels are expressed in most excitable cells and facilitate Ca\(^{2+}\) entry in response to membrane depolarization. Among the 10 types of Ca\(_v\) channels, R-type CaV2.3 channels belong to the high voltage-activated (HVA) calcium channel family and are broadly expressed in the brain area, including the hippocampus, amygdala, olfactory bulb, and frontal cortex (Niidome et al., 1992; Soong et al., 1993; Williams et al., 1994). The CaV2.3 channels play important roles in neurotransmitter release, pain transmission, and fear (Lee et al., 2002; Saequsa et al., 2000; Wu et al., 1998). Despite high sequence homology among \(\alpha_1\) subunits of the CaV2 family, CaV2.3 channels have different gating properties and pharmacological characteristics from those of CaV2.1 and CaV2.2 channels. CaV2.3 channels are activated at a lower voltage than other CaV2 channels. In addition, the kinetics for activation and inactivation of CaV2.3 currents are faster than those of CaV2.2 channels. From a pharmacological perspective, CaV2.3 channels are insensitive to CaV2.1 and CaV2.2 channel blockers (Soong et al., 1993; Williams et al., 1994). Another significant difference between CaV2.3 channels and other CaV2 channels is the modulation by G-protein-coupled receptors (GPCRs). As a G\(_\alpha\)-protein-coupled receptor, M1 muscarinic receptor (M1R) activation results in degradation of plasma membrane PI(4,5)P\(_2\). According to previous studies, CaV2.3 channel gating is enhanced by M1R stimulation, probably through the activation of Ca\(^{2+}\)-independent protein kinase C (PKC) (Bannister et al., 2004; Melliti et al., 2000; Tai et al., 2006). On the other hand, CaV2.1 and CaV2.2 channels are known to be suppressed by M1R activation, and this suppression turned out to be owing to G\(\beta\gamma\)-mediated signaling pathways and/or PI(4,5)P\(_2\) depletion (Gamper et al., 2004; Kammereimer et al., 2000; Keum et al., 2014; Melliti et al., 2001; Perez-Burgos et al., 2008; 2010; Shapiro et al., 1999). One previous study reported that CaV2.3 channels were slowly inhibited by G\(_{\alpha}p11\)-coupled neurokinin 1 receptors (Meza et al., 2007). The molecular mechanism of slow inhibition was not identified, but they proposed that the depletion of membrane PI(4,5)P\(_2\) may be involved in the inhibitory pathway.

In this paper, we further investigated whether CaV2.3 channels are sensitive to plasma membrane PI(4,5)P\(_2\) depletion. PI(4,5)P\(_2\) hydrolysis by M1R-mediated phospholipase C (PLC) activation results in the generation of several intracellular secondary molecules, such as inositol 1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol (DAG), as well as the increase of intracellular Ca\(^{2+}\) concentration and PKC activity. Hence, here we employed voltage-sensitive phosphatase from zebrafish (Dr-VSP) and chemically inducible dimerization (CID) systems, which directly and selectively dephosphorylate PI(4,5)P\(_2\) in the plasma membrane without producing any other second messengers. By using these methods, we observed that CaV2.3 channels are modulated by M1R through the modification of the membrane PI(4,5)P\(_2\) level. Together, our data demonstrate that CaV2.3 channels are regulated by M1R through dual modulatory pathways: activation through PKC activation and inhibition through PI(4,5)P\(_2\) depletion.
MATERIALS AND METHODS

Materials
The following cDNAs weregifted to us: rat α1E (accession number NM_019294) from Terrance P. Snutch, University of British Columbia; rat α1B (accession number NM_001195199), J3 (accession number NM_012828), and α2δ1 (accession number NM_012919) from Diane Lipscombe, Brown University, Providence, RI; rat M1-muscarinic receptor (accession number NM_080773) from Neil N. Nathanson, University of Washington, WA; Dr-VSP with EGFP from Yasushi Okamura, Osaka University, Osaka, Japan; Lyn11-FRB, PJ-Dead, PJ-Sac, INPP5E, PJ, and PH-PLCδ-GFP from Bertil Hille, University of Washington School of Medicine, Seattle, Washington.

Cell culture and transfection
Human embryonic kidney cell-derived tsA201 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA), and 0.2% penicillin/streptomycin (Invitrogen, USA) in 100-mm culture dishes. In all experiments, for calcium channel expression, the α1B or α1E of CaV, J3, and α2δ1 sub-units were transiently transfected into tsA201 cells in a 1:1:1 ratio. In some cases, 1 μg of M1R or 1 μg of Dr-VSP was co-transfected. For the rapamycin-inducible dimerization experiment, 200 ng of Lyn11-FRB and 300 ng of translocatable enzymes (PJ-Dead, PJ-Sac, INPP5E, and PJ) were co-transfected. In addition, for the confocal experiment, 200 ng of PH-PLCδ-GFP was co-transfected. The cells were allowed to grow on a 35-mm culture dish and transfection was performed when the confluence of cells reached 60-70%. Lipofectamine 2000 (10 μl; Invitrogen, USA) was added to 250 μl of DMEM and then left for 5 min. DNA was applied with another 250 μl DMEM. Both solutions were mixed and incubated for 15 min in a dark space, then the transfectant mixture was added to cells. After 4 h, fresh culture media containing FBS and antibiotics was exchanged. Transfected cells were plated on the poly-L-lysine-coated (0.1 mg/ml, Sigma-Aldrich, USA) chip 48 h later for the electrophysiological experiment or 24 h later for the confocal experiment after transfection.

Solutions
The bath solution used to record Ba2+ currents contained (in mM) 10 BaCl2, 150 NaCl, 1 MgCl2, 10 HEPES, and 8 glucose (adjusted to pH 7.4 with NaOH). The pipette solution contained (in mM) 175 CsCl, 5 MgCl2, 5 HEPES, 0.1 1,2-bis(2-aminophenyl)ethane N,N,N,N'-tetraacetic acid (BAPTA), 3 Na2 ATP, and 0.1 Na2GTP (adjusted to pH 7.4 with CsOH). The external solution for confocal imaging contained (in mM) 160 NaCl, 2.5 KCl, 2 CaCl2·H2O, 1 MgCl2, 10 HEPES, and 8 glucose (adjusted to pH 7.4 with NaOH). The bath solutions were stored in a refrigerator at 4°C. The pipette solution was stored in a freezer at -20°C. BAPTA, Na2ATP, Na2GTP, CsOH, and BaCl2 reagents were obtained from Sigma-Aldrich (USA). HEPES was from Calbiochem (USA), and other chemicals were obtained from Merck (Germany).

Chemicals
Oxotremorine-M (Oxo-M, Sigma-Aldrich, USA) was dissolved in sterile water to make a 10 μM stock and was stored at -20°C. Both phorbol 12-myristate 13-acetate (PMA, Enzo life sciences, USA) and rapamycin (LC Laboratories, USA) were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) to make 100 μM and 5 mM stocks, respectively. All chemicals were stored at -20°C. They were diluted with bath solution before being applied to cells.

Current recording
All currents were obtained at room temperature (22-25°C). Patch pipettes (1-4 MΩ) were pulled from borosilicate glass micropipette capillaries (1.5 mm outer diameter; 1.1 mm inner diameter; and 10 cm length) (Sutter Instrument Company, USA). The whole-cell configuration was used to record Ba2+ currents. In cell attached mode, a gigahorn seal was formed, and the plasma membrane was ruptured by negative pressure. Series resistance was 3.6-6 MΩ and was compensated by 60%. A HEKA EPC-10 amplifier with pulse software (HEKA Elektronik) was used for current recording. Ba2+ currents were recorded with a membrane holding potential of -80 mV, and a 100-ms test pulse (+10 mV for CaV2.2 channels and 0 mV for CaV2.3 channels) was applied every 4 s.

For Dr-VSP experiments, the following protocol was used. First, test pulse a (+10 mV for CaV2.2 channels and 0 mV for CaV2.3 channels) was applied for 10 ms. This current became the baseline. Then, +120 mV was generated for 1 s to activate Dr-VSP and to deplete PI(4,5)P2. Following the large depolarizing pulse, -150 mV hyperpolarizing pulse was applied for 400 ms to remove calcium channel inactivation. Finally, test pulse b was applied. Currents a and b, before and after PI(4,5)P2 depletion by Dr-VSP activation, were compared to calculate the ratio of current inhibition.

Confocal imaging
Confocal images were obtained with a Carl Zeiss Inverted LSM 700 confocal microscope (Carl Zeiss AG, GFP by argon-ion laser and mRFP by blue diode laser) at room temperature (22-25°C). In time course, images were obtained by scanning cells with a 40× (water) objective lens at 512 × 512 pixels, and were taken every 10 s for 5 min. For the single image, cells were scanned with a 40× (water) objective lens at 1024 × 1024 pixels. Cytosolic fluorescence intensity was measured by using ZEN2010 software (Carl Zeiss) and was processed with Microsoft Office Excel 2010 (Microsoft) or Igor Pro (WaveMetrics, Inc.).

Data analysis
For data acquisition and analysis, a HEKA EPC-10 amplifier (HEKA Elektronik) was used. Additional data processing was accomplished with Igor Pro (WaveMetrics, Inc.) and Microsoft Office Excel 2010 (Microsoft). Time constants for the responses were obtained by fitting the data to a single-exponential function. All quantitative data were expressed as the mean ± standard error of the mean (SEM). Student's t-test was used for comparisons between two groups. One-way ANOVA was used for comparisons between more than two groups.

RESULTS
To record calcium channel currents, tsA201 cells were transfected with α1B for CaV2.2 currents or α1E for CaV2.3 currents plus auxiliary subunits J3 and α2δ1. We used Ba2+ as a charge carrier instead of Ca2+ to rule out calcium-dependent inactivation and other unexpected events triggered by Ca2+ ions (Liang et al., 2003). In all experiments, we measured the channel activity of both CaV2.2 and CaV2.3, where the CaV2.2 current was measured as a control for PI(4,5)P2 regulation because it is known to be inhibited by M1R activation (Kim et al., 2015; Suh et al., 2012). To obtain the peak currents, +10 mV and 0 mV were applied.
Fig. 1. Differential regulation of CaV2.2 and CaV2.3 currents by M1R activation. TsA201 cells co-transfected with M1 muscarinic receptor (M1R) and either CaV2.2 or CaV2.3 channels were treated with 10 μM of Oxo-M for 60 s. (A) Left: Time course of CaV2.2 current regulation. Right: Protocol generating CaV2.2 currents (Upper) and selected current traces designated in left graph (Lower). (B) Left: Time course of CaV2.3 current regulation. Right: Protocol generating CaV2.3 currents (Upper) and selected current traces designated in the left graph (Lower). (C) Summary of % inhibition by Oxo-M treatment in CaV2.2 (n = 13) and CaV2.3 (n = 9) channels. Data are mean ± SEM.

Differential modulation of CaV2.2 and CaV2.3 channels by M, muscarinic receptors

Whereas most HVA calcium channels are known to be inhibited by M1R activation (Melliti et al., 2000; Suh et al., 2010). When tsA201 cells co-transfected with M1R and either CaV2.2 or CaV2.3 channels were treated with muscarinic receptor agonist Oxo-M (10 μM) for 60 s, CaV2.2 (N-type) currents were rapidly decreased by 55 ± 2% (n = 13, Figs. 1A and 1C), while CaV2.3 (R-type) currents were increased by 83 ± 7% (n = 9, Figs. 1B and 1C). These differential results were consistent with those of previous studies (Melliti et al., 2000; Perez-Burgos et al., 2008; Perez-Rosello et al., 2004; Suh et al., 2010).

According to previous studies, phosphorylation of CaV α1 subunits by PKC could activate CaV2.3 channels (Fang et al., 2005; Kamatchi et al., 2003; 2004; Rajagopal et al., 2008; Stea et al., 1995). Based on these studies, we decided to verify the effect of PKC on both CaV2.2 and CaV2.3 currents. The bath solution containing 1 μM phorbol 12-myristate 13-acetate (PMA), which is a DAG analog recruiting PKC to the plasma membrane, was applied to the cells for 120 s. While CaV2.2 currents were not significantly changed by PMA application (Fig. 2A), CaV2.3 currents increased almost two-fold (Fig. 2B). Interestingly, we found that after full activation of CaV2.3 channels by PKC activation, M1R activation decreased the CaV2.3 currents by 52 ± 8% (n = 5), similarly to CaV2.2 currents (47 ± 5%, n = 9) (Fig. 2C). The time constants for Oxo-M-induced inhibition of CaV2.2 currents and CaV2.3 currents were 4 ± 0 s (n = 9) and 12 ± 2 s (n = 5), respectively (Fig. 2D). Collectively, our results showed that after full activation of PKC, CaV2.3 channels were also inhibited by M1R activation, which is very similar to CaV2.2 channels.

CaV2.3 currents are decreased by Dr-VSP activation

Since M1 muscarinic inhibition of voltage-gated calcium channels (VGCCs) is known to be partially due to PI(4,5)P2 depletion through PLCβ enzyme activation (Gamper et al., 2004), we decided to test the effect of PI(4,5)P2 depletion on CaV2.3 channels. Dr-VSP was used to transiently dephosphorylate PI(4,5)P2 in the plasma membrane in response to membrane depolarization and to exclude the effects of the other secondary signaling molecules generated by M1R activation (Okamura et al., 2009; Suh et al., 2010). The protocols used for activating Dr-VSP are represented in Fig. 3A. In tsA201 cells expressing both CaV2.2 channels and Dr-VSP, CaV2.2 currents were decreased by 40 ± 4% (n = 9) after a 1-s depolarizing pulse. In contrast, there was no significant change in the control (−Dr-VSP) (Figs. 3B left and 3C). Similarly, CaV2.3 currents in cells expressing Dr-VSP were decreased by 38 ± 1% (n = 6) in response to PI(4,5)P2 depletion, while the control cells were not (Figs. 3B right and 3D). These results suggest that the depl-
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Fig. 3. PI(4,5)P₂ depletion by Dr-VSP decreases both Ca₂⁺₂ and Ca₂⁺₃ currents. TsA201 cells were co-transfected with Dr-VSP and either Ca₂⁺₂ or Ca₂⁺₃ channels. (A) Standard protocol for Dr-VSP activation. Cells received a test pulse (a), then a depolarization to +120 mV for 1 s to activate the Dr-VSP, a hyperpolarization to -150 mV for 0.4 s to remove the voltage-dependent inactivation, and a second test pulse (b). Ca₂⁺₂ or Ca₂⁺₃ currents were measured before and after the Dr-VSP activation at +10 mV or 0 mV, respectively. (B) Left, Ca₂⁺₂ current regulation by a membrane depolarization to +120 mV for 1 s in control (-Dr-VSP, n = 8) and cells expressing Dr-VSP (n = 6). Right, Ca₂⁺₃ current regulation in control (n = 6) and cells expressing Dr-VSP (n = 6). (C, D) Summary of % inhibition by Dr-VSP-induced PI(4,5)P₂ depletion in Ca₂⁺₂ (C) and Ca₂⁺₃ (D) channels. Data are mean ± SEM. *** P < 0.001, compared with -Dr-VSP.

Ca₂⁺₃ channels are inhibited by rapamycin-inducible pseudokinase systems
To further examine the regulatory effects of membrane PIs on Ca₂⁺₃ currents, we employed the recently developed rapamycin-inducible translocatable PI phosphatase system (Hammond et al., 2012). In the system, the PI phosphatase is conjugated with FK506-binding protein 12 (FKBP), one of the dimerization subunits. The phosphatase-containing subunit can be recruited to the plasma membrane by application of rapamycin to the cells expressing the plasma membrane-targeting LDR subunit. By using this method, we can selectively and irreversibly deplete the specific PIs in the plasma membrane. Three constructs were used to manipulate the plasma membrane PIs: PJ-Sac, INPP5E, and PJ (Fig. 4A). PJ-Sac is 4-phosphatase from S. cerevisiae sac1. This enzyme dephosphorylates PI(3)P, PI(4)P, and PI(3,5)P₂, but not PI(4,5)P₂ (Guo et al., 1999). INPP5E, inositol polyphosphate-5-phosphatase E, is 5-phosphatase, and its substrates are PI(4,5)P₂ and PI(3,4,5)P₃ (Bielas et al., 2009). PJ contains both active PJ-Sac and INPP5E domains; thus, this translocatable enzyme can deplete both PI(4,5)P₂ and PI(4)P by sequentially dephosphorylating 5- and 4-phosphates. Unlike PJ, PJ-Dead is inactive in both phosphatases. Lyn₁₁₁, a plasma membrane-targeting motif (Inoue et al., 2005), is fused with FKBP-rapamycin binding protein (FRB). When rapamycin is added, FKBP and FRB form a ternary complex with rapamycin. Hence, the phosphatase conjugated to FKBP is recruited to the plasma membrane and dephosphorylates its substrates (Fig. 4B).

The movement of translocatable enzymes was monitored using confocal microscopy every 10 s. TsA201 cells were co-transfected with Lyn₁₁₁-FRB and one of the following four translocatable enzymes tagged with mRFP: PJ-Dead, PJ-Sac, INPP5E, or PJ. The cells were also transfected with the pleckstrin homology (PH) domain of PLCδ labeled with GFP (PH-PLCδ-GFP) as a PI(4,5)P₂-specific probe. The PH domain of PLCδ binds to the head group of PI(4,5)P₂ so we can detect plasma membrane PI(4,5)P₂ in live cells.

Cells expressing both PH-PLCδ-GFP (green) and translocatable enzymes (red) are shown in Fig. 4C. At first, PH-PLCδ-GFP is present in the plasma membrane, while the translocatable enzymes, including PJ-Dead, PJ-Sac, INPP5E, and PJ, exist in the cytosol. After the application of 1 μM rapamycin, all the translocatable enzymes were commonly translocated to the plasma membrane. However, the movement of PH-PLCδ-GFP from the plasma membrane to the cytosol was different depending on the translocatable enzymes. In cells co-transfected with PJ-Dead, the cytosolic fluorescence intensity of PH-PLCδ-GFP was almost the same before and after the rapamycin application (± 2%, n = 4) (Figs. 4D and 4E). However, in cells expressing PJ-Sac, PH-PLCδ-GFP was significantly dissociated from plasma membrane and the cytosolic fluorescence intensity was increased by 24 ± 4% (n = 10). When the INPP5E or PJ systems were applied, the increase in cytosolic fluorescence intensity by INPP5E (44 ± 2%, n = 7) and PJ (48 ± 7%, n = 9) was further increased (Figs. 4D and 4E). We also measured time constants (τ) for the translocation of enzymes as well as PH-PLCδ-GFP. As in a previous study by Dickson et al. (2004), we also used time-series images taken every 10 s for resolving the τ value. There was no difference in τ value between translocatable enzymes. However, when the PJ-Sac transfected, the time constant of the rapamycin-induced PI(4,5)P₂ dephosphorylation in cytosolic PH-PLCδ-GFP intensity was 25 ± 3 s (n = 10), relatively slower than those of INPP5E 17 ± 1 s (n = 8) and PJ 15 ± 3 s (n = 9) (Fig. 4F). In summary, our results show that PJ-Sac might be involved in PI(4,5)P₂ depletion, but the rate of PI(4,5)P₂ dephosphorylation by PJ-Sac was slower than those of INPP5E and PJ. Based on these data, it suggests that PJ-Sac dephosphorylates PI(4)P, and dephosphorylation of PI(4)P induces PI(4,5)P₂ depletion.

We then measured the Ca₂⁺₂ and Ca₂⁺₃ current changes when the translocatable enzymes moved to the plasma membrane and dephosphorylated their PI substrates. The tsA201 cells were transfected with Ca₂⁺₂ channel, Lyn₁₁₁-FRB, and one of the following phosphatases: PJ-Dead, PJ-Sac, INPP5E, and PJ. The external solution containing 1 μM of rapamycin was perfused for 60 s. The recruitment of PJ-Dead had no significant effects on the currents (Figs. 5A and 5B). Ca₂⁺₂ currents in cells expressing PJ-Sac were decreased by 39 ± 5% (n = 9)
and the currents expressing INPP5E were decreased by 37 ± 3% (n = 5). When the cells were co-transfected with PJ, the currents were inhibited by 56 ± 4% (n = 11). Because of the irreversibility of rapamycin-induced FKBP-FRB dimerization (Suh et al., 2006), the current amplitudes were not recovered and remained stable even after washout of rapamycin. The inhibition of CaV2.2 currents by the recruitment of PJ-Sac took longer time (29 ± 2 s, n = 9) than that of INPP5E (10 ± 1 s, n = 5) or PJ (7 ± 4 s, n = 11) (Fig. 5C).

We also examined the effects of the translocation of pseudojanin constructs on CaV2.3 channel regulation. The tendency for a decrease in CaV2.3 current was similar to that of CaV2.2 channels. The translocation of PJ-Dead had no significant effect on the CaV2.3 currents (3 ± 5%, n = 3). The membrane recruitment of PJ-Sac decreased the CaV2.3 currents by 37 ± 4% (n = 5), while INPP5E decreased the currents by 53 ± 3% (n = 6). Lastly, PJ induced the strongest decrease in CaV2.3 current (66 ± 3%, n = 7) (Figs. 6A and 6B). The time constant for decreasing the CaV2.3 currents by translocation of PJ-Sac was much slower (39 ± 3 s, n = 5) than that of INPP5E (11 ± 1 s, n = 6) or PJ (9 ± 1 s, n = 7) (Fig. 6C). These results also suggested that CaV2.3 currents were suppressed mostly by depletion of PI(4,5)P2 in the plasma membrane.

**DISCUSSION**

Even though PI(4,5)P2 is known as a crucial regulator of many types of ion channels, including high-voltage activated CaV channels (Hilgemann et al., 2001; Huang, 2007; Rohacs, 2009; Suh and Hille, 2005; 2008), it is not clear whether PI(4,5)P2 in the plasma membrane can regulate CaV2.3 channels. In this study, we showed that CaV2.3 channel can be suppressed by depletion of PI(4,5)P2. This inhibition was proved by direct and selective dephosphorylation of PI(4,5)P2 in the plasma membrane by Dr-VSP (Fig. 3) and rapamycin-induced translocatable (CID) systems (Figs. 5 and 6).

The α1E gene used in our experiments is rbE-II extracted from rat brain (Soong et al., 1993). The amino-terminus of rbE-II is insensitive to voltage-dependent, membrane-delimited inhibition by Gβγ subunits (Page et al., 1998). In our study, we showed that α1E isoform can be regulated by the voltage-independent, PI(4,5)P2-dependent...
pathway. Owing to the sequence homology between mammalian α1E (over 93%) (Williams et al., 1994), we speculate that the modulation pattern by M1R activation might be similar.

As shown in Fig. 1B, when M1R is activated, the CaV2.3 current is increased in two phases; an initial steep increase followed by a slow increase. The data suggest that there are two factors involved in CaV2.3 channel regulation. Previous studies showed that M1R induces an increase in the CaV2.3 current through PKC-mediated phosphorylation. However, in our study, we observed that CaV2.3 is also regulated by PI(4,5)P2 depletion. The reason that the inhibitory effect of PI(4,5)P2 depletion on CaV2.3 currents was hidden might be owing to the stronger effect of PKC on CaV2.3 currents. In other words, the PKC effect seems to be masked by PKC-induced potentiating effects. Why is the PKC effect on CaV2.3 channels stronger than on other types of CaV2 channels? That may be owing to several potential phosphorylation sites in the α1E subunit. As mentioned in the introduction, CaV2.3 channels are potentiated by PKC activation. Previous studies showed that both CaV2.2 and CaV2.3 channels have possible sites for phosphorylation by PMA (Hamid et al., 1999; Zamponi et al., 1997), which are embedded in the II-loop of the α1 subunit (Fan et al., 2005; Kamatchi et al., 2003; 2004). However, CaV2.3 channels have more phosphorylation sites than CaV2.2 channels in the II-III loop. Indeed, the sequences of the II-III loop between CaV2.3 channels and CaV2.1 or CaV2.2 channels show many differences (Soong et al., 1993). Application of acetyl-β-methylcholine (MCh), another PKC activator, induced phosphorylation in the II-III loop and further increased the CaV2.3 currents (Kamatchi et al., 2004; Rajagopal et al., 2008).

According to our results, the inhibition ratio of CaV2.2 and CaV2.3 currents by the translocation of PJ was greater than that of INPP5E (Figs. 5B and 6B), but the time constants of inhibition by INPP5E and PJ were similar (Figs. 5C and 6C). This might be owing to the rapid turnover between PI(4)P and PI(4,5)P2 (Oude Weernink et al., 2004; Wuttke et al., 2010). In the plasma membrane, PI(4,5)P2 is continuously and rapidly

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**Fig. 5.** CaV2.2 currents were suppressed by depletion of PI(4,5)P2. TsA201 cells were co-transfected with CaV2.2 channels, Lyn11-FRB (plasma membrane anchoring protein), and one of the following four constructs: PJ-Dead, PJ-Sac, INPP5E, or PJ. Rapamycin was applied for 60 s. (A) Time courses of CaV2.2 currents in cells expressing PJ-Dead, PJ-Sac, INPP5E, or PJ. (B) Summary graph of % inhibition by rapamycin addition in CaV2.2 currents (n = 6 for PJ-Dead; n = 9 for PJ-Sac; n = 5 for INPP5E; and n = 11 for PJ). (C) Summary graph of the time constant for rapamycin-induced inhibition in CaV2.2 currents (n = 9 for PJ-Sac; n = 5 for INPP5E; and n = 11 for PJ). Data are mean ± SEM. * P < 0.05, ** P < 0.01, and *** P < 0.001, with one-way ANOVA followed by Bonferroni post-hoc test.

**Fig. 6.** CaV2.3 currents were suppressed by depletion of PI(4,5)P2. CaV2.3 channels were expressed in tsA201 cells with Lyn11-FRB and one of the following four constructs: PJ-Dead, PJ-Sac, INPP5E, or PJ. Rapamycin was added for 60 s. (A) Time courses of CaV2.3 currents in cells expressing PJ-Dead, PJ-Sac, INPP5E, or PJ. (B) Summary graph of % inhibition by rapamycin addition in CaV2.3 currents (n = 3 for PJ-Dead; n = 5 for PJ-Sac; n = 6 for INPP5E; and n = 7 for PJ). (C) Summary graph of the time constant for rapamycin-induced inhibition in CaV2.3 currents (n = 5 for PJ-Sac; n = 6 for INPP5E; and n = 7 for PJ). Data are mean ± SEM. * P < 0.05, and *** P < 0.001, with one-way ANOVA followed by Bonferroni post-hoc test.
Regulated by membrane phosphoinositide. PI(4,5)P2 level in the plasma membrane. This study might confirm that depletion of PI(4)P, a precursor of PI(4,5)P2, indirectly affects the CaV2.3 channel activity by slowly decreasing the amount of PI(4)P and PI(4,5)P2, and thus that PI(4,5)P2 is maintained at an almost 1:1 ratio by inositol polyphosphate 5-phosphatases (Kwiatkowska, 2010). Altogether, it seems that PI(4)P depletion by PJ-Sac breaks the balance between the amount of PI(4)P and PI(4,5)P2, and thus that PI(4,5)P2 is dephosphorylated to keep the balance between them.

Another regulator of HVA channels is CaVβ subunits. They regulate the physiological properties and expression levels of HVA channels. They also regulate the channel sensitivity to PI(4,5)P2, where the sensitivity is different depending on the types of CaVβ subunits and their subcellular localization. For example, in cells expressing both CaV2.2 channels and Dr-VSP, currents with β3 subunits were markedly decreased, while currents with β2a subunits showed little effect (Keum et al., 2014; Suh et al., 2012). Therefore, it is meaningful to test the effect of CaVβ subunits on the regulation of CaV2.3 channels by PI(4,5)P2 to better understand the regulation mechanism of CaV2.3 channels.

In summary, our study reports that CaV2.3 channels can be regulated by plasma membrane PI(4,5)P2. Like other types of HVA CaV channels, our data demonstrate that CaV2.3 channels are inhibited by PI(4,5)P2 depletion. The present results also show that depletion of PI(4)P, a precursor of PI(4,5)P2, indirectly affects the CaV2.3 channel activity by slowly decreasing the level in the plasma membrane. This study might contribute to extend our knowledge about regulation of CaV2.3 channels by membrane phosphoinositide.

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