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A role of PI(4,5)P_2 for maintaining the activity of TRPC4β

TRPC4β의 활성 유지를 위한 PI(4,5)P_2의 역할

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서울대학교 대학원
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김 하 나

TRPC4β의 활성 유지를 위한 $PI(4,5)P_2$ 의 역할

지도교수 서 인 석

이 논문을 의학 석사 학위논문으로 제출함

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A role of PI(4,5)P_2 for maintaining the activity of TRPC4β

by

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A Thesis Submitted to the Department of Biomedical Sciences in Partial Fulfillment of the Requirements for the Master Degree in Medical Science at the Seoul National University College of Medicine

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Approved by thesis committee:

Professor _____ (Chairman)

Professor _____ (Vice chairman)

Professor _____

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논문제목 : A role of PI(4,5)P_2 for maintaining the activity of TRPC4β

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Abstract

A role of PI(4,5)P_2 for maintaining the activity of TRPC4β

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The Transient Receptor Potential Canonical 4 (TRPC4) channel is a Ca^{2+}-permeable, non-selective cation channel in mammalian cells and mediates a number of cellular functions. Our previous study showed that the TRPC4 current was inhibited by co-expression of a constitutively active form of $G\alpha_q$ ($G\alpha_q^{Q209L}$). It may have caused a shortage of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) because a constitutively active $G\alpha_q$ would have persistently activated PLCβ. Therefore, we used an inducible system to regulate PI(4,5)P_2 specifically and acutely. The TRPC4β current was reduced by inducible $G\alpha_q^{Q209L}$ but not by the mutants whose binding ability to PLCβ is impaired. Depletion of PI(4,5)P_2 using the inositol polyphosphate 5-phosphatase (Inp54p) inducible system led to an irreversible inhibition of TRPC4 currents after application of rapamycin to HEK293 cells that were co-expressing TRPC4 with Inp54p. On the other hand, phosphatidylinositol 4-phosphate 5-kinase (PIP5K) inducible system did not activate the initial gating of TRPC4β channel. Even in the case of $G\alpha_{i2}$-activated TRPC4β currents, the acute depletion of PI(4,5)P_2 led to reduced TRPC4β currents. A PI(4,5)P_2 increase, however, did not induce any changes in TRPC4β activation. Therefore, we suggested that PI(4,5)P_2 is not the activator for TRPC4 activation but it is still necessary for regulating TRPC4 activation. Especially, TRPC4 desensitization might be a result of hydrolysis of PI(4,5)P_2 since TRPC4 desensitization through muscarinic receptor 3 which activates $G\alpha_q$-PLC pathway disappeared by adding PI(4,5)P_2 and nonhydrolysis PI(4,5)P_2. These findings indicate an essential role of PI(4,5)P_2 for maintaining the activity of TRPC4β.

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keywords : TRPC4; PI(4,5)P_2; GPCR; rapamycin inducible system

Student Number **: 2011-21937**

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Introduction

Transient Receptor Potential Canonical (TRPC) channels are considered as the molecular candidates for receptor-operated Ca^{2+}-permeable non-selective cation channels. Phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), localized mainly in the cytoplasmic leaflet of the plasma membrane, is required for the function of many ion channels and transporters (McLaughlin et al, 2002). $PI(4,5)P_2$ and related phosphoinositides exert positive effects on TRPC6 (Tseng et al, 2004; Kwon et al, 2007). For TRPC5, a TRP channel very similar to TRPC4, we showed that $PI(4,5)P_2$ exerts a positive role (Kim et al, 2008), whereas other reports showed both inhibitory and positive roles (Zeng et al, 2006; Trebak et al, 2009). In our previous study, we showed the inhibition of TRPC4 and TRPC5 channels induced by expression of a constitutively active $G\alpha_q$ protein ($G\alpha_q^{Q209L}$), although $G\alpha_q$-PLC is believed to be the primary pathway for activation of all TRPC channels (Schaefer et al, 2000; Jeon et al, 2012). Moreover, that intracellular $PI(4,5)P_2$ could recover that inhibition. This result suggests that the role of $PI(4,5)P_2$ is important to regulate TRPC4. However, constitutively active $G\alpha_q$ can elicit chronic effect on downstream effector as well as on PLCβ.

Therefore, we considered the inducible system as the strategy to regulate $PI(4,5)P_2$ because recently, rapamycin-inducible systems have become widely used to see the acute effect of G proteins and enzymes related to the metabolism of $PI(4,5)P_2$, such as Inp54p or PIP5K. Thus, we used an

inducible system to examine the direct role of $PI(4,5)P_2$ on the positive mechanism of TRPC4. The inducible system is accomplished by heterodimerization of protein domains from the FK506 binding protein (FKBP) and from mTOR rapamycin-binding domain (FRB) through the rapamycin (Spencer et al, 1993; Inoue et al, 2005). For regulation of $PI(4,5)P_2$, we used inositol polyphosphate 5-phosphatase (Inp54p) and phosphatidylinositol 4-phosphate 5-kinase (PIP5K). Inp54p is the one of inositol polyphosphate 5-phosphatases that regulate cellular $PI(4,5)P_2$ levels by hydrolyzing the 5-position phosphate from the inositol ring, forming phosphatidylinositol 4-phosphate (PI(4)P) (Majerus, 1996; Mitchell et al, 1996), and moreover, Inp54p specifically converts $PI(4,5)P_2$ into PI(4)P at the plasma membrane (Raghu et al, 2000). PIP5K synthesizes $PI(4,5)P_2$ from PI(4)P (Loijens et al, 1996). In this study, we found that $PI(4,5)P_2$ is essential for the maintaining the activity of TRPC4.

Material and Method

Cell Culture, cloning and Transient transfection

Human embryonic kidney (HEK293) cells (HEK293, ATCC, U.S.A.) were maintained according to the supplier's recommendations. We cloned the mTRPC4-green fluorescent protein (TRPC4-GFP) fusion protein and mouse $G\alpha_{i2}^{Q205L}$-mRFP, a constitutively active form. Human mRFP-FKBP-$(GKK)_3$-$G\alpha_q^{Q209L}$, an inducible form, was given by Dr. Carsten Schultz. For the transient transfection, the cells were seeded in 12-well plates. The following day, 0.5g/well of mouse TRPC4 was transfected using the transfection reagent FuGENE 6 (FuGENE 6, Roche Molecular Biochemicals, Switzerland), as detailed in the manufacturer's protocol. Co-expression of TRPC4 channels with the rapamycin system was achieved using a channel to rapamycin system transfection ratio of 1:2. After 30-40 h, the cells were trypsinized and transferred to a small recording chamber (RC-11, Warner Instruments, U.S.A.) for whole-cell recording (Jeon et al, 2008; Jeon et al, 2012).

Regulation of $PI(4,5)P_2$ levels using rapamycin

Regulation of $PI(4,5)P_2$ was achieved by the Inp54p-rapamycin system and the PIP5K-rapamycin system. The plasma membrane-targeted FRB domain of mTOR can be heterodimerized with FKBP using rapamycin (20 nM). For the $PI(4,5)P_2$ rapamycin system, we used CFP-FKBP-PIP5K, CFP-FKBP-Inp54p, FKBP-mRFP, and Lyn-FRB.

Whole-cell patch-clamp experiment

The cells were transferred to a small chamber on the stage of an inverted microscope (TE2000S, Nikon, Japan) and attached to coverslips in the small chamber for 15 minutes prior to the patch recording. Glass microelectrodes with 2-2.5 megohm resistance were used to obtain gigaohm seals. The bath solutions were constantly perfused with a physiological salt solution at a rate of 1-2 ml/min. The currents were recorded using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Molecular Devices, U.S.A.). The whole-cell configuration was used to measure the TRPC channel current in the HEK cells, as described previously (So and Kim, 2003; Kim et al, 2006; Kim et al, 2008). After establishing the whole-cell configuration, the external solution was changed from the Normal Tyrode to the Cs^+-rich external solution. The current was recorded for 500-ms duration ramps from +100 to -100 mV and with a holding membrane potential of -60 mV. pCLAMP software v.10.3 and a Digidata 1440A (Axon Instruments, Molecular Devices, U.S.A.) were used for data acquisition and application of the command pulses. The data were filtered at 5 kHz and displayed on a computer monitor. The data were analyzed using pCLAMP v.10.3 and Origin software (Microcal Origin v.7.5, OriginLab Corporation, USA).

Solutions and Drugs

For recordings of the TRPC4 channel, we used a physiological salt solution (PSS) and a Cs^+-rich external solution. The PSS contained 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES with a pH that was adjusted to 7.4 using NaOH. The Cs^+-rich external solution contained equimolar CsCl rather than NaCl and KCl. The internal solution

contained 140 mM CsCl, 10 mM HEPES, 0.2 mM Tris-GTP (Tris-guanosine 5'-triphospate), 0.5 mM EGTA, and 3 mM Mg-ATP (adenosine 5'-triphosphate) with a pH that was adjusted to 7.3 with CsOH. We used 0.2 mM GTPγS that was purchased from Sigma, 20 nM rapamycin for the rapamycin treatment. 20 μM of diC8-PI(4,5)P_2 20 μM of α-fluorophosphonate (ms-PI(4,5)P_2) from Echelon biosciences.

Statistics

All data are expressed as the means ± S.E. (error bar). The statistical significance was determined using paired or unpaired Student's *t*-tests. P values of less than 0.05 were considered statistically significant. *n* represents the number of cell recordings.

Results

To study the role of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) on the activity of the TRPC4 channel, we used an inducible system in which TRPC4 is co-expressed in HEK 293 cells. The inducible system is based on the heterodimerization of protein domains: FKBP and FRB are chemically heterodimerized by rapamycin. In this system, the FRB, which is anchored to the plasma membrane via the Lyn N-terminal sequence (aa 1-11), forms the FRB-rapamycin-FKBP complex irreversibly upon application of rapamycin. This dimerization leads to plasma membrane recruitment of the FKBP fusion protein from the cytosol and the subsequent activation of signal cascades (Spencer et al, 1993; Inoue et al, 2005).

$PI(4,5)P_2$ is related with $G\alpha_q$ PLC pathway for regulating TRPC4.

$PI(4,5)P_2$ is related with $G\alpha_q$ PLC pathway for regulating TRPC4.

We first checked the effects of the acute translocation of active $G\alpha_q$ on TRPC4 currents. In co-expression of the inducible $G\alpha_q^{Q209L}$ construct with TRPC4β-GFP construct, TRPC4β currents activated by intracellular GTPγS application were attenuated about 80.8 ± 4.3% through the $G\alpha_q^{Q209L}$-inducible system after rapamycin treatment (Fig. 1A, B and E; n = 8, 68.8 ± 9.7 pA/pF: peak currents and 11.1 ± 1.3 pA/pF: after rapamycin treatment). However, the control experiment did not show increased currents after rapamycin treatment (Fig. 1 C, D; TRPC4β: n = 8, 121.4 ± 10.8 pA/pF: peak currents and 137.0 ± 8.2 pA/pF: after rapamycin treatment) and plasma membrane translocation of

the GTPase-deficient $G\alpha_q$ constructs ($G\alpha_q^{Q209L}$) carrying additional mutations – H218A or L254A – that abolished the interaction of $G\alpha_q$ with PLCβ (Putyrskil et al, 2011) did not reduce TRPC4β currents after application of rapamycin (Fig. 2A-E, $hG\alpha_q^{Q209L/H218A}$ with TRPC4β: n = 5, peak currents & after rapamycin treatment : 64.6 ± 12.4 pA/pF & 61.1 ± 13.8 pA/pF and $hG\alpha_q^{Q209L/L254A}$ with TRPC4β: n = 5, peak currents & after rapamycin treatment : 86.5 ± 14.3 pA/pF & 82.4 ± 15.7 pA/pF). The $G\alpha_q$-PLC pathway activated by the rapamycin-inducible system inhibited activation of TRPC4β rather than activating it further. Therefore, these results suggest that reduction of TRPC4β currents is attributable to the hydrolysis of $PI(4,5)P_2$ by the $G\alpha_q$-PLC pathway (Fig. 3).

To check whether the reduction of the current by Gq-PLC resulted in desensitization of TRPC4 by PKC, we made a TRPC4 (T877A) mutant which is not phosphorylated by protein kinase C (PKC). Current of TRPC4β (T877A) mutant elicited by GTPγS infusion and its current amplitude showed no difference compared to wild type of TRPC4β without $G\alpha_q^{Q209L}$ co-expression (Fig. 4A, B and E, opened and closed column : n = 4 and n = 5, 161.5 ± 36.9 pA/pF and 194.4 ± 20.6 pA/pF). The mutant was inhibited by co-expression of $G\alpha_q^{Q209L}$ (Fig. 4C-E; opened and closed column: n = 5 and n = 5, 1.8 ± 0.6 pA/pF and 1.2 ± 0.8 pA/pF). Thus, the inhibited TRPC4 current by $G\alpha_q^{Q209L}$ was not induced by PKC activation.

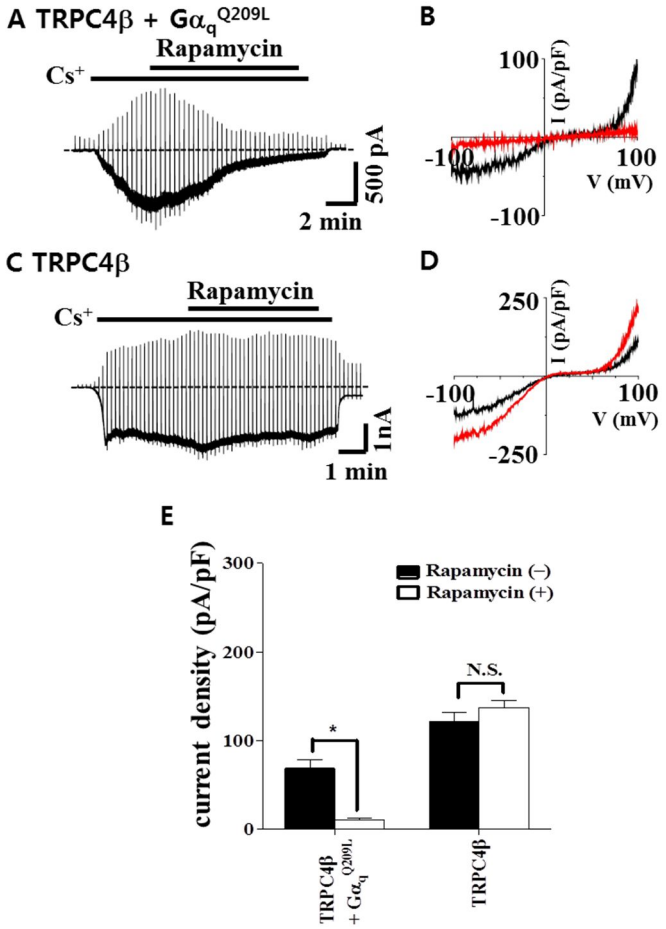

Figure 1. Regulation of TRPC4 gating properties by $G\alpha_q$.
(A) The representative current traces of GTPγS-induced TRPC4β currents were recorded in HEK cells with 140 mM Cs^+ external solution and rapamycin treatment (20 nM, 5 minutes). mTRPC4β-GFP and Lyn-FRB co-expressed with mRFP-FKBP$(GKK)_3$-h$G\alpha_q^{Q209L}$. (B), (D) The representative current (*I*)-voltage *(V)* relationship curves of GTPγS (0.2 mM)-induced TRPC4β currents were recorded by voltage ramps from +100 to -100 mV for 500 ms, while the cells were held at -60 mV. The black line and the red line in curves indicate before rapamycin treatment and after rapamycin treatment, respectively. The black line and the red line in curves indicate before rapamycin treatment and after rapamycin treatment, respectively. (C) In HEK cells which is expressing mTRPC4β-GFP, GTPγS-induced TRPC4 currents were recorded with 140 mM Cs^+ external solution and rapamycin treatment (20 nM, 5 minutes). (E) Summarized effects of rapamycin-inducible $G\alpha_q$ proteins in GTPγS-induced TRPC4β currents. Current density means maximal current peaks at -60 mV in Cs^+ external solution (means ± S.E.). Statistical significance was denoted by * ($p<0.05$). N.S. = not significant.

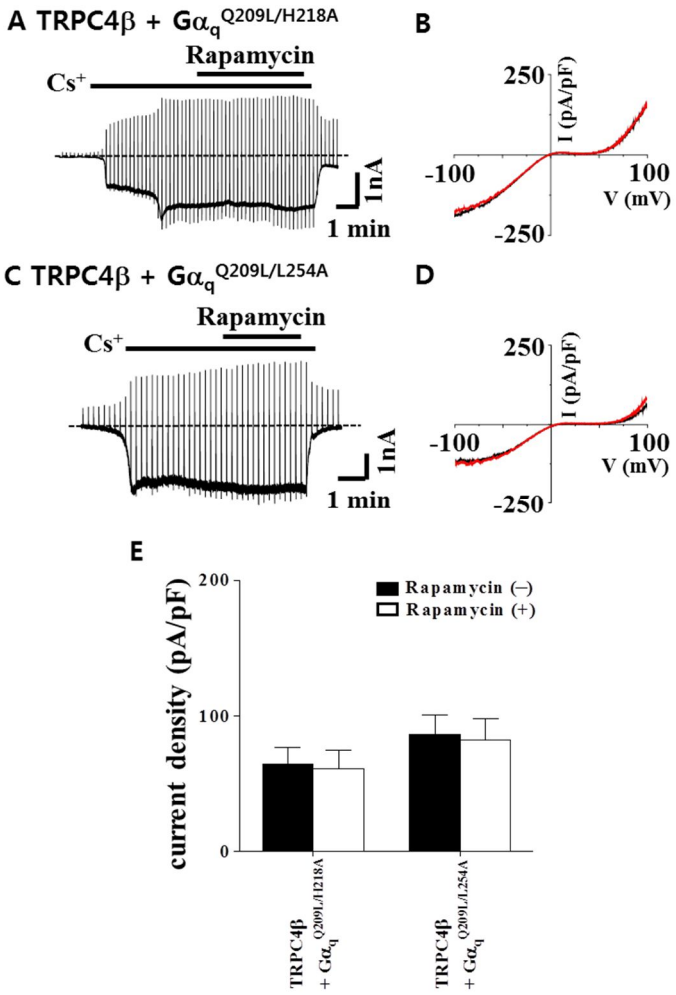

Figure 2. Regulation of TRPC4 gating properties by $G\alpha_q$-mutants which are impaired binding with PLC.

(A), (C) The representative current traces of GTPγS-induced TRPC4β currents were recorded in HEK cells with 140 mM Cs^+ external solution and rapamycin treatment (20 nM, 5 minutes). mTRPC4β-GFP and Lyn-FRB co-expressed with mRFP-FKBP$(GKK)_3$-h$G\alpha_q^{Q209L/H218A}$ or mRFP-FKBP-$(GKK)_3$-h$G\alpha_q^{Q209L/L254A}$, respectively. (B), (D) The representative current *(I)*-voltage *(V)* relationship curves of GTPγS (0.2 mM)-induced TRPC4β currents were recorded by voltage ramps from +100 to -100 mV for 500 ms, while the cells were held at -60 mV. The black line and the red line in curves indicate before rapamycin treatment and after rapamycin treatment, respectively. (E) Summarized effects of rapamycin-inducible $G\alpha_q$ proteins in GTPγS-induced TRPC4β currents. Current density means maximal current peaks at -60 mV in Cs^+ external solution (means ± S.E.).

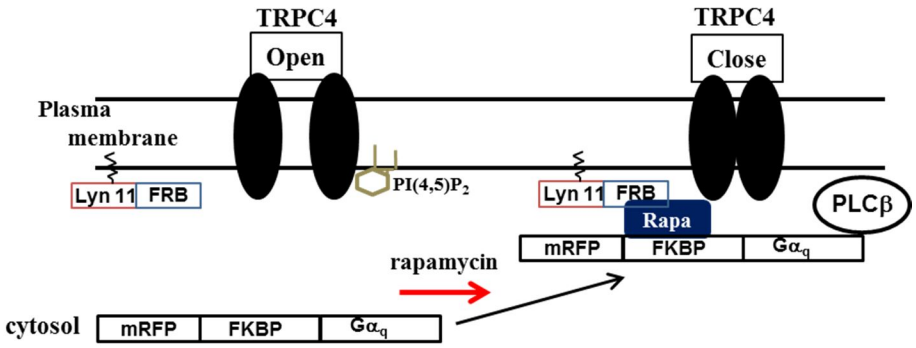

Figure 3. Schematic diagram that shows inhibition of TRPC4β currents by the translocation of rapamycin-inducible $G\alpha_q^{Q209L}$.

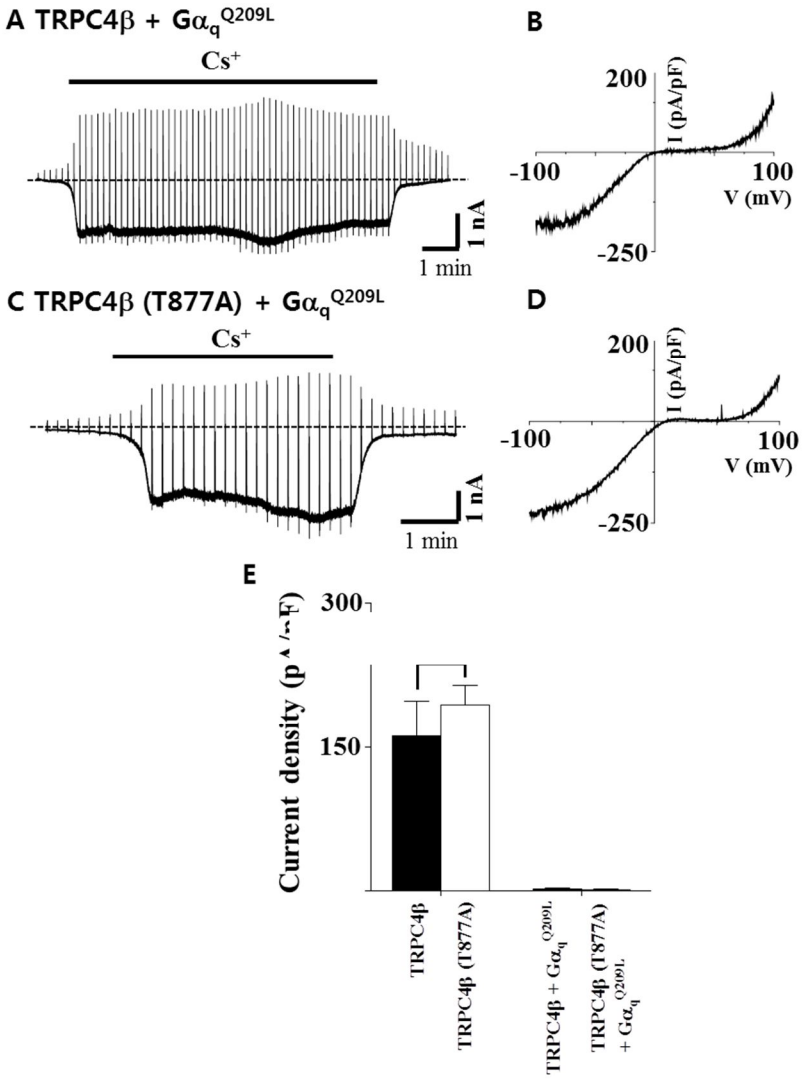

Figure 4. TRPC4β gating properties with PKC regulation of $G\alpha_q$-PLC downstream.

(A) TRPC4 currents which were induced by GTPγS were recorded in HEK cells expressing mTRPC4β with 140 mM Cs^+ external solution. (B), (D) The representative *I-V* curves of TRPC4β-GFP currents by 0.2 mM GTPγS were recorded by voltage ramps from +100 to -100 mV for 500 ms, while the cells were held at -60 mV. (C) TRPC4 currents which were induced by GTPγS were recorded in HEK cells expressing mTRPC4β (T877A) with 140 mM Cs^+ external solution. (E) Summary bar graphs of TRPC4β currents. Current density means maximal current peaks at -60 mV in Cs^+ external solution (means ± S.E.). N.S. = not significant.

$PI(4,5)P_2$ is necessary for maintaining TRPC4 activity.

We used a $PI(4,5)P_2$-specific phosphatase, Inp54p, to exclude the possibility that increases of the many signaling molecules downstream of PLC or other changes in phosphoinositides are essential for maintaining channel activity (Suh et al, 2006). Inp54p specifically cleaves the phosphate at the 5 position of $PI(4,5)P_2$ (Raghu et al, 2000). Depletion of $PI(4,5)P_2$ using the Inp54p-inducible system led to an irreversible inhibition of TRPC4β currents about 99.4 ± 1.3% after application of rapamycin to HEK293 cells that were co-expressing TRPC4β with Inp54P (Fig. 5C, D; n = 7, 65.9 ± 12.9 pA/pF: peak currents and 0.7 ± 1.0 pA/pF: after rapamycin treatment). The simple FKBP-mRFP constructs did not exhibit any changes in the TRPC4 currents after rapamycin application (Fig. 5A, B; n = 10, 49.1 ± 8.6 pA/pF: peak currents and 43.8 ± 18.4 pA/pF: after rapamycin treatment). When the application of $PI(4,5)P_2$ by an internal solution was used to compensate for the effect of the Inp54p-inducible system, the TRPC4β currents were not reduced by application of rapamycin (Fig. 5E, F; n = 5, 56.2 ± 6.9 pA/pF: peak currents and 58.7 ± 8.7 pA/pF: after rapamycin treatment). Therefore, these data indicate that $PI(4,5)P_2$ is essential for maintaining the activated state of TRPC4β (Fig. 6).

TRPC4 inhibition by $PI(4,5)P_2$ application shown in another study was reproduced (Otsuguro et al, 2008), TRPC4α was reduced to 30.9 ± 5.6% by application of diC8-$PI(4,5)P_2$ (20 μM) via the pipette solution (Fig. 7A, B and E; n = 9, 26.0 ± 4.7 pA/pF) compared with the activated TRPC4α current with GTPγS (Fig. 7C-E; n = 9, 84.1 ± 15.1 pA/pF). On the other hand, TRPC4β was not effected by infusion of $PI(4,5)P_2$ (Fig. 8, TRPC4β : n = 5, 121.7 ±

14.1 pA/pF and TRPC4β with $PI(4,5)P_2$: n = 6, 121.9 ± 13.2 pA/pF). In this study, the inhibition of the TRPC4 current was induced by $PI(4,5)P_2$ depletion. The role of $PI(4,5)P_2$ on TRPC4 activity shows an opposite results in TRPC4 variants. These data indicate that TRPC4 variant shows different regulation by $PI(4,5)P_2$

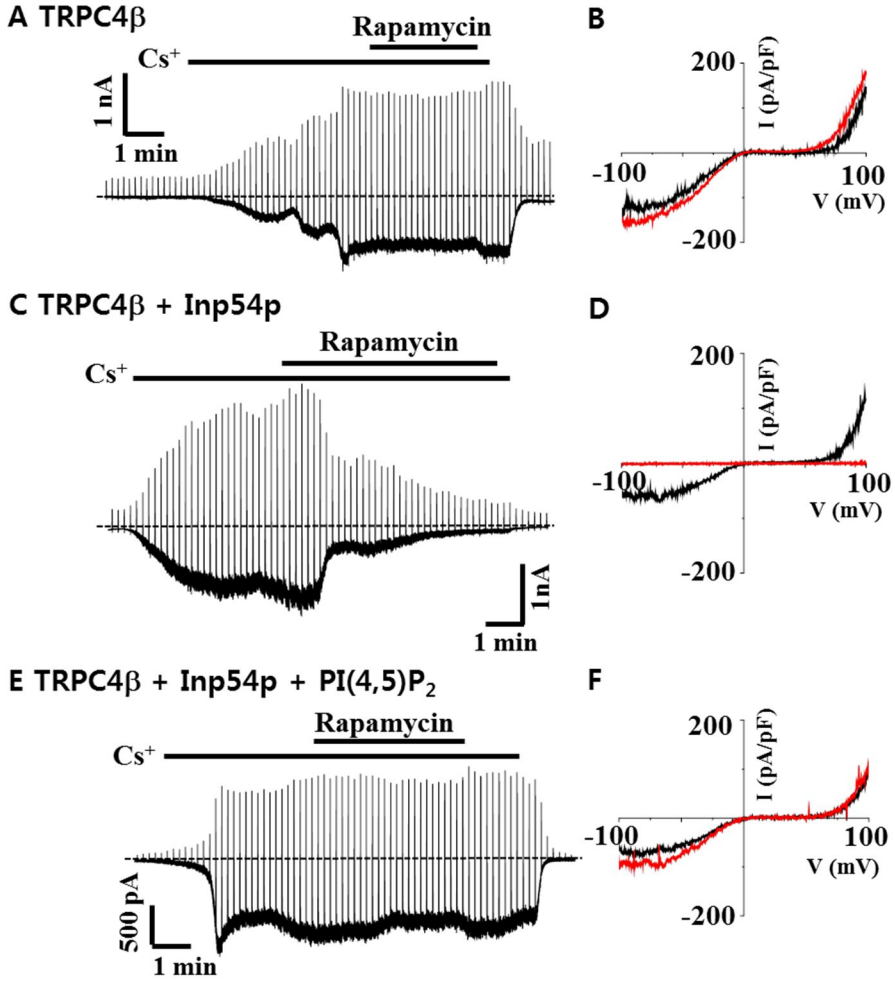

Figure 5. TRPC4β gating properties by GTPγS is regulated by PI(4,5)P_2. (A) GTPγS-activated TRPC4β currents were recorded in HEK cells expressing mTRPC4β-GFP, mRFP-FKBP, and Lyn-FRB with 140 mM Cs^+ external solution and rapamycin treatment (20 nM, 5 minutes). (B), (D), (F) The representative *I-V* curves of TRPC4β currents by 0.2 mM GTPγS were recorded by voltage ramps from +100 to -100 mV for 500 ms, while the cells were held at -60 mV. The black line and the red line in curves indicate before rapamycin treatment and after rapamycin treatment, respectively. (C) GTPγS-activated TRPC4β currents were recorded in HEK cells expressing mTRPC4β-GFP, FKBP-Inp54p, and Lyn-FRB with 140 mM Cs^+ external solution and rapamycin treatment (20 nM, 5 minutes). (E) TRPC4 currents induced by GTPγS-were recorded in HEK cells expressing mTRPC4β-GFP, FKBP-Inp54p, and Lyn-FRB with the internal solution including 20 μM PI(4,5)P_2 with 140 mM Cs^+ external solution and rapamycin treatment (20 nM, 5 minutes).

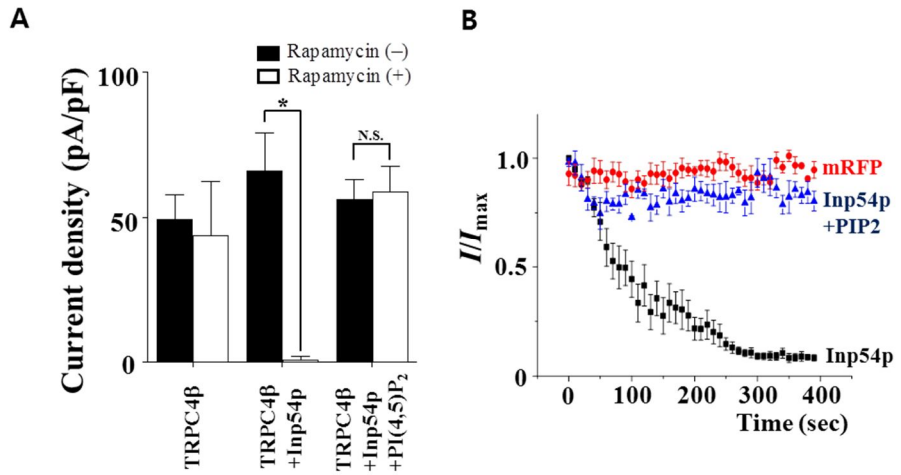

Figure 6. Graphs about regulation of TRPC4β by PI(4,5)P_2.
(A) Summary bar graphs of TRPC4β currents. Current density means maximal current peaks at -60 mV in Cs^+ external solution (means ± S.E.). Statistical significance was denoted by * (p<0.05). N.S. = not significant. (B) TRPC4β currents (I/I_{max}) over time for comparing the changes of control, PIP5K, and Inp54p with coexpression of $G\alpha_{i2}^{Q205L}$.

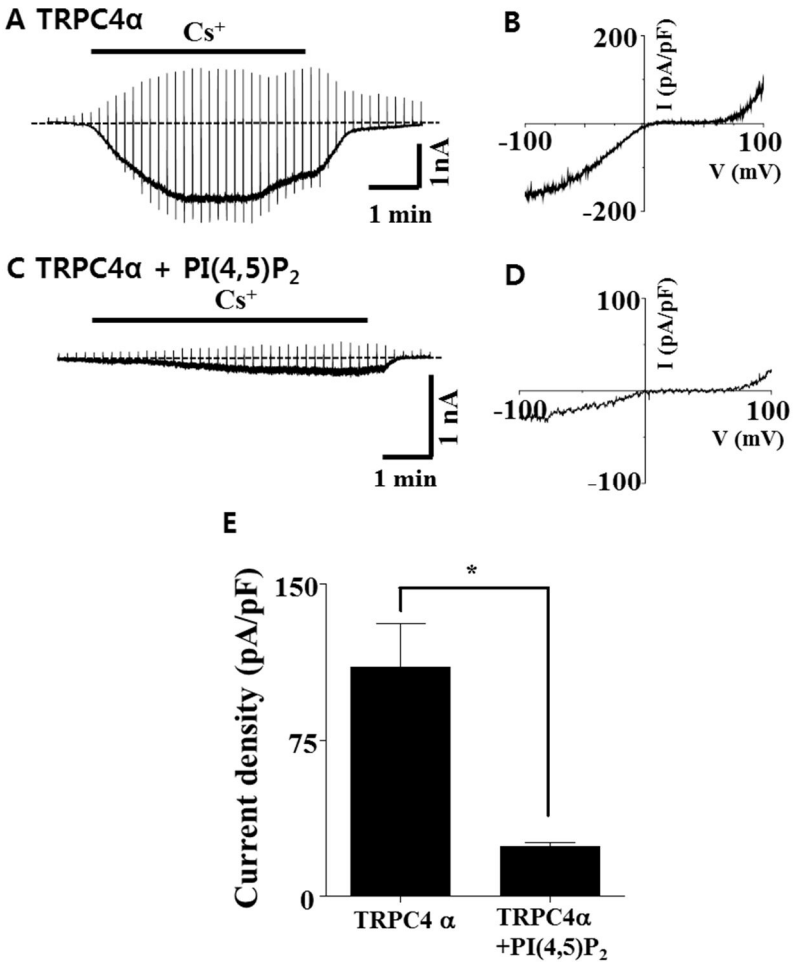

Figure 7. Regulation of TRPC4α by $PI(4,5)P_2$ infusion.
(A) TRPC4 currents were recorded using GTPγS in HEK cells expressing mTRPC4α with 140 mM Cs^+ external solution. (B), (D) The representative *I-V* curves of TRPC4α currents by 0.2 mM GTPγS were recorded by voltage ramps from +100 to -100 mV for 500 ms, while the cells were held at -60 mV. (C) In HEK cells expressing mTRPC4α, GTPγS-activated TRPC4 currents were recorded with the internal solution including 20 μM $PI(4,5)P_2$ with 140 mM Cs^+ external solution and rapamycin treatment (20 nM, 5 minutes). (E) Summary bar graphs of TRPC4β currents. Current density means maximal current peaks at -60 mV in Cs^+ external solution (means ± S.E.).

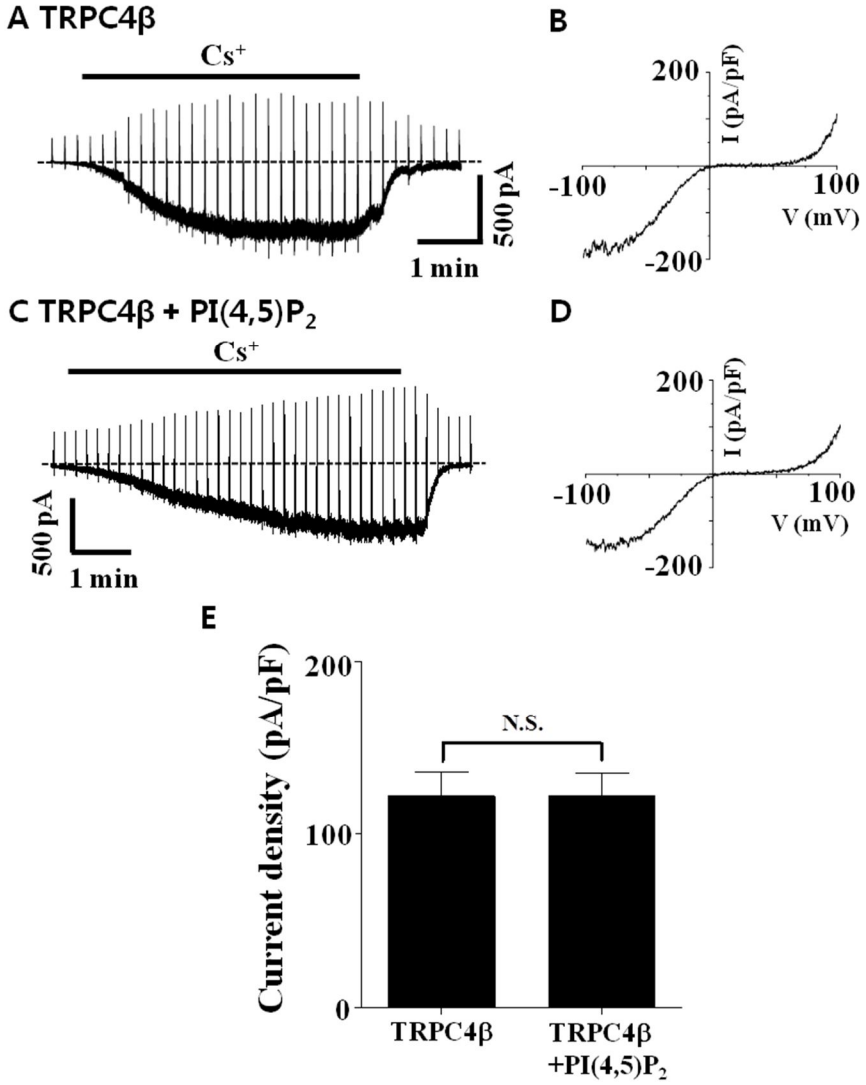

Figure 8. Regulation of TRPC4β by $PI(4,5)P_2$ infusion.
(A) TRPC4 currents were recorded using GTPγS in HEK cells expressing mTRPC4β with 140 mM Cs^+ external solution. (B), (D) The representative *I-V* curves of TRPC4β currents by 0.2 mM GTPγS were recorded by voltage ramps from +100 to -100 mV for 500 ms, while the cells were held at -60 mV. (C) In HEK cells expressing mTRPC4β, GTPγS-activated TRPC4 currents were recorded with the internal solution including 20 μM $PI(4,5)P_2$ with 140 mM Cs^+ external solution and rapamycin treatment (20 nM, 5 minutes). (E) Summary bar graphs of TRPC4β currents. Current density means maximal current peaks at -60 mV in Cs^+ external solution (means ± S.E.). Statistical significance was denoted by * ($p<0.05$). N.S. = not significant.

$PI(4,5)P_2$ is not an activator of TRPC4

We next checked whether the TRPC4β current that is activated by $G\alpha_{i2}$ (Jeon et al, 2008; Jeon et al, 2012) is regulated by an increase or decrease in $PI(4,5)P_2$ levels. Constitutively active $G\alpha_{i2}^{Q205L}$, which was co-expressed with TRPC4β, elicited activation of TRPC4β (Fig. 9A, B; n = 7, 161.7 ± 20.5 pA/pF: peak currents and 152.8 ± 20.9 pA/pF: after rapamycin treatment). In the presence of constitutively active $G\alpha_{i2}^{Q205L}$, the depletion of $PI(4,5)P_2$ using the Inp54p-inducible system also led to inhibition of TRPC4β currents about 93.3 ± 3.9% after application of rapamycin (Fig. 9C, D and Fig. 10A; n = 8, 72.2 ± 0.9 pA/pF: peak currents and 4.8 ± 2.8 pA/pF: after rapamycin treatment). However, the increase of $PI(4,5)P_2$ production using the PIP5K-rapamycin system, did not lead to a further opening of TRPC4β after application of rapamycin when constitutively active $G\alpha_{i2}^{Q205L}$ is present (Fig. 9E, F and Fig. 10A; n = 6, 102.3 ± 15.8 pA/pF: peak currents and 106.5 ± 36.1 pA/pF: after rapamycin treatment). The TRPC4 current activated by $G\alpha_{i2}^{Q205L}$ was reduced by $PI(4,5)P_2$ specific depletion using an inducible system (Fig. 10 and Fig. 11).

To investigate whether $PI(4,5)P_2$ is the activator of the TRPC4β as well as an essential factor, we used a PIP5K-inducible system to enhance the effect of $PI(4,5)P_2$ on TRPC4β activity; PIP5K restores $PI(4,5)P_2$ by converting the PI(4)P to PI(4,5)P2, which increases $PI(4,5)P_2$ levels (Loijens et al, 1996). We investigated whether increasing $PI(4,5)P_2$ had an effect on activating TRPC4β without any activators, such as the $G\alpha_q$-PLC pathway or the $G\alpha_{i2}$ protein. Increasing $PI(4,5)P_2$ production only using the PIP5K-inducible system did not lead to any apparent opening of the TRPC4β channels (Fig. 12). To study

whether $PI(4,5)P_2$ acts on TRPC4β as an opening factor, we used three experimental conditions. First, activation of a PIP5K-inducible system with rapamycin treatment did not elicit a further increase of TRPC4β currents, despite an intracellular application of 0.2 mM GTPγS, in the Cs^+ external solution that was used to amplify TRPC4 currents (Fig. 12A-C; n=11, 64.8 ± 20.8 pA/pF: peak currents and 61.5 ± 13.4 pA/pF: after rapamycin treatment). Second, a PIP5K-inducible system with internal solution including 0.2 mM GTPγS did not activate TRPC4β in the Normal Tyrode's (NT) external solution after application of rapamycin (Fig. 12A; n=16, 0.4 ± 0.3 pA/pF: peak currents and 1.0 ± 0.3 pA/pF: after rapamycin treatment). These data raised a question on whether $PI(4,5)P_2$ is an activator of the TRPC4β channel. Third, in the absence of GTPγS, a PIP5K-inducible system using a 140 mM Cs^+ external solution did not activate TRPC4β after application of rapamycin (Fig. 12A; n=8, 1.5 ± 0.3 pA/pF: peak currents and 1.9 ± 0.3 pA/pF: after rapamycin treatment). These results suggest that, even if the $PI(4,5)P_2$ level increases, $PI(4,5)P_2$ was not acting as an effective activator of the TRPC4β channel.

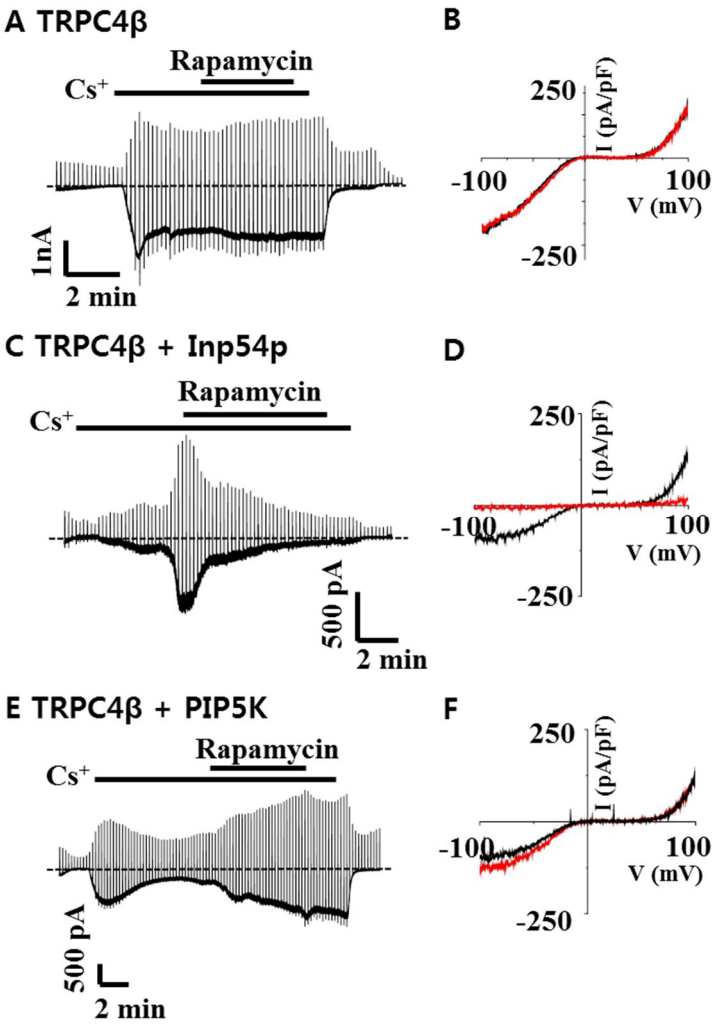

Figure 9. The necessity of PI(4,5)P_2 for maintaining the steady state of $G\alpha_{i2}$-activated TRPC4β.

(A) $G\alpha_{i2}^{Q205L}$-activated TRPC4β currents were recorded in HEK cells expressing mTRPC4β and $G\alpha_{i2}^{Q205L}$ with 140 mM Cs^+ external solution and rapamycin treatment (20 nM, 5 minutes). (B), (D), (F) The representative *I-V* curves of TRPC4β currents by $G\alpha_{i2}^{Q205L}$ were recorded by voltage ramps from +100 to -100 mV for 500 ms, while the cells were held at -60 mV. The black line and the red line indicate before rapamycin treatment and after rapamycin treatment, respectively. (C), (E) In HEK cells, the representative current traces of TRPC4 currents which is induced by $G\alpha_{i2}^{Q205L}$ were recorded with 140 mM Cs^+ external solution. mTRPC4β-GFP and Lyn-FRB co-expressed with either FKBP-Inp54p or FKBP-PIP5K.

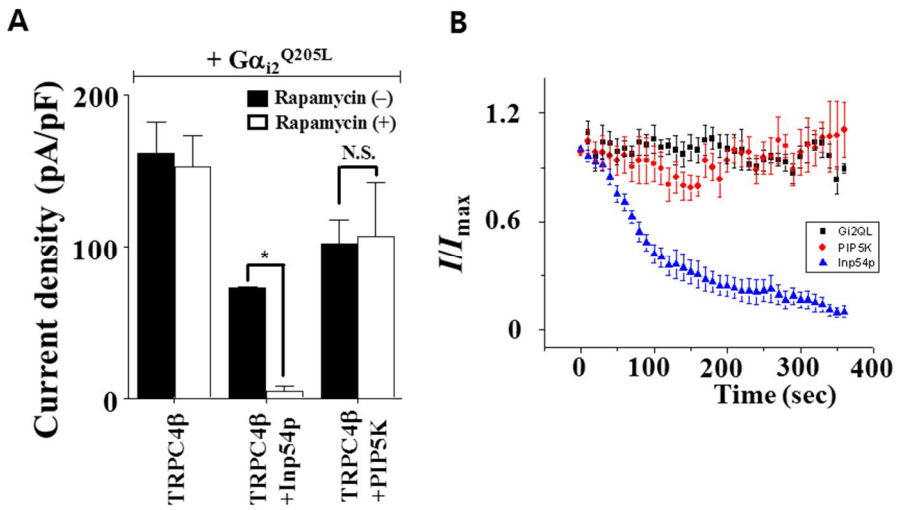

Figure 10. Graphs about the necessity of PI(4,5)P_2 to maintain the Gα_{i2}^{Q205L} induced TRPC4β.

(A) Summary bar graphs of TRPC4β currents. Current density means maximal current peaks at -60 mV in Cs^+ external solution (means ± S.E.). Statistical significance was denoted by * (p<0.05). N.S. = not significant. (B) Schematic diagram that shows the reduction of Gα_{i2}^{Q205L}-induced TRPC4β currents by the translocation of rapamycin-inducible Inp54p.

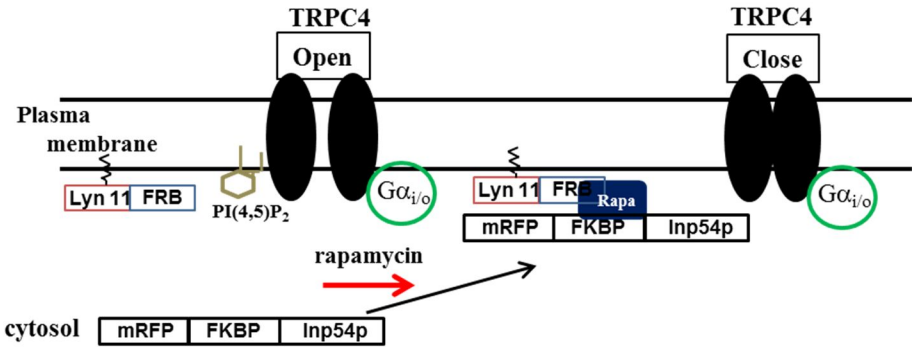

Figure 11. Schematic diagram that shows reduction of $G\alpha_{i2}^{Q205L}$-induced TRPC4β currents by the translocation of rapamycin-inducible Inp54p.

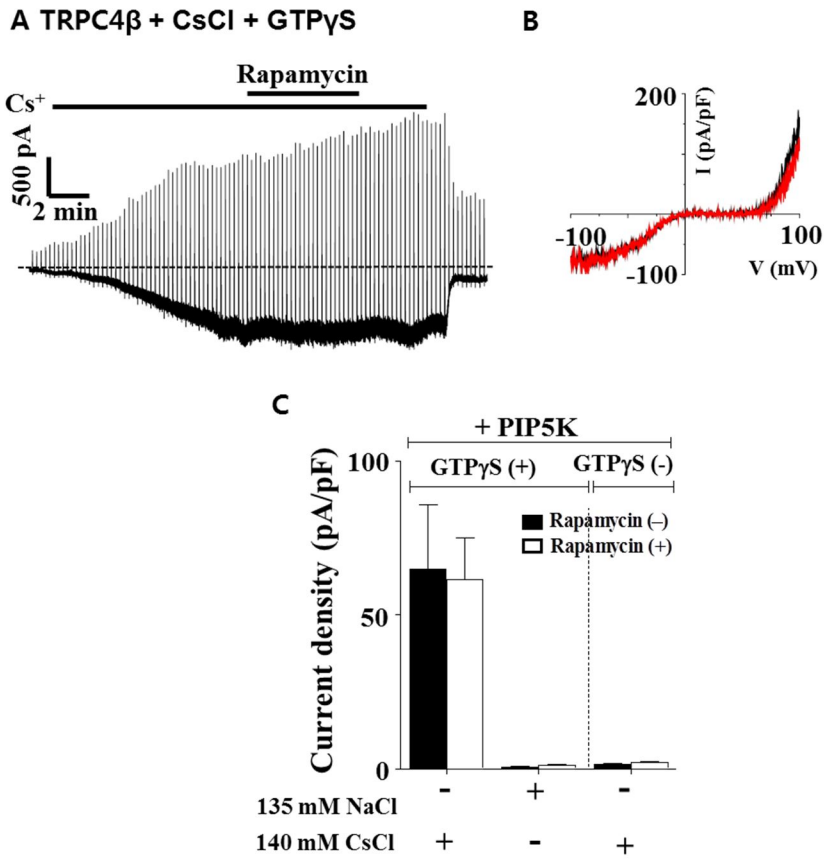

Figure 12. The possibility of PI(4,5)P_2 to be the initial activator of the TRPC4β.

(A) TRPC4β currents activated by GTPγS were recorded in HEK cells expressing mTRPC4β, FKBP-PIP5K, and Lyn-FRB with 140mM Cs^+ external solution with 140 mM Cs^+ external solution and rapamycin treatment (20 nM, 5 minutes). (B) The representative *I-V* curves of TRPC4β currents by 0.2 mM GTPγS were recorded by voltage ramps from +100 to -100 mV for 500 ms, while the cells were held at -60 mV. The black line and the red line indicate before rapamycin treatment and after rapamycin treatment, respectively. (C) Summary bar graphs of TRPC4β currents. Current density means maximal current peaks at -60 mV in Cs^+ external solution (means ± S.E.).

$PI(4,5)P_2$ regulates a desensitization in M3-induced TRPC4 activation.

We tested the role of $PI(4,5)P_2$ in muscarinic receptor 3 (M3)-induced TRPC4 currents. M3-induced TRPC4 currents showed desensitization that caused by PKC activation. The activation of TRPC4 by M3 with carbachol (CCh) was desensitized within 1 minute (Fig. 13 A, B and Fig.14; n = 9, 177.1 ± 25.5 pA/pF: peak currents and 19.8 ± 6.9 pA/pF: after CCh treatment). In comparison, the desensitization was not detected by using intracellular application of $PI(4,5)P_2$ and ms-$PI(4,5)P_2$ (20 μM) which is a non-hydrolysable analogue of $PI(4,5)P_2$ in M3 with TRPC4 expressing HEK293 cells (Fig. 7 C-F and Fig.14; n = 10, 198.6 ± 42.0 pA/pF: peak currents and 115.2 ± 15.8 pA/pF: after CCh treatment and n = 9, 229.5 ± 25.6 pA/pF: peak currents and 152.7 ± 14.0 pA/pF: after CCh treatment).

Therefore, these results suggest that $PI(4,5)P_2$ is essential for sustaining the activated state of TRPC4β but is not the factor needed for the initial activation. In summary, the $G\alpha_q$-PLC pathway regulates $PI(4,5)P_2$ which is necessary for activating the channel, and channel activation by $G\alpha_{i2}$ proteins requires $PI(4,5)P_2$ to sustain the activation.

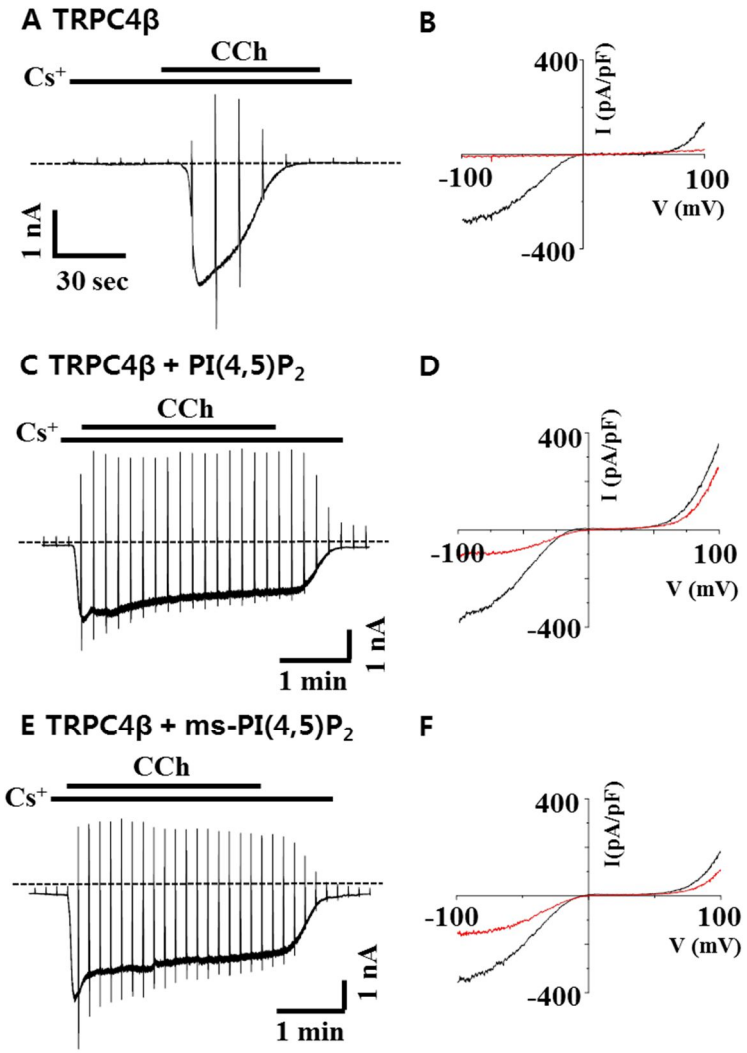

Figure 13. The relationship between PI(4,5)P_2 and TRPC4β. desensitization. (A) M3-activated TRPC4 currents were recorded in HEK cells expressing mTRPC4β and M3 with 140 mM Cs^+ external solution and activated by 100 μM CCh.. (B), (D), (F) The representative *I-V* of M3-activated TRPC4 currents were measured by voltage ramps from +100 to -100 mV for 500 ms, while the cells were held at -60 mV. The black line indicates *I-V* curve of maximal peak current at CCh-activated currents and the red line indicates *I-V* curve of current at the 120 sec application of CCh (100 μM). (C), (E) Representative current trace of M3-acitvated TRPC4 currents. M3-activated TRPC4 currents did not show desensitization in the presence of 20 μM PI(4,5)P_2 and 20 μM ms-PI(4,5)P_2 with 140 mM Cs^+ external solution.

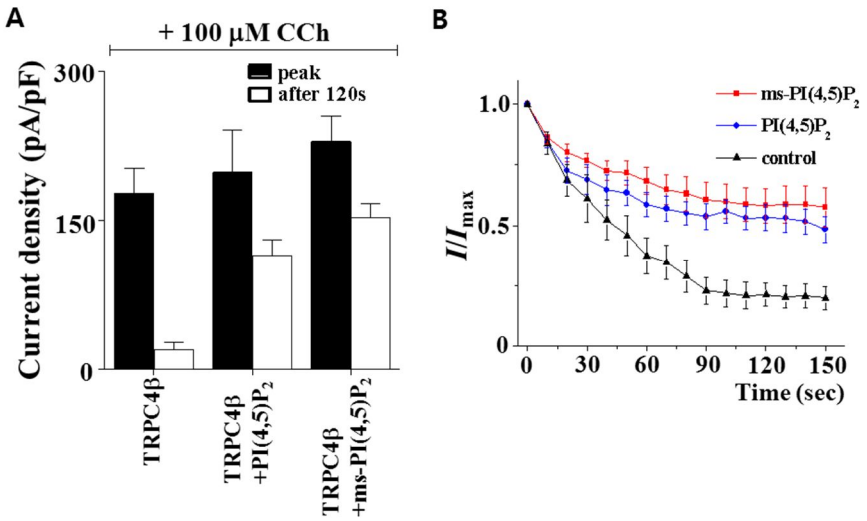

Figure 14. Compared graphs about the relationship between $PI(4,5)P_2$ and TRPC4β desensitization.

(A) Summary bar graphs of TRPC4β currents. Current density means maximal current peaks at -60 mV in Cs^+ external solution (means ± S.E.). Statistical significance was denoted by * ($p<0.05$). (B) TRPC4β currents (I/I_{max}) over time for comparing the changes of mock, $PI(4,5)P_2$ and ms-$PI(4,5)P_2$.

Discussion

Many channels are positively and negatively regulated by $PI(4,5)P_2$ (Suh and Hille, 2008). $PI(4,5)P_2$ dependence also occurs in the TRP channels (Rohacs, 2007; Qin, 2007). Another group has previously reported that by applied $PI(4,5)P_2$ inhibits TRPC4α, not TRPC4β (Otsuguro et al, 2008) whereas the other displayed the stimulation of TRPC4 and TRPC5 channels by $PI(4,5)P_2$ (Kim et al, 2008; Jeon et al, 2008). Our results suggest a new role of $PI(4,5)P_2$ in the regulation of TRPC4β channels. We thought that the difference between TRPC4α and TRPC4β is caused by the known binding domain of $PI(4,5)P_2$ on TRPC4α, 84 amino acid in the C-terminal region (Schaefer et al, 2000).

In our previous study, $G\alpha_q^{Q209L}$ mutants inhibited TRPC4 and TRPC5 currents, although the $G\alpha_q$-PLC pathway was considered as a main activation pathway. In the $G\alpha_q$-inducible system, $G\alpha_q$ inhibited TRPC4 currents, but PLCβ interaction-impaired mutants did not inhibit TRPC4 currents. Considering the $G\alpha_q$-PLC pathway, the $G\alpha_q$-PLC signal can activate its downstream signals and can deplete $PI(4,5)P_2$ levels. Our experimental findings ruled out the effects of changes in PKC on TRPC4 activation, and pointed to an indirect effect of $G\alpha_q$ on channel function.

Thus, we used a $PI(4,5)P_2$-specific rapamycin system. Depletion of $PI(4,5)P_2$ by the Inp54p-rapamycin system led to the reduction of TRPC4 currents whether TRPC4 currents were activated by GTPγS or by a $G\alpha_{i2}^{Q205L}$ protein, which was compensated with an internal solution containing $PI(4,5)P_2$. Enhancing $PI(4,5)P_2$ production by the PIP5K-rapamycin system, however, did not further activate TRPC4 currents. $PI(4,5)P_2$ alone did not activate

TRPC4 channels as it did not in GIRK2 channel (Kim et al, 2008, Jeon et al, 2012; Otsuguro et al, 2008). These data suggest that $PI(4,5)P_2$ is an essential factor for keeping TRPC4β open but is not an initial gating factor for TRPC4β activation.

TRPC4 is a molecular candidate for the nonselective cation channels (NSCC) that are activated by muscarinic stimulation-cation currents (I_{mCaT}) in the gastrointestinal (GI) tract (Lee et al, 2005; Tsvilovskyy et al, 2009). I_{mCaT} is known to be regulated by both $G\alpha_{i/o}$ and the $G\alpha_q$ pathway in native GI tissues. Our present results showed that $PI(4,5)P_2$ is necessary for regulating TRPC4 through $G\alpha_q$-PLC pathway and through $G\alpha_{i2}$. The regulation of NSCC currents and GI motility by muscarinic receptor 2 (M2) and muscarinic receptor 3 (M3) is similar to that of TRPC4 by muscarinic receptors (So and Kim, 2003). In GI native tissues, the activation of I_{mCaT} was related to $G\alpha_{i/o}$ proteins, whereas the desensitization of I_{mCaT} was related to $G\alpha_q$ protein due to the activation of PKC and $PI(4,5)P_2$ hydrolysis.

As the inhibition of inwardly rectifying K^+ channels by receptor-induced depletion of $PI(4,5)P_2$ (Cho et al, 2005), we thought that depletion of $PI(4,5)P_2$ by $G\alpha_q$-PLC pathway leads to inactivation of TRPC channel. It does not mean $G\alpha_q$-PLC pathway is inactivation pathway because in the normal condition, severe depletion of $PI(4,5)P_2$ did not occurred. We hypothesize that $PI(4,5)P_2$ plays an important role for regulating TRPC4 in that the presence of $PI(4,5)P_2$ affects the coupled G loop gate and a second inner helix gate so that both gates open on the GIRK channel (Whorton and MacKinnon, 2011).

In conclusion, $PI(4,5)P_2$ is an essential lipid for the steady activation of TRPC4 whether it is activated by the $G\alpha_q$-PLC pathway or $G\alpha_{i/o}$ proteins.

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국 문 초 록

칼슘 투과성을 가진 비선택적 양이온 통로인 Transient Receptor Potential Canonical (TRPC) channels은 동물 세포에 존재하고 많은 세포의 기능과 관련있다. 많은 연구들이 $G\alpha_q$-PLC 경로가 TRPC 이온통로들을 활성화시키는 기전이라고 주장하고 있다. 하지만, 이전 논문을 통해 우리는 $G\alpha_q$의 활성화 형태($G\alpha_q^{Q209L}$)가 TRPC 전류를 억제하는 것을 보고했다. 이 보고를 하면서 우리는 $G\alpha_q^{Q209L}$가 계속적으로 포스포리파아제β(PLCβ)를 활성화시키기에 포스파티딜이노시톨 4,5-비스인산($PI(4,5)P_2$; phosphatidylinositol 4,5-bisphosphate)이 부족해지는 것이 원인일 것이라고 생각했다. 그래서 우리는 포스파티딜이노시톨 4,5-비스인산을 특별하고 정확하게 조절할 수 있는 유도계(inducible system)를 사용하였다. $G\alpha_q^{Q209L}$유도계에 의해서 TRPC4β 전류가 감소하였지만 PLCβ와의 결합을 하지 못하게 만든 돌연변이에 의해서는 TRPC4β 전류가 감소하지 않았다. 이노시톨폴리인산 5-인산가수분해효소(Inp54p; inositol polyphosphate 5-phosphatase) 유도계를 이용해서 $PI(4,5)P_2$를 고갈시켰을 때는 TRPC4 이온통로의 활성이 비가역적으로 억제되는 것을 볼 수 있었다. 반면에, 다른 활성요소들을 제외시키고 포스파티딜이노시톨 4-인산 5-인산화효소(PIP5K; phosphatidyl inositol 4-phosphate 5-kinase) 유도계를 작용시켰을 때 TRPC4β를 활성화시키지 못했기에 TRPC4β의 직접적인 활성화 인자는 아니라고 생각했다. 하지만 TRPC4β의 탈감각화(desensitization)가 일어날 때 $PI(4,5)P_2$를 적용하면 탈감각화 현상이 줄어드는 것을 통해서 TRPC4β의 탈감각화(desensitization) 조절에 $PI(4,5)P_2$가 중요하다는 것을 알았다. 이 논문을 통해서, 우리는 $PI(4,5)P_2$가 TRPC4 활성을 시키지는 못하지만 활성을 조절하기 위한 필수요소라는 것을 주장한다.

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주요어 : TRPC4; $PI(4,5)P_2$; GPCR; rapamycin inducible system

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