

Original Article

Negative self-regulation of transient receptor potential canonical 4 by the specific interaction with phospholipase C- δ 1

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ABSTRACT Transient receptor potential canonical (TRPC) channels are non-selective calcium-permeable cation channels. It is suggested that TRPC4 β is regulated by phospholipase C (PLC) signaling and is especially maintained by phosphatidylinositol 4,5-bisphosphate (PIP₂). In this study, we present the regulation mechanism of the TRPC4 channel with PIP₂ hydrolysis which is mediated by a channel-bound PLC δ 1 but not by the G_qPCR signaling pathway. Our electrophysiological recordings demonstrate that the Ca²⁺ via an open TRPC4 channel activates PLC δ 1 in the physiological range, and it causes the decrease of current amplitude. The existence of PLC δ 1 accelerated PIP₂ depletion when the channel was activated by an agonist. Interestingly, PLC δ 1 mutants which have lost the ability to regulate PIP₂ level failed to reduce the TRPC4 current amplitude. Our results demonstrate that TRPC4 self-regulates its activity by allowing Ca²⁺ ions into the cell and promoting the PIP₂ hydrolyzing activity of PLC δ 1.

INTRODUCTION

Transient receptor potential (TRP) channels are nonselective cation channels which are permeable to Ca²⁺. Among TRP channels, this canonical family is composed of 7 types, and they are subdivided into two groups based on their amino acid sequence and structure; TRPC1, 4, 5 and TRPC3, 6, 7. Interestingly, TRPC4 is functionally analogous to TRPC5, but only TRPC5 shows constitutive activity even without stimulation. However, the mechanism that makes this difference still remains obscure. The activity of TRPC4 and TRPC5 are closely related to G_q-phospholipase C (PLC) signaling, and many studies also demonstrated the effect of downstream molecules on TRPC channels, which include PLC, phosphatidylinositol 4,5-bisphosphate (PIP₂) [1-4], diacylglycerol (DAG) [5,6], protein kinase C (PKC) [7] and Ca²⁺. We previously emphasized the self-limiting mechanism of G_q pathway

on channels where G_q protein solely activates TRPC1/4 and TRPC1/5 channels, and PIP₂ depletion, PKC or Ca²⁺ inhibit them [8,9]. These data implicate that TRPCs are strongly activated by G_q, hence fast and tight regulation is necessary afterwards.

PLC is an enzyme that cleaves PIP₂ into inositol triphosphate (IP₃) and DAG. There are ten kinds of PLC isozymes that are largely classified into three families; β , γ , δ [10-13]. Among them, primordial PLC δ family has basic structure with the shortest length. The δ subtypes have about 10-fold higher sensitivity to calcium than the other isozyme [14,15]. The precise activation mechanism of PLC δ 1 is not known, but it is expected that the Ca²⁺ concentration mainly controls their activity [16], and IP₃, the product of PIP₂ hydrolysis, is involved in its negative regulation [17].

PIP₂ is the most important component in TRPC channel's regulation as they are concerned with channel maintenance.



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Author contributions: J. Ko designed the study, performed experiments, generated figures, analyzed data, and wrote the manuscript. J. Kim. generated figures and wrote the manuscript. J.M. performed confocal imaging, and M.K. performed Co-IP experiment. I.S. provided the overall experimental advice and coordinated the study. All authors reviewed the manuscript.

Other TRP channels are also known to be regulated by PIP_2 and the effect of PLC δ subtypes on this action has been proposed. PLC $\delta 3$ co-expression facilitated the desensitization of TRPV1 in oocytes [18], and subsequent PIP_2 reductions by PLC δ isoform promoted reduction in TRPM8 currents [19]. Thus, understanding the regulatory mechanism that alters TRPC activity related to the change in PIP_2 levels will provide insights on how channels are tightly regulated by PLC signaling, and how PIP_2 functions differently on TRPC channel functions.

In the present study, we set out to determine an effect of PLC δ on TRPC channel activity, and their working condition in this regulation. We verified that PLC $\delta 1$ directly interacts with TRPC4 but not with TRPC5, and it works as a key component of channel's negative feedback regulation. PLC $\delta 1$ is activated by the calcium *via* open TRPC4, and active PLC $\delta 1$ catalyzes the hydrolysis of PIP_2 . These series of events reduced the current amplitude of TRPC4. We suggest the possibility that the involvement of PLC $\delta 1$ in channel regulation contribute to functional difference between TRPC4 and TRPC5.

METHODS

Cell culture and transfection

Human embryonic kidney (HEK)293 cells (ATCC) were maintained in Dulbecco's Modified Eagle's Medium (HyClone) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin (HyClone) at 37°C in a 5% CO_2 humidified incubator. Cells were seeded in glass bottom dish for imaging, 12-well plate for whole-cell patch clamp recordings, and 6-well plate for Western blot. The following day, transfection was carried out by using the FuGENE 6 Transfection Reagent (Promega) for patch clamp and imaging experiments, and Lipofectamine 2000 (Invitrogen) for western blotting according to the manufacturer's instructions. All experiments were performed 20–30 h after transfection. cDNA construct for mouse TRPC4 (GenBank ID: U50921.1, UniProt ID: Q9QUQ5-2) was kindly donated by Dr. V. Flockerzi and Dr. M. Schaefer, and that for human PLC $\delta 1$ (GenBank ID: U09117.1, UniProt ID: P51178-1) and human PLC $\delta 3$ (GenBank ID: BC072384.1, UniProt ID: Q8N3E9) by Dr. M. Zhu. The mutants of PLC $\delta 1$ were generated using a QuickChange site-directed mutagenesis kit (Agilent Technologies) following the manufacturer's protocol. The sequence was verified by sequencing.

Solutions and drugs

The recording pipette containing standard intracellular solution; 140 mM CsCl, 10 mM HEPES, 10 mM BAPTA, variable CaCl_2 , 3 mM Mg-ATP, 0.2 mM Tris-GTP, was balanced to pH 7.3 with CsOH. The free Ca^{2+} concentration was calculated using

CaBuf software (G.Droogmans). External solution was perfused constantly as follows; 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM MgCl_2 , 2 mM CaCl_2 , 10 mM glucose, pH 7.4 with NaOH. Rapamycin was purchased from Sigma-Aldrich, and (-)-Englerin A (EA) was purchased from PhytoLab.

Electrophysiology

The cells were transferred onto a small chamber on the stage of an inverted microscope (IX70; Olympus), and attached to coverslip in the small chamber for 10 min prior to the recording. Transiently transfected cells were identified by their fluorescence tagging. Recording pipettes were pulled from glass capillaries (Harvard Apparatus) using puller (PC-10; Narishige). Whole-cell currents were recorded using Axopatch 200B amplifier (Molecular Devices) and Digidata 1550B interface (Molecular Devices). Experiments were performed at room temperature (18°C–22°C). The recording chamber was continuously perfused at a flow rate of 1–2 ml/min. Glass microelectrodes with 2–2.5 megaohms resistance were used to obtain gigaohm seals. The whole cell configuration was used to measure the TRPC4 β channel currents in HEK293 cells. Voltage ramps ranging from +100 to –100 mV over a period of 500 ms were imposed every 10 sec with a holding membrane potential of –60 mV. pCLAMP v.10.2 (OriginLab) was used for data acquisition and the data were analyzed using the OriginPro 8 (OriginLab).

Image acquisition and FRET measurements

HEK293 cells were cultured in a 35-mm glass bottom dishes for imaging. For the confocal images, we used confocal laser scanning microscopy (LSM 710; Zeiss) equipped 63x oil objective lens. To obtain the FRET images, we used an inverted microscope (IX70; Olympus) equipped with 60x oil objective lens (UPlanSApo; Olympus). Each image was captured on an EMCCD camera (iXon3; Andor) and the light at 440 nm and 500 nm wavelengths was illuminated with LED light source (pE-2; CoolLED) under the control of MetaMorph 7.6 software (Molecular Devices). Based on this imaging system, FRET measurements were made by the three-cube FRET method [20] (excitation, dichroic mirror, filter) *via* a fixed collimator. The illumination of specific wavelength and the emission filter were rotated sequentially, and the rotation period for each filter cube was ~0.5 sec. All of the images were obtained within a second. Every image was analyzed using MetaMorph 7.6 software (Molecular Devices).

FRET efficiency computation

We did FRET efficiency computation following methods in Ko *et al.* [3]. FRET Ratio (FR) is equal to the fractional increase in YFP emission due to FRET and calculated as:

$$FR = \frac{FA_D}{FA} = \frac{[S_{FRET}(DA) - R_{D1} \cdot SCFP(DA)]}{RA_1 [S_{YFP}(DA) - R_{D2} \cdot SCFP(DA)]}$$

Here, S_{CUBE} (SPECIMEN) denotes an intensity measurement, where CUBE indicates the filter cube (CFP, YFP, or FRET), and SPECIMEN indicates whether the cell is expressing the donor (D; ECFP), acceptor (A; EYFP), or both (DA). $R_{D1} = S_{FRET}(D)/(S_{CFP}(D))$. $R_{D2} = (S_{YFP}(D)/(S_{CFP}(D)))$, and $R_{A1} = S_{FRET}(A)/S_{YFP}(A)$ are predetermined constants from measurements applied to single cells expressing only ECFP or EYFP-tagged molecules. Although three-cube FRET does not require that ECFP and EYFP fusion constructs preserve the spectral features of the unattached fluorophores, similar ratios and recorded spectra furnished two indications that the spectral features of the fluorophores were largely unperturbed by fusion. Since the FR relies on EYFP emission, EYFP should be attached to the presumed limiting moiety in a given interaction. Subsequent quantitative calculations based on FR relied on a presumed 1:1 interaction stoichiometry. The effective FRET efficiency (E_{EFF}) was determined as:

$$E_{EFF} = E \times A_b = (FR - 1)[E_{YFP}(440)/E_{CFP}(440)]$$

where E is the intrinsic FRET efficiency when fluorophore-tagged molecules are associated with each other, A_b is the fraction of EYFP-tagged molecules that are associated with ECFP-tagged molecules, and the bracketed term is the ratio of EYFP and ECFP molar extinction coefficients scaled for the FRET cube excitation filter [21]. We determined this ratio to be 0.094 based on maximal extinction coefficients for ECFP and EYFP and excitation spectra [22].

Western blotting, co-immunoprecipitation (Co-IP) analysis

We performed Western blotting following the methods in Kwak *et al.* [23], except with different antibodies. After transfection for 20–30 h, the cells were harvested as follows. Lysates were prepared in lysis buffer (0.5% Triton X-100, 120 mM NaCl, 50 mM HEPES, 2 mM MgCl₂, 2 mM EDTA, pH 7.5) by being passed through a 26-gauge needle ten to twenty times. Lysates were centrifuged at 13,000 × g for 10 min at 4°C, and the protein concentration in the supernatants was determined. In the Co-IP experiments for detection of TRPC-PLCδ, 500 μl of cell lysates (500–1,000 μg) were incubated with 1 μg of anti-GFP antibody and 30 μl of protein G-agarose beads at 4°C overnight with gentle rotation. After the beads were washed three times with wash buffer (0.1% Triton X-100), the precipitates were eluted with 30 μl of 2x Laemmli sample buffer and subjected to western blot analysis. The proteins extracted in sample buffer were loaded onto 8% Tris-glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. The proteins were transferred onto a nitrocellulose membrane. The following commercial antibodies

were used: anti-GFP (A-11122; Thermo Fisher Scientific); anti-Flag (F3165; Sigma-Aldrich); and anti-β-tubulin (T-4026; Sigma-Aldrich).

Statistical analysis

All statistical analysis and graph generation were done with OriginPro8 (OriginLab). Results were compared using Student's t-test. A probability value (p) less than 0.05 was considered statistically significant. Data are presented as means ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

RESULTS

TRPC4β directly interacts with PLCδ1

TRPC4β is functionally very comparable to TRPC5, but only TRPC5 shows constitutive activity even without the stimulation. Previously, we demonstrated that PIP₂ binds to TRPC4 and TRPC5 with different affinities [3]. Thus, we hypothesized that TRPC4 and TRPC5 channels have different mechanisms controlling the PIP₂ level. To support this hypothesis, we began studying the interaction of PLCδ enzyme with TRPC4 and TRPC5 channels. To identify the expression patterns, we performed fluorescence imaging experiments in a HEK293 cell line transiently co-transfected with ECFP tagged channel (TRPC4β or TRPC5) and EYFP tagged PLCδ (PLCδ1 or PLCδ3) enzyme. In the cases of expressing EYFP-PLCδ alone, PLCδ1 was expressed in the plasma membrane and was also distributed in the cytoplasm, and PLCδ3 was detected in the plasma membrane and the nuclear fraction (Supplementary Fig. 1). However, their expression pattern changed when they were co-expressed with TRPC4β. As shown in Fig. 1A, EYFP-PLCδ1 showed high fluorescence on the same location where TRPC4β-ECFP showed up as puncta on the membrane. The overlay image and line scan in this location showed an analogous fluorescence pattern (Fig. 1A upper). On the other hand, when TRPC4β-ECFP and EYFP-PLCδ3 were expressed together, PLCδ3 was observed mainly on the membrane, but it was not present in the TRPC4β expressing puncta region (Fig. 1A bottom). In other words, the distribution pattern of PLCδ3 was a total opposite to that of TRPC4β. We observed that TRPC5 did not colocalize with any of the PLCδ subtypes in the cells expressing ECFP-TRPC5 and EYFP-PLCδ1 or EYFP-PLCδ3 (Fig. 1C).

We used FRET imaging technique to determine whether colocalization of TRPC4β and PLCδ1 resulted from direct interaction or just close distances between these proteins. We used ECFP (donor)-TRPC ion channel and EYFP (acceptor)-PLCδ enzyme constructs to measure FRET efficiencies between them. As it was with the fluorescence imaging experiments, TRPC4β with PLCδ1 showed high FRET efficiency (Fig. 1B), but TRPC4β with PLCδ3, TRPC5 with PLCδ1 and TRPC5 with PLCδ3 showed low FRET

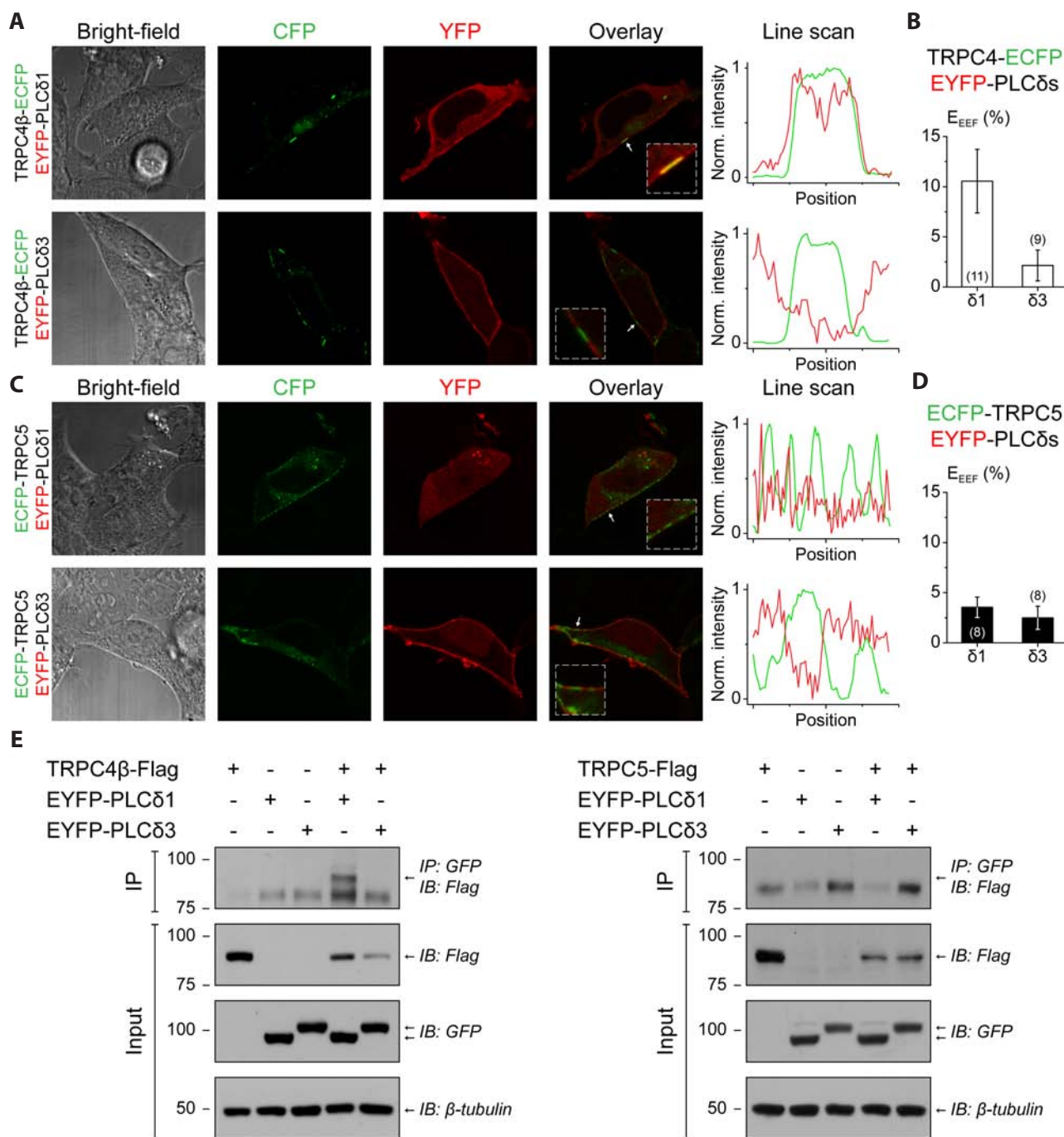


Fig. 1. TRPC4 β and PLC δ 1 colocalize together. (A, C) Localizations of TRPC channel and PLC δ enzyme. Upper panel: Channel with PLC δ 1; Lower panel: Channel with PLC δ 3. ECFP-tagged channel and EYFP-tagged PLC δ were co-expressed in HEK293 cells. The line scanned position is indicated by arrow in overlay images. Line scan graph shows TRPC4 β colocalized with PLC δ 1, but not with PLC δ 3. Original magnification, $\times 63$. (B, D) Summaries of FRET efficiency in the same expression conditions. The numbers in parentheses refer to cell numbers. (E) Representative blots of Co-IP experiments. HEK293 cells were co-expressed with Flag-tagged channel and EYFP-tagged PLC δ . Proteins from each condition were subjected to immunoprecipitation using anti-GFP antibody and probed with anti-Flag antibody. TRPC4 β interacts with PLC δ 1 directly but not with another subtype. TRPC5 interacts with neither. Data are expressed as mean \pm SEM. TRPC, transient receptor potential canonical; PLC, phospholipase C; Co-IP, Co-immunoprecipitation.

efficiency (Fig. 1D).

As a final approach to identify the binding between TRPC and PLC δ , we expressed Flag tagged TRPC ion channel and EYFP tagged PLC δ enzyme and analyzed them *via* Co-IP. Protein was

immunoprecipitated with anti-GFP antibody and then probed with anti-Flag antibody. The IP band was observed only in the cells expressing TRPC4 β and PLC δ 1, which indicates that only TRPC4 β interacts with PLC δ 1 (Fig. 1E).

PLC δ 1 decreases TRPC4 β currents amplitude and cytosolic Ca $^{2+}$ determines the activity of PLC δ 1

To determine the function of PLC δ 1 on TRPC4 β , we measured the changes in channel current. We used rapamycin-inducible system to translocate PLC δ 1 to the membrane. HEK293 cells were transfected with CFP-FKBP-PLC δ 1 and Lyn-FRB, and 50 nM of rapamycin was used, which is sufficient to translocate the PLC δ 1 to the membrane (Fig. 2A). To investigate the changes in the TRPC4 β current, we performed whole-cell patch clamp experiments with ramp pulse protocol from +100 to -100 mV every 10 sec in cells expressing TRPC4 β , CFP-FKBP-PLC δ 1, and Lyn-FRB. First, cells were treated with 50 nM rapamycin to translocate PLC δ 1 with an efficient amount, then TRPC4 β was stimulated by a channel agonist, EA (100 nM). In the cells not expressing Lyn-FRB, rapamycin did not show any current changes compared to basal current, and EA-evoked TRPC4 β currents showed the characteristic double-rectifying current-voltage relationship (Fig. 2B). The presence of Lyn-FRB showed the characteristics of TRPC4 β , however, the currents with EA stimulation were significantly decreased (Fig. 2C, D). Thus, these results suggest that PLC δ 1 functions as a negative regulator of TRPC4 β , reducing the current amplitudes.

We also investigated the Ca $^{2+}$ -dependent effect of PLC δ 1 on TRPC4 β channel currents. TRPC channels are potentiated by intracellular calcium [24], and especially, Ca $^{2+}$ is the main regula-

tor of PLC δ 1 activity [16]. Thus, we performed whole-cell patch clamp experiments by using different internal Ca $^{2+}$ buffering solutions. We used 10 mM BAPTA in recording pipette to tightly buffer the concentration of free calcium to be 50, 100 and 500 nM. HEK293 cells were expressed with TRPC4 β alone or together with PLC δ 1, and channels were stimulated with 100 nM EA. Channel currents were recorded with ramp pulse protocol from +100 to -100 mV every 10 sec. With 50 nM [Ca $^{2+}$] $_i$ buffered internal solution, TRPC4 β alone or TRPC4 β and PLC δ 1 expressing cells produced currents slowly after EA stimulation (Fig. 3A). In this condition, the presence of PLC δ 1 did not significantly decrease the TRPC4 β channel's peak current density (Fig. 3B). By increasing [Ca $^{2+}$] $_i$ to 100 nM, TRPC4 β expressing cells showed relatively faster time course to reach the peak current (Fig. 3C). Notably, the EA-evoked current was significantly decreased in the cells expressing PLC δ 1 together with TRPC4 β (Fig. 3D). These aspects of decreased current in the cells expressing PLC δ 1 were also observed in the experiments with 500 nM [Ca $^{2+}$] $_i$ pipette solutions (Fig. 3F). Interestingly, they showed robust current activation and desensitized quickly after reaching the peak current (Fig. 3E). Exceptionally, the time course of TRPC4 with or without PLC δ 1 was different only in 100 nM calcium buffering condition. In the cells only expressing TRPC4 β , higher internal calcium concentration potentiates channel to show a faster time course, and 500 nM [Ca $^{2+}$] $_i$ is sufficient to activate endogenous PLC to cause desensitization. PLC δ 1 reduced the overall TRPC4 β

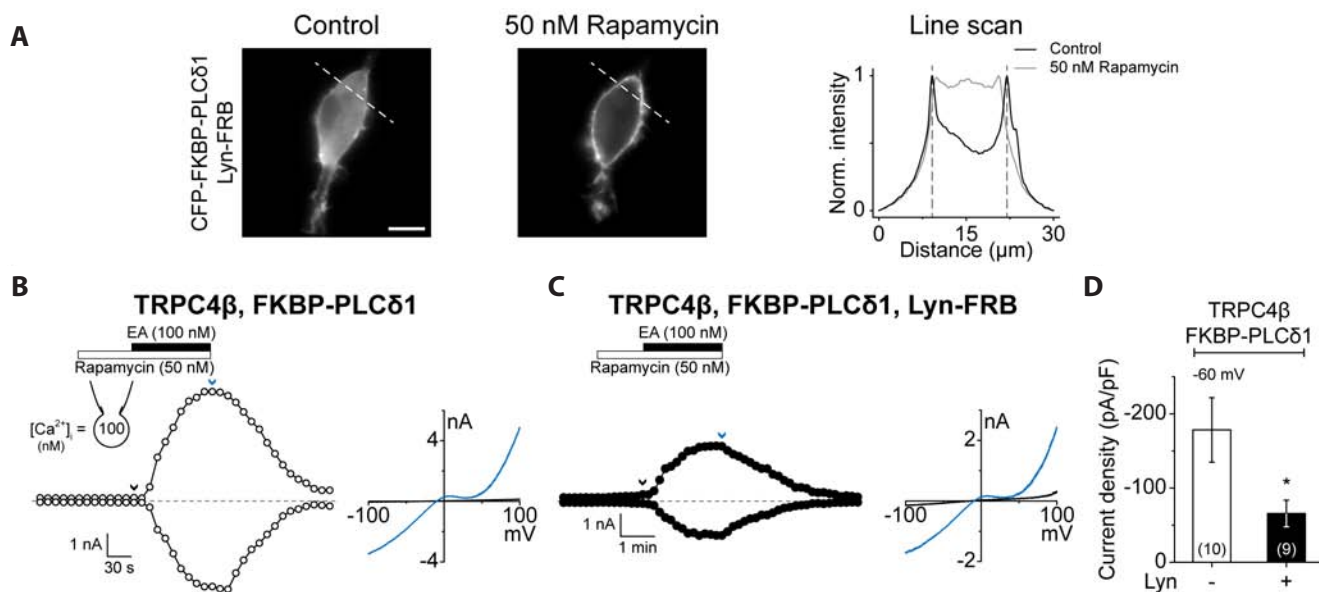


Fig. 2. PLC δ 1 inhibits TRPC4 β currents. (A) Rapamycin-induced translocation of CFP-FKBP-PLC δ 1 to the plasma membrane. CFP-FKBP-PLC δ 1 and Lyn-FRB were co-expressed in HEK293 cells, and 50 nM rapamycin was used. The line scanned region is indicated by dashed line. Scale bars, 10 μ m. (B, C) Representative whole-cell current recordings of HEK293 cells co-expressing with TRPC4 β , FKBP-PLC δ 1 in the absence (B) or presence of Lyn-FRB (C). Left panel: Time course of currents at \pm 100 mV every 10 sec; Right panel: I-V relationship for selected time points. Stippled lines indicate zero currents. Applications of 50 nM rapamycin and 100 nM (-)-Englerin A (EA) are indicated. The pipette solution contained 100 nM free Ca $^{2+}$. (D) Summaries of peak current densities at -60 mV induced by rapamycin and EA. Rapamycin-induced PLC δ 1 translocation to plasma membrane significantly reduced TRPC4 β currents. Data are expressed as mean \pm SEM. TRPC, transient receptor potential canonical; PLC, phospholipase C. * p < 0.05 by t-test. The numbers in parentheses refer to cell numbers.

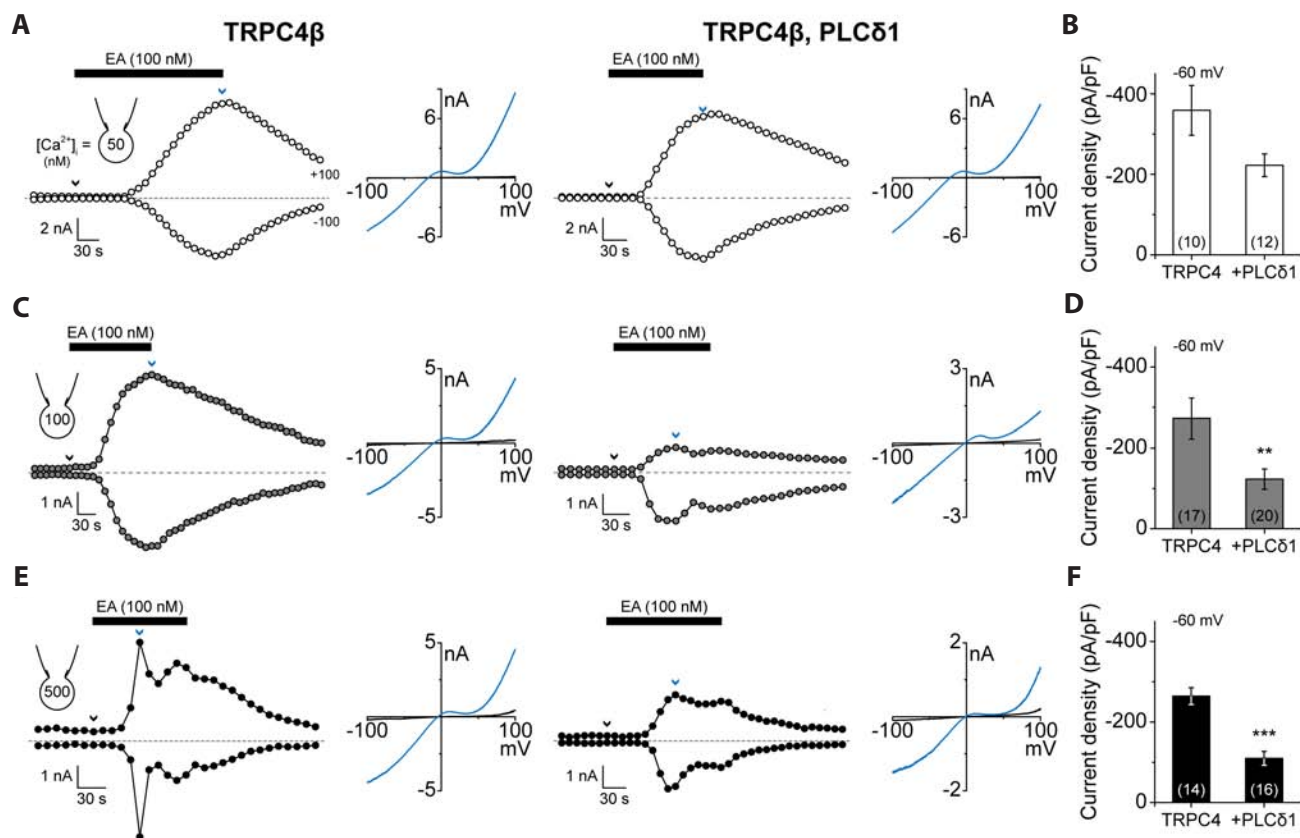


Fig. 3. Ca^{2+} -dependent activation of PLC δ 1 occurs in physiological intracellular calcium range. (A, C, E) Representative whole-cell current recordings of HEK293 cells co-expressed with TRPC4 β in the absence or presence of PLC δ 1 using 50 nM (A), 100 nM (C), and 500 nM (E) free Ca^{2+} recording pipette solutions. Left panel: Time course of currents at ± 100 mV every 10 sec; Left panel: I-V relationship for selected time points. Stippled lines indicate zero currents. Applications of 100 nM (-)-Englerin A (EA) are indicated. (B, D, F) Summaries of peak current densities at -60 mV induced by EA. The PLC δ 1 inhibited TRPC4 currents when using 100 nM and 500 nM $[\text{Ca}^{2+}]_i$ recording solutions. Data are expressed as mean \pm SEM. TRPC, transient receptor potential canonical; PLC, phospholipase C. ** $p < 0.01$, *** $p < 0.001$ by t-test. The numbers in parentheses refer to cell numbers.

currents in 100 nM and 500 nM free Ca^{2+} . Therefore, we suggest that physiological calcium concentration is sufficient to activate PLC δ 1 after channel stimulation, and the activity of PLC δ 1 is tightly regulated in a calcium dependent manner.

TRPC4 β -mediated increase in intracellular Ca^{2+} concentration activates PLC δ 1, and it accelerates PIP_2 depletion

PLC is an enzyme that cleaves phospholipids in a Ca^{2+} -dependent manner. TRPC4 channel is a nonselective cation channel which is permeable to calcium as well as monovalent cations. Therefore, Ca^{2+} influx-induced by TRPC4 β activation may act as a stimulator of PLC δ 1. To identify the activity of PLC, we used PH-domain of PLC δ 1 as a PIP_2 sensor to monitor the PIP_2 changes. We co-transfected TRPC4 β , PLC δ 1, and CFP-tagged PH domain in HEK293 cells, and TRPC4 β was stimulated by 100 nM EA. At first, CFP-PH was observed on the membrane, and line scan showed dense fluorescence intensity in the membrane before channel stimulation. In the cells expressing TRPC4 β without

PLC δ 1, CFP-PH still showed similar intensity in the membrane even after 30 sec of EA stimulation and PIP_2 started to deplete after 60 sec (Fig. 4A). On the other hand, TRPC4 β and PLC δ 1 co-expressed cells showed relatively faster PIP_2 depletion from membrane. In these cells, 30 sec of EA stimulation was enough to deplete the membrane PIP_2 (Fig. 4B). These results suggest that calcium influx via TRPC4 β activates PLC δ 1, and it causes the acceleration of PIP_2 depletion.

As a final approach, we evaluated the effect of PLC δ 1 on TRPC4 β . We used two mutant forms of PLC δ 1 (Supplementary Fig. 2). The first mutant is PLC δ 1 (K30A/K32A) which interrupted the PIP_2 binding, as Lys30 and Lys32 residues are located in the PH-domain [25]. And the second one is PLC δ 1 (H311A) which shows abolished phospholipase activity since His311 residue is located in the X-region of catalytic domain (Fig. 5C) [26]. We measured TRPC4 β currents in the cells expressing TRPC4 β and PLC δ 1 (K30A/K32A) or PLC δ 1 (H311A) mutant, respectively. Whole-cell patch clamp experiments were performed using the same protocol as mentioned before and the intracellular free calcium concentration was buffered to be 100 nM. In the cells

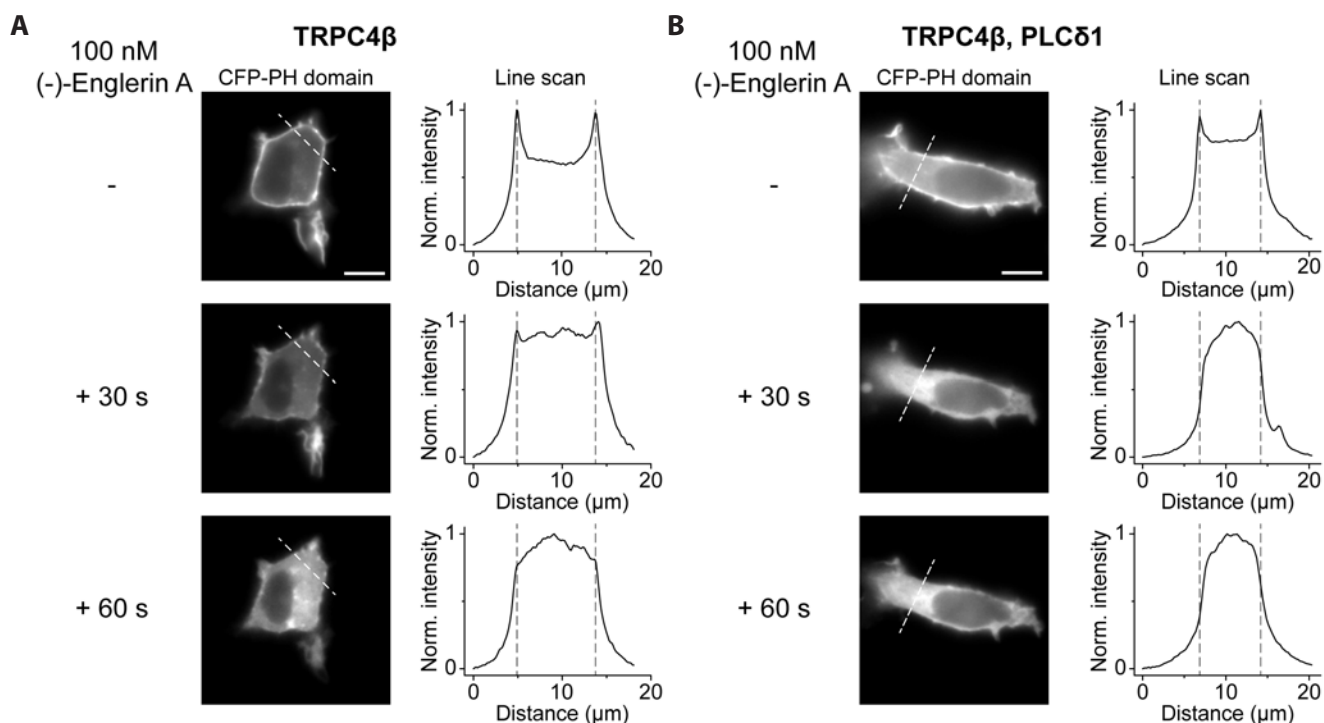


Fig. 4. Channel calcium-activated PLCδ1 accelerates PIP₂ depletion. (A, B) PIP₂ depletion from plasma membrane to cytosol after (-)-Englerin A stimulation. Imaging was performed in the cells expressing TRPC4β and CFP-PH domain in the absence (A) or presence of PLCδ1 (B). PIP₂ changes are monitored by CFP-PH domain. Left panel: Images of CFP-PH domain for selected time points; Right panel: Line scan graph of each image. The line scanned regions are indicated by dashed line. Scale bars, 10 μm. TRPC, transient receptor potential canonical; PLC, phospholipase C; PIP₂, phosphatidylinositol 4,5-bisphosphate.

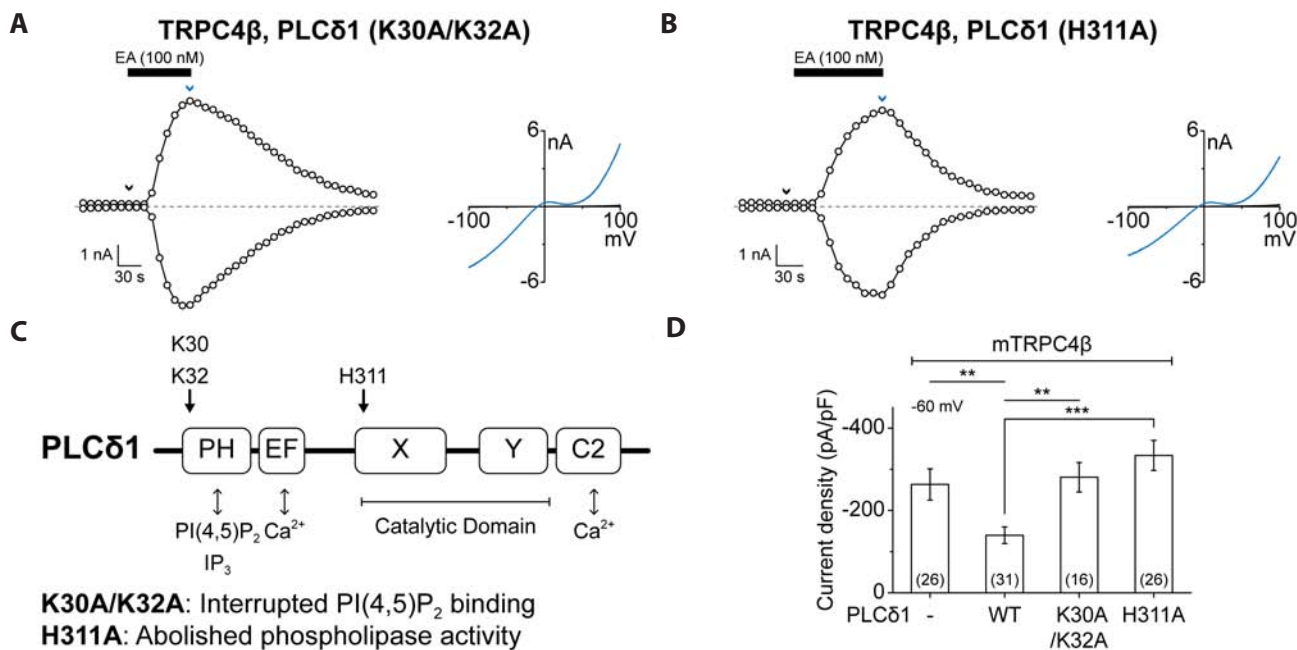


Fig. 5. Nonfunctional PLCδ1 mutants on PIP₂ level have no effect on TRPC4 currents. (A) Representative whole cell current recordings of HEK293 cells co-expressed with TRPC4β and PLCδ1 (K30A/K32A) (A) or PLCδ1 (H311A) (B). Left panel: Time course of currents at ±100 mV every 10 sec; Right panel: I-V relationship for selected time points. Stippled lines indicate zero currents. Application of 100 nM (-)-Englerin A (EA) are indicated. The pipette solution contained 100 nM free Ca²⁺. (C) Schematization of PLCδ1. The mutation sites are indicated with arrow. (D) Summaries of peak current densities at -60 mV induced by EA. PLCδ1 mutants had no effect on TRPC4β currents as the PLCδ1 non-expressing cells. Data are expressed as mean ± SEM. TRPC, transient receptor potential canonical; PLC, phospholipase C. **p < 0.01, ***p < 0.001 by t-test. The numbers in parentheses refer to cell numbers.

expressing both mutants, the TRPC4 β currents showed a similar time course as it would with cells expressing TRPC4 β alone (Fig. 5A, B). Furthermore, EA-evoked currents in the cells expressing PLC δ 1 (K30A/K32A) and PLC δ 1 (H311A) with TRPC4 β were significantly higher than in the cells expressing wild type PLC δ 1, similar to the case when TRPC4 β was expressed alone (Fig. 5D). In other words, PLC δ 1 mutants which cannot regulate PIP₂ levels did not have any effect on the TRPC4 β activity. Therefore, these results suggest that TRPC4 β -bound PLC δ 1 regulates PIP₂ level to decrease TRPC4 β currents.

DISCUSSION

Activity of TRPC4 ion channel is closely controlled by PIP₂. In this study, we found that PLC δ 1 binds to TRPC4 and contribute to channel regulation. The conclusions of this study are as follows: 1. TRPC4 directly interacts with PLC δ 1, but TRPC5 has no interaction with any PLC δ subtypes, 2. PLC δ 1 causes inhibition of TRPC4 currents in a calcium dependent manner, 3. TRPC4-bound PLC δ 1 responds to the calcium influx by the channel, which in turn depletes PIP₂. Altogether, we propose a negative feedback regulation of TRPC4 by PLC δ 1 in a Ca²⁺ and PIP₂ dependent manner.

Previously, we suggested that G α_q -PLC pathway has a self-limiting activation on TRPC channels. The G α_q protein strongly activates TRPC heteromeric channels [8], and sequential events such as PIP₂ depletion, PKC activation, and Ca²⁺ increase result in channel inhibition [9]. We emphasized several times that regulation of PIP₂ level is the most important factor as they are a requisite for channel activity. Accordingly, this important role of PLC and PIP₂ on TRPC4 channel is not surprising. Several TRP channels are also known to be regulated *via* signaling cascade including PLC and PIP₂. For instance, PLC-mediated PIP₂ decrease is shown to be involved in the desensitization of TRPM4, 5, and 8, hence PIP₂ appears to play a key role and ever-present regulator of TRPM channels [19,27-29]. Furthermore, TRPM8 neurons express Ca²⁺-sensitive PLC δ isozymes [30], and *in vivo* data showed that the activity is regulated by PLC δ 4 [31]. Also, it is suggested that PIP₂ is necessary for the normal function of TRPV1 channel. In this context, the desensitization of the TRPV1 currents is accelerated and eventually completely suppressed when PLC δ 3 is expressed together, and even such desensitization disappeared in PLC δ 4-/- [32]. Based on the followings, we have studied the effect of PLC with δ subtype, which is most sensitive to Ca²⁺ versus other PLC subfamilies. Here, we provide an evidence that PLC δ 1 directly interact with TRPC4 β based on the results of FRET and Co-IP experiments (Fig. 1). The inclusion of PLC δ 1 in regulation of TRPC4 β channel activity has been reported as an underlying concept of inhibitory PIP₂. They suggested that TRPC4 β activity was affected by PLC δ 1 in preference to PLC δ 3, and our data are consistent with this result that it might be from their direct inter-

action.

The direct interaction between TRPC4 β and PLC δ 1 also appeared clearly in expression patterns. PLC δ 1 is expressed in both membrane and cytoplasm, and PLC δ 3 is expressed in membrane. In our hands, PLC δ 1 showed denser fluorescence intensity where TRPC4 β is present, and they showed co-localization. Interestingly, PLC δ 3 was observed to be empty in the channel puncta region when it was expressed with TRPC4 β or TRPC5 (Fig. 1A, C). As PLC is a membrane-associated enzyme and TRPC channels are transmembrane channels, this expression pattern implies that the membrane resident proteins are present on the membrane competitively. Also, this difference presumably reflects that TRPC4 β channel has specificity in PLC interaction, and membrane molecules cannot co-exist on the membrane unless they are interacting together.

In this study, we highlight that PLC δ 1 binds to TRPC4 β but not TRPC5, and it affects PIP₂ level to regulate channel activity. TRPC4 and TRPC5 have a high similarity in amino acid sequence, and thus their structures are almost identical. However, they have some differences in their characteristics. First, TRPC5 shows constitutive activity while TRPC4 does not show channel activity in the absence of stimulation. Second, TRPC4 has a higher affinity for PIP₂ than TRPC5. In a preceding study, TRPC5 showed robust current inhibition with weak voltage stimulation which activates voltage-sensitive phosphatase. Based on our data, we suggest that PIP₂ and PLC δ 1 are possible candidates underlying these features [3]. TRPC4 β -bound PLC δ 1 would continuously regulate PIP₂ level in the vicinity of the channel even without the stimulation, and it would attribute to TRPC4 β showing no basal activity. In our hands, TRPC5 interacts with neither PLC δ 1 nor PLC δ 3, thus relatively abundant PIP₂ pool would exist around TRPC5. The detailed knowledge of mechanism causing differences between TRPC4 β and TRPC5 is important in understanding how they differently function physiologically in human body, but it should be elucidated in more detail.

Electrophysiological experiments suggest that PLC δ 1 activity produces decreased TRPC4 β currents in a calcium dependent manner. In the presence of PLC δ 1, EA-evoked TRPC4 β currents were significantly reduced (Fig. 3). Whether calcium increase alone causes the optimal activation of PLC δ 1 is still controversial. It was suggested that high extracellular potassium, or stimulations like thapsigargin or ionomycin also causes an increase in IP₃, but much less than the amount that is increased by the fully activated PLC δ subtypes [14,15,33]. Through experiments with differing intracellular calcium concentrations, our data agree with the notion that the channel-induced calcium influx within the physiological calcium range is sufficient to activate PLC δ 1. In our hands, 50 nM of [Ca²⁺]_i condition was not enough to fully activate PLC δ 1, but PLC δ 1 significantly reduced channel currents in higher calcium conditions (100 and 500 nM). Thus, we mainly used 100 nM [Ca²⁺]_i buffering solution in electrophysiological experiments which showed prominent differences including re-

duced current amplitude and altered time course of currents in the presence of PLC δ 1. Interestingly, the time course of channel currents was changed depending on the presence of PLC δ 1 (Fig. 3C) or intracellular calcium concentration (Fig. 3E). It is well known that the TRPC4 and TRPC5 channels show desensitization after they are activated by G_qPCR pathway [34-36]. Therefore, such desensitization with PLC δ 1 and calcium might also explain the inclusion of PIP₂ in this negative regulation mechanism. Our data showed that the kinetics of the process was much slower with low calcium which has a latency to respond to the channel agonist (Fig. 3). This observation is coincident with the fact that intracellular calcium strongly potentiates TRPC channels [24]. However, this reaction rate was not influenced by the presence of PLC δ 1 in the same calcium condition, hence we concluded that when PIP₂ levels are diminished with PLC δ 1, equivalent stimulus intensity will be less effective.

By monitoring the PIP₂ level changes after TRPC4 β stimulation, we suggest that PLC δ 1 accelerates PIP₂ hydrolysis in response to calcium influx (Fig. 4). As a second approach to demonstrate the involvement of PIP₂ in this mechanism, we used PLC δ 1 mutant which cannot hydrolyze PIP₂ (Fig. 5). We expected that the PLC δ 1-reduced TRPC4 β currents might not be observed when PLC δ 1 is unable to degrade PIP₂. In electrophysiological experiments, these PLC δ 1 mutants did not affect the channel currents. Therefore, it is likely that the PIP₂ is the main source of negative feedback regulation by PLC δ 1, and we emphasize the PIP₂ as a requisite component for TRPC4 β activation in this negative feedback mechanism of PLC.

Until now, we only focused on PIP₂ as an indispensable component for TRPC4 channel activity, and the channel seems to fall into desensitization when PIP₂ level is decreased. It should be noted that other groups have proposed the inhibitory effect of PIP₂ on TRPC4 [6,37]. Recent evidence suggests that PIP₂ has a dual regulatory role, and yet it remains an open question as to how PIP₂ decides its role. TRPV1 has also been proposed to be regulated by PIP₂ with a duality in function. Initially, TRPV1 channel was suggested to be potentiated after PIP₂ hydrolysis by releasing inhibitory PIP₂ from the channel [18,38-40].

Previously, it is suggested that the activity of PLC δ 1 does not exhibit receptor-specific activation [41]. In the electrophysiological experiments, we used channel-specific and strong agonist, hence we suggest that PLC δ 1 is activated by channel-mediated calcium. However, it is still obscure how EA is involved in the signaling pathway that activates TRPC4 β . The other group specified that PLC δ 1 is involved in the G $\alpha_{i/o}$ -mediated TRPC4 activation. It should be elucidated whether the activation of PLC δ 1 is confined to the specific pathway or the calcium through the channel itself is enough.

Collectively, our data reveal that TRPC4 β -interacting PLC δ 1 is sensitively activated by the calcium influx through TRPC4 β and in turn, hydrolyzes TRPC4 β -PI(4,5)P₂ which maintains TRPC4 β activity. Consequently, PLC δ 1 is a negative regulator of TRPC4 β .

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary data including two figures can be found with this article online at <https://doi.org/10.4196/kjpp.2023.27.2.187>.

REFERENCES

1. Kim BJ, Kim MT, Jeon JH, Kim SJ, So I. Involvement of phosphatidylinositol 4,5-bisphosphate in the desensitization of canonical transient receptor potential 5. *Biol Pharm Bull.* 2008;31:1733-1738.
2. Kim H, Jeon JP, Hong C, Kim J, Myeong J, Jeon JH, So I. An essential role of PI(4,5)P₂ for maintaining the activity of the transient receptor potential canonical (TRPC)4 β . *Pflugers Arch.* 2013;465:1011-1021.
3. Ko J, Myeong J, Shin YC, So I. Differential PI(4,5)P₂ sensitivities of TRPC4, C5 homomeric and TRPC1/4, C1/5 heteromeric channels. *Sci Rep.* 2019;9:1849.
4. Otsuguro K, Tang J, Tang Y, Xiao R, Freichel M, Tsvilovskyy V, Ito S, Flockerzi V, Zhu MX, Zholos AV. Isoform-specific inhibition of TRPC4 channel by phosphatidylinositol 4,5-bisphosphate. *J Biol Chem.* 2008;283:10026-10036.
5. Mederos Y, Schnitzler M, Gudermann T, Storch U. Emerging roles of diacylglycerol-sensitive TRPC4/5 channels. *Cells.* 2018;7:218.
6. Storch U, Forst AL, Pardatscher F, Erdogmus S, Philipp M, Gregoritz M, Mederos Y, Schnitzler M, Gudermann T. Dynamic NHERF interaction with TRPC4/5 proteins is required for channel gating by diacylglycerol. *Proc Natl Acad Sci U S A.* 2017;114:E37-E46.
7. Zhu MH, Chae M, Kim HJ, Lee YM, Kim MJ, Jin NG, Yang DK, So I, Kim KW. Desensitization of canonical transient receptor po-

- tential channel 5 by protein kinase C. *Am J Physiol Cell Physiol.* 2005;289:C591-C600.
8. Myeong J, Ko J, Kwak M, Kim J, Woo J, Ha K, Hong C, Yang D, Kim HJ, Jeon JH, So I. Dual action of the Gαq-PLCβ-PI(4,5)P₂ pathway on TRPC1/4 and TRPC1/5 heterotetramers. *Sci Rep.* 2018;8:12117.
 9. Ko J, Myeong J, Kwak M, Jeon JH, So I. Identification of phospholipase C β downstream effect on transient receptor potential canonical 1/4, transient receptor potential canonical 1/5 channels. *Korean J Physiol Pharmacol.* 2019;23:357-366.
 10. Berridge MJ. Inositol trisphosphate and calcium signalling. *Nature.* 1993;361:315-325.
 11. Noh DY, Shin SH, Rhee SG. Phosphoinositide-specific phospholipase C and mitogenic signaling. *Biochim Biophys Acta.* 1995;1242:99-113.
 12. Rhee SG, Choi KD. Regulation of inositol phospholipid-specific phospholipase C isozymes. *J Biol Chem.* 1992;267:12393-12396.
 13. Lee SB, Rhee SG. Significance of PIP₂ hydrolysis and regulation of phospholipase C isozymes. *Curr Opin Cell Biol.* 1995;7:183-189.
 14. Kim YH, Park TJ, Lee YH, Baek KJ, Suh PG, Ryu SH, Kim KT. Phospholipase C-delta1 is activated by capacitative calcium entry that follows phospholipase C-beta activation upon bradykinin stimulation. *J Biol Chem.* 1999;274:26127-26134.
 15. Allen V, Swigart P, Cheung R, Cockcroft S, Katan M. Regulation of inositol lipid-specific phospholipase cdelta by changes in Ca²⁺ ion concentrations. *Biochem J.* 1997;327(Pt 2):545-552.
 16. Rebecchi MJ, Pentylala SN. Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiol Rev.* 2000;80:1291-1335.
 17. Cifuentes ME, Delaney T, Rebecchi MJ. D-myo-inositol 1,4,5-trisphosphate inhibits binding of phospholipase C-delta 1 to bilayer membranes. *J Biol Chem.* 1994;269:1945-1948.
 18. Lukacs V, Thyagarajan B, Varnai P, Balla A, Balla T, Rohacs T. Dual regulation of TRPV1 by phosphoinositides. *J Neurosci.* 2007;27:7070-7080.
 19. Rohács T, Lopes CM, Michailidis I, Logothetis DE. PI(4,5)P₂ regulates the activation and desensitization of TRPM8 channels through the TRP domain. *Nat Neurosci.* 2005;8:626-634.
 20. Erickson MG, Alseikhan BA, Peterson BZ, Yue DT. Preassociation of calmodulin with voltage-gated Ca²⁺ channels revealed by FRET in single living cells. *Neuron.* 2001;31:973-985.
 21. Epe B, Steinhäuser KG, Woolley P. Theory of measurement of Förster-type energy transfer in macromolecules. *Proc Natl Acad Sci U S A.* 1983;80:2579-2583.
 22. Patterson G, Day RN, Piston D. Fluorescent protein spectra. *J Cell Sci.* 2001;114(Pt 5):837-838.
 23. Kwak M, Hong C, Myeong J, Park EYJ, Jeon JH, So I. Gα_i-mediated TRPC4 activation by polycystin-1 contributes to endothelial function via STAT1 activation. *Sci Rep.* 2018;8:3480.
 24. Blair NT, Kaczmarek JS, Clapham DE. Intracellular calcium strongly potentiates agonist-activated TRPC5 channels. *J Gen Physiol.* 2009;133:525-546.
 25. Garcia P, Gupta R, Shah S, Morris AJ, Rudge SA, Scarlata S, Petrova V, McLaughlin S, Rebecchi MJ. The pleckstrin homology domain of phospholipase C-delta 1 binds with high affinity to phosphatidylinositol 4,5-bisphosphate in bilayer membranes. *Biochemistry.* 1995;34:16228-16234.
 26. Ellis MV, U S, Katan M. Mutations within a highly conserved sequence present in the X region of phosphoinositide-specific phospholipase C-delta 1. *Biochem J.* 1995;307(Pt 1):69-75.
 27. Zhang Z, Okawa H, Wang Y, Liman ER. Phosphatidylinositol 4,5-bisphosphate rescues TRPM4 channels from desensitization. *J Biol Chem.* 2005;280:39185-39192.
 28. Liu D, Liman ER. Intracellular Ca²⁺ and the phospholipid PIP₂ regulate the taste transduction ion channel TRPM5. *Proc Natl Acad Sci U S A.* 2003;100:15160-15165.
 29. Liu B, Qin F. Functional control of cold- and menthol-sensitive TRPM8 ion channels by phosphatidylinositol 4,5-bisphosphate. *J Neurosci.* 2005;25:1674-1681.
 30. Daniels RL, Takashima Y, McKemy DD. Activity of the neuronal cold sensor TRPM8 is regulated by phospholipase C via the phospholipid phosphoinositol 4,5-bisphosphate. *J Biol Chem.* 2009;284:1570-1582.
 31. Yudin Y, Lutz B, Tao YX, Rohacs T. Phospholipase C δ4 regulates cold sensitivity in mice. *J Physiol.* 2016;594:3609-3628.
 32. Lukacs V, Yudin Y, Hammond GR, Sharma E, Fukami K, Rohacs T. Distinctive changes in plasma membrane phosphoinositides underlie differential regulation of TRPV1 in nociceptive neurons. *J Neurosci.* 2013;33:11451-11463.
 33. Banno Y, Okano Y, Nozawa Y. Thrombin-mediated phosphoinositide hydrolysis in Chinese hamster ovary cells overexpressing phospholipase C-delta 1. *J Biol Chem.* 1994;269:15846-15852.
 34. Tsvilovskyy VV, Zholos AV, Aberle T, Philipp SE, Dietrich A, Zhu MX, Birnbaumer L, Freichel M, Flockerzi V. Deletion of TRPC4 and TRPC6 in mice impairs smooth muscle contraction and intestinal motility in vivo. *Gastroenterology.* 2009;137:1415-1424.
 35. Dresviannikov AV, Bolton TB, Zholos AV. Muscarinic receptor-activated cationic channels in murine ileal myocytes. *Br J Pharmacol.* 2006;149:179-187.
 36. Lee KP, Jun JY, Chang IY, Suh SH, So I, Kim KW. TRPC4 is an essential component of the nonselective cation channel activated by muscarinic stimulation in mouse visceral smooth muscle cells. *Mol Cells.* 2005;20:435-441.
 37. Thakur DP, Tian JB, Jeon J, Xiong J, Huang Y, Flockerzi V, Zhu MX. Critical roles of Gi/o proteins and phospholipase C-δ1 in the activation of receptor-operated TRPC4 channels. *Proc Natl Acad Sci U S A.* 2016;113:1092-1097.
 38. Klein RM, Ufret-Vincenty CA, Hua L, Gordon SE. Determinants of molecular specificity in phosphoinositide regulation. Phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P₂) is the endogenous lipid regulating TRPV1. *J Biol Chem.* 2008;283:26208-26216.
 39. Liu B, Zhang C, Qin F. Functional recovery from desensitization of vanilloid receptor TRPV1 requires resynthesis of phosphatidylinositol 4,5-bisphosphate. *J Neurosci.* 2005;25:4835-4843.
 40. Rohacs T, Thyagarajan B, Lukacs V. Phospholipase C mediated modulation of TRPV1 channels. *Mol Neurobiol.* 2008;37:153-163.
 41. Murthy SN, Lomasney JW, Mak EC, Lorand L. Interactions of G(h)/transglutaminase with phospholipase Cdelta1 and with GTP. *Proc Natl Acad Sci U S A.* 1999;96:11815-11819.