Regulation of transient receptor potential canonical 4 activity by phospholipase C-δ1

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Abstract

Transient receptor potential canonical (TRPC) channels are non-selective calcium-permeable cation channels. It is suggested that TRPC4β and TRPC5 channels are regulated by phospholipase C (PLC) signaling, and are especially maintained by phosphatidylinositol 4,5-bisphosphate (PIP$_2$). The PLCδ subtype is the most Ca$^{2+}$-sensitive form among the isozymes which cleaves phospholipids to respond to the calcium rise. In this study, we investigated the regulation mechanism of TRPC channel by Ca$^{2+}$, PLCδ1 and PIP$_2$ signaling cascades. The interaction between TRPC4β and PLCδ1 was identified through the Föster resonance energy transfer (FRET) and co-immunoprecipitation (Co-IP). With the electrophysiological experiments, we found that TRPC4β-bound PLCδ1 reduces the overall whole-cell current of channel. The Ca$^{2+}$-via opened channel promotes the activation of PLCδ1, which subsequently decreases PIP$_2$ level. By comparison TRPC4β activity with or without PLCδ1 using differently [Ca$^{2+}$], buffered solution, we demonstrated that PLCδ1 functions in normal condition with physiological calcium range. The negative regulation effect of PLCδ1 on TRPC4β helps to elucidate the roles of each PIP$_2$ binding residues whether they are concerned in channel maintenance or inhibition of channel activity.
Introduction

Transient Receptor Potential (TRP) channels are nonselective cation channels which are permeable to Ca\(^{2+}\).

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 structures; TRPC1, 4, 5 and TRPC3, 6, 7. Especially, TRPC4 and TRPC5 are expressed in specific tissues like smooth muscle, brain and neurons and their activities are closely related to Ga\(_q\)-phospholipase C (PLC) signaling. Interestingly, TRPC4 is functionally very comparable to TRPC5, but only TRPC5 shows constitutive activity even without stimulation. However, which mechanism makes this difference is still obscure. Many researches demonstrated the effect of downstream molecules on TRPC channels, which includes PLC, phosphatidylinositol 4,5-biphosphate (PIP\(_2\))\(^{1-4}\), diacylglycerol (DAG)\(^{5,6}\), protein kinase C (PKC)\(^7\) and Ca\(^{2+}\). We previously emphasized the self-limiting mechanism of Ga\(_q\) pathway on channels that Ga\(_q\) protein solely activates TRPC1/4 and TRPC1/5 channels, and then PIP\(_2\) depletion and PKC or Ca\(^{2+}\) inhibits them\(^8,9\). These data implicate that TRPCs are strongly activated by Ga\(_q\), hence fast and tight regulation is necessary afterwards.

The PLC is an enzyme that cleaves PIP\(_2\) into inositol triphosphate (IP\(_3\)) and DAG. There are ten kinds of PLC isozymes that are largely classified into three families; \(\beta, \gamma, \delta\)\(^10,13\). Among them, primordial PLC\(\delta\) family has basic structure with the shortest length. \(\delta\) subtypes have about 10-folds higher sensitivity to calcium than the other isozyme\(^14,15\). The PLC\(\delta1\)subtype is abundantly expressed in brain, heart, lung, smooth muscle and spleen\(^16\). The precise activation mechanism of PLC\(\delta1\) is not known, but it is expected that the Ca\(^{2+}\) concentration mainly controls their activity\(^17\), and IP\(_3\), the product of PIP\(_2\) hydrolysis is involved in its negative regulation\(^18\).

PIP\(_2\) is, above all else, the most important component in TRPC channel’s regulation as they are concerned with channel maintenance. Other TRP channels are also known to be regulated by PIP\(_2\) and the effect of PLC\(\delta\) subtypes on this action has been proposed. The PLC\(\delta3\) co-expression facilitated the desensitization of TRPV1 in oocytes\(^19\), and also, subsequent PIP\(_2\) reductions by PLC\(\delta\) isoform promoted reduction in TRPM8 currents\(^20\). Until now, we have only focused on this effect, however others have shown that PIP\(_2\) exert along with negative effect on TRPC activities. In understanding the main role of PIP\(_2\) as an inhibitor of TRPC4, Thakur et al. suggested that PLC\(\delta1\) mediately facilitates TRPC4\(\beta\) activation with Ga\(_{io}\) signaling pathway\(^21\) and the apparent correlation between TRPC4\(\beta\) and PLC\(\delta1\) enzyme. Thus, understanding the regulatory mechanism that alters TRPC activity related to PIP\(_2\) level change will provide the insights of how channels are tightly regulated by PLC signaling, and also it could be demonstrated that how PIP\(_2\) on TRPC channel functions differently.

In the present study, we set out to determine the series of events with PLC\(\delta1\) that regulates TRPC4\(\beta\) activity tightly in view of negative feedback. We identified that PLC\(\delta1\) interacts with TRPC4\(\beta\) but not with TRPC5. The calcium influx induced by TRPC4\(\beta\) activates PLC\(\delta1\) and this effect was dependent on physiological cytosolic calcium range. The activated PLC\(\delta1\) hydrolyzes PIP\(_2\), hence TRPC4\(\beta\) which was maintained by PIP\(_2\) shows relatively inhibited currents. By comparing the effect of PLC\(\delta1\) on TRPC4\(\beta\) mutant which interacts with PIP\(_2\), we suggest that each residue affects the channel activity differently by binding with PIP\(_2\).
Results

**TRPC4β directly interacts with PLCδ1**

TRPC4β is functionally very comparable to TRPC5, but only TRPC5 shows constitutive activity even without stimulation. Previously, we also demonstrated that the PIP2 binds to TRPC4 and TRPC5 with different affinity. Thus, we hypothesized that TRPC4 and TRPC5 channels have different mechanism to control 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 Human Embryonic Kidney (HEK)293 cell line transiently co-transfected with ECFP tagged channel (TRPC4β or TRPC5) and YFP tagged PLCδ (PLCδ1 or PLCδ3) enzyme. In the cases of expressing EYFP-PLCδ alone, PLCδ1 expressed on the plasma membrane and also distributed in the cytoplasm, PLCδ3 is detected in membrane and nuclear fraction (see Supplementary Fig. S1 online). However, their expression pattern was changed when they were co-expressed with TRPC4β. As shown in Figure 1a, EYFP-PLCδ1 showed high fluorescence on the same position where TRPC4β-ECFP showed puncta on the membrane. The overlay image and line scan in this position showed 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 the TRPC4β expressed puncta region was observed to be empty (Fig. 1a bottom). In other words, the distribution of PLCδ3 showed totally opposite to the TRPC4β. We observed that TRPC5 was not colocalized with any 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 is resulted from direct interaction or just close location of these proteins. We used ECFP (donor)-TRPC ion channel and YFP (acceptor)-PLCδ enzyme constructs, and measured FRET efficiency between them. As same as fluorescence imaging, TRPC4β with PLCδ1 showed high FRET efficiency (Fig. 1b), but TRPC4β with PLCδ3, TRPC5 with PLCδ1 and TRPC5 with PLCδ3 showed low FRET efficiency (Fig. 1d).

As a final approach to identify the binding between TRPC and PLCδ, we expressed Flag tagged TRPC ion channel and YFP tagged PLCδ enzyme and analyzed them via co-immunoprecipitation (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 TRPC4β and PLCδ1, which indicates that only TRPC4β interacts with PLCδ1 (Fig. 1e).

[Figure 1, near here]

**PLCδ1 decreases TRPC4β currents amplitude and cytosolic Ca2+ determines the activity of PLCδ1**

To determine the function of PLCδ1 on TRPC4β, we measured the changes in channel current. We used rapamycin-inducible system to translocate PLCδ1 with an efficient amount enough to the membrane. HEK293 cells were transfected with CFP-FKBP-PLCδ1 and Lyn-FRB, and 50 nM of rapamycin was used which is sufficient to translocate the PLCδ1 to the membrane (Fig. 2a). To investigate the TRPC4β current changes, we performed whole-cell patch clamp experiments with ramp pulse protocol from +100 to -100 mV every 10 s in the cells expressing TRPC4β, CFP-FKBP-PLCδ1 and Lyn-FRB. First, cells were treated with 50 nM rapamycin to translocate PLCδ1 with an efficient amount, then TRPC4β was stimulated by a channel agonist, (+)-Englerin A (EA, 100 nM). In control cells expressing without Lyn-FRB, rapamycin did not show any current changes and (+)-EA-evoked TRPC4β currents showed characteristic double-rectifying current-voltage relationship (Fig. 2b). Also, the presence of Lyn-FRB showed the characteristics of TRPC4β, however the currents with EA stimulation was significantly decreased (Fig. 2c and d). Thus, these results suggest that PLCδ1 functions as a negative regulator of TRPC4β to reduce the currents.

[Figure 2, near here]

We also investigated the Ca2+-dependent effect of PLCδ1 on TRPC4β channel currents. TRPC channels are potentiated by intracellular calcium22, and especially, the Ca2+ is the main source in the regulation of PLCδ1 activity17. Thus, we performed whole-cell patch clamp experiments by using different internal Ca2+ buffering solution. 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 are expressed with TRPC4β alone or PLCδ1 together, and channels are stimulated with 100 nM (-)-EA. Channel currents were recorded with ramp pulse protocol from +100 to -100 mV every 10 s. With 50 nM [Ca2+], buffered internal solution, TRPC4β alone or TRPC4β and PLCδ1 expressed cells elicited current slowly after EA stimulation (Fig. 3a). In this condition, the presence
of PLCδ1 decreased the TRPC4β channel’s peak current density but it was not significantly different in the cells expressing TRPC4β alone (Fig. 3b). By increasing [Ca\(^{2+}\)]\_i to 100 nM, TRPC4β 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 PLCδ1 together (Fig. 3d). These aspects of current decrease with the cells expressing PLCδ1 together were also observed in the experiments with 500 nM [Ca\(^{2+}\)]\_i pipette solutions. Interestingly, they showed robust current activation and desensitized quickly after reaching the peak current (Fig. 3e). Besides, the presence of PLCδ1 significantly decreased the TRPC4β currents (Fig. 3f). Exceptionally, the presence of PLCδ1 changed the time course of currents recording in 100 nM calcium condition, only. In the cells only expressing TRPC4β, higher internal calcium concentration potentiates channel to show the faster time course and also, 500 nM [Ca\(^{2+}\)]\_i is sufficient to activate endogenous PLC to cause the current desensitization. PLCδ1 reduced the overall TRPC4β currents in 100 nM and 500 nM free Ca\(^{2+}\). Therefore, we suggest that physiological calcium concentration is sufficient to activate the PLCδ1 after channel stimulation, and the activity of PLCδ1 is tightly regulated by the calcium concentration.

[Figure 3, near here]

**TRPC4β-induced Ca\(^{2+}\) activates PLCδ1, and it accelerates PIP\(_2\) depletion**

PLC is an enzyme that cleaves phospholipids in Ca\(^{2+}\)-dependent manner. TRPC4 channel is a nonselective cation channel which is permeable to calcium as well as monovalent. Therefore, Ca\(^{2+}\) influx-induced by TRPC4β activation may act as a stimulator of PLCδ1. To identify the activity of PLC, we used PH-domain of PLCδ1 as a PIP\(_2\) sensor to monitor the PIP\(_2\) changes. We co-expressed TRPC4β, PLCδ1 and CFP-tagged PH domain in HEK293 cells, and TRPC4β was stimulated by 100 nM EA. At first, CFP-PH was observed on the membrane, and also, line scan showed higher fluorescence intensity in the membrane than cytosol before channel stimulation. In the cells expressing TRPC4β without PLCδ1, CFP-PH still showed similar intensity in the membrane and cytosol even after 30 seconds of EA stimulation. On the other hand, TRPC4β and PLCδ1 co-expressed cells showed relatively faster PIP\(_2\) depletion from membrane to cytosol. In these cells, 30 seconds of EA stimulation was enough to show higher fluorescence intensity of CFP-PH in the cytosol than the membrane (Fig. 4b). These results suggest that calcium influx via TRPC4β activates PLCδ1, and it causes the acceleration of PIP\(_2\) depletion.

[Figure 4, near here]

As a final approach, we evaluated the effect of PLCδ1 on TRPC4β. We used two mutant forms of PLCδ1 (see Supplementary Fig. S3 online). The first mutant is PLCδ1 (K30A/K32A) which interrupted the PIP\(_2\) binding, as Lys30 and Lys32 residues are located in the PH-domain\(^{21}\). And the second one is PLCδ1 (H311A) which shows abolished phospholipase activity because His311 residue is located in the X-region of catalytic domain (Fig. 5c)\(^{22}\). We measured TRPC4β currents in the cells expressing TRPC4β and PLCδ1 (K30A/K32A) or PLCδ1 (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 expressing both mutants, the TRPC4β currents showed similar time course as TRPC4β alone (Fig. 5a and b). Furthermore, EA-evoked currents in the cells expressing PLCδ1 (K30A/K32A) and PLCδ1 (H311A) with TRPC4β were significantly higher than in the cells expressing wildtype PLCδ1, and it was to the extent of TRPC4β channel alone (Fig. 5d). In other words, PLCδ1 which cannot regulate the PIP\(_2\) level did not have any effect on the TRPC4β activity. Therefore, these results suggest that TRPC4β-bound PLCδ1 regulates PIP\(_2\) level to decrease TRPC4β currents.

[Figure 5, near here]

**Each PIP\(_2\) binding residue of TRPC4β has distinct role on TRPC4β activity**

TRPC4 channels are known to be maintained by PIP\(_2\), but also the possibility of inhibition by PIP\(_2\) was suggested\(^{21}\). Previously, we determined two PIP\(_2\) binding pockets of TRPC4β based on its structure and patch clamp recordings\(^5\). We suggested the five PIP\(_2\) interaction residues such as Lys419A, Arg511A, Lys518, His630 and Lys664. However, the role of each residue is still unclear. Therefore, we investigated whether mutants of these residues are affected by PLCδ1. HEK293 cells are transiently transfected with the mutant of PIP\(_2\) binding residue of TRPC4β alone, or PLCδ1 together, and channels were stimulated by 100 nM EA until they reached the peak current. We used 100 nM free calcium recording pipette solution and channel currents were recorded with the same ramp pulse protocol as in previous experiments. All of these mutants
showed TRPC4’s typical current-voltage relationship. TRPC4β (K419A) and TRPC4β (K518A) mutants showed smaller EA-induced currents than the wildtype (Fig. 6a and e). In the cells expressing TRPC4β (K419A) with PLCδ1, they showed decreased channel activity, but it was not significantly different (Fig. 6b). On the contrary, TRPC4β (K518A) with PLCδ1 showed relatively increased currents, but it was not significantly different (Fig. 6f). The amplitude of TRPC4β (H630A) currents was smaller than the wildtype as well, but it was not as much as TRPC4β (K419A) and TRPC4β (K518A) (Fig. 6g). And additionally, PLCδ1 expressed with TRPC4β (H630A) did not decrease currents (Fig. 6h). Exceptionally, TRPC4β (R511A) mutant elicited currents as much as TRPC4β wildtype after EA stimulation, and there were no significant differences between the wildtype and the mutant Arg511A, even in the presence of PLCδ1. Interestingly, EA stimulation potentiated more than wildtype in the cells expressing TRPC4β (K664A) (Fig. 6i), and current amplitude was decreased with PLCδ1 which is almost 2-folds smaller than in TRPC4β and PLCδ1 expressed cells (Fig. 6j). As a result, mutation of Lys419, Lys518 and His630 residue itself showed reduced channel activity, and PLCδ1 did not affect their currents. Instead, mutation of Lys664 residue showed increased currents, and PLCδ1 inhibited channel current extremely. TRPC4β (R511A) did not show any difference in comparison to wildtype TRPC4β. Thus, we suggest that Lys419, Lys518 and His630 contribute to maintain TRPC4β by binding with PIP2, and Lys664 contributes to channel inhibition. Exceptionally, Arg511 has no effect on TRPC4β currents.

Collectively, our findings suggest that TRPC4β interacts with PLCδ1 directly, and TRPC4β-bound PLCδ1 shows negative feedback regulation. The mechanism is as followed. When TRPC4β is activated, Ca\textsuperscript{2+} influx via TRPC4β activates PLCδ1. And then, activated PLCδ1 hydrolyzes PIP2 to IP3 and DAG. As TRPC4β channels are maintained by PIP2, TRPC4β is inhibited. Ultimately, the intracellular calcium level tightly regulates TRPC4β activity through PLCδ1.

[Figure 6, near here]
Discussion

Activity of TRPC4 ion channel is closely controlled by PIP2. In this study, we found that PLCδ1 is involved in TRPC4 regulation. The conclusions of this study are as follow: 1. TRPC4 directly interacts with PLCδ1, but TRPC5 has no interaction with any PLCδ subtypes. 2. PLCδ1 causes inhibition in TRPC4 currents in a calcium dependent manner. 3. TRPC4-bound PLCδ1 responds to the calcium influx by the channel, which in turn depletes PIP2. 4. Each PIP2 interaction residue of TRPC4 has a distinct role of inhibiting or maintaining the channel by PIP2 binding. Altogether, we propose a negative feedback regulation of TRPC4 by PLCδ1 in calcium and PIP2 dependent manner.

Previously, we suggested that Gaβ3-PLC pathway has a self-limiting activation on TRPC channels. The Gaβ3 protein strongly activates TRPC heteromeric channels, and sequential events such as PIP2 depletion, PKC and Ca2+ increase results in channel inhibition. We emphasized several times that regulation of PIP2 level is most important as they are 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 the PIP2 appears to play a key role and ever-present regulator of TRPM channels. Furthermore, TRPM8 neurons express Ca2+-sensitive PLCδ isozymes, and in vivo data showed that the activity is regulated by PLCδ4. Also, TRPV1 is suggested that PIP2 is necessary for the normal function of channel. In this context, the desensitization of the TRPV1 currents is accelerated and eventually completely suppressed when PLCδ3 is expressed together, and even desensitization was disappeared in PLCδ4/. 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 PLCδ1 directly interact with TRPC4β, based on the results of FRET and Co-IP experiments (Fig. 1). The inclusion of PLCδ1 in regulation of TRPC4β channel activity has been reported as an underlying concept of inhibitory PIP2. They suggested that TRPC4β activity was affected by PLCδ1 in preference to PLCδ3, and our data are consistent with this result that it might be from their direct interaction.

It also appeared clearly in expression patterns. The PLCδ1 is expressed in both membrane and cytoplasm, and PLCδ3 is expressed in membrane. In our hands, PLCδ1 showed denser fluorescence intensity where TRPC4β is present, and they co-localized together. Interestingly, PLCδ3 was observed to be empty in that channel puncta region when it was expressed with the TRPC4β or TRPC5 (Fig.1a and c). As PLC is a membrane-associated enzyme and TRPC channels are transmembrane channel, this expression pattern implies that the membrane resident proteins are present on the membrane competitively. Also, this difference presumably reflects that TRPC4β 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 PLCδ1 binds to TRPC4β but not TRPC5, and it affects PIP2 level to regulates 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 characteristic. 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 precedent study, TRPC5 relatively showed robust current inhibition with the weak voltage stimulation which activates voltage-sensitive phosphatase. Based on our data, we suggest that PIP2 and PLCδ1 are a possible candidate underlying these features. TRPC4β-bound PLCδ1 would continuously regulates PIP2 level in the vicinity of the channel even without the stimulation, and it would attribute TRPC4β to show no basal activity. In our hands, TRPC5 interacts with neither PLCδ1 nor PLCδ3, thus relatively abundant PIP2 pool would exist around TRPC5. The detailed knowledge of mechanism causing differences between TRPC4β 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 PLCδ1 activity produces decreased TRPC4β currents in calcium dependent manner. In the presence of PLCδ1, EA-evoked TRPC4β currents were significantly reduced (Fig. 3). Whether calcium increase alone causes the optimal activation of PLCδ1 is still controversial. It was suggested that high potassium in extracellular, or stimulations like thapsigargin or ionomycin also causes an increase in IP3, but much less than the amount that increased by the fully activated PLCδ subtypes. Through experiments with differing intracellular calcium concentration, our data agree with the notion that the channel-induced calcium influx with physiological calcium range is sufficient to activates PLCδ1. In our hands, 50 nM of [Ca2+]i condition was not enough to fully activate PLCδ1, but PLCδ1 significantly reduced channel currents with the higher calcium condition with 100 and 500 nM. Thus, we
mainly used the 100 nM [Ca\(^{2+}\)], buffering solution in electrophysiological experiments which shows prominent difference including reduced currents amplitude and alteration in the time course of currents in the presence of PLCζ1. Interestingly, the time course of channel currents was changed depending on the PLCζ1 presence (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\(_i\)PCR pathways\(^{22-34}\). Therefore, such desensitization with PLCζ1 and calcium might also explain the inclusion of PIP\(_2\) in this negative regulation mechanism. Our data showed that kinetics of the process was much slower with low calcium which have latency to respond to the channel agonist (Fig.3). This observation is coincident with the fact that intracellular calcium strongly potentiates TRPC channels\(^{22}\). However, this reaction rate was not influenced by the presence of PLCζ1 in same calcium condition, hence we concluded that when PIP\(_2\) levels are diminished with PLCζ1, equivalent stimulus intensity will be less effective.

By monitoring the PIP\(_2\) level changes after TRPC4β stimulation, we suggest that PLCζ1 accelerates PIP\(_2\) hydrolysis in response to calcium influx (Fig. 4). As a second approach to demonstrate the involvement of PIP\(_2\) in this mechanism, we used PLCζ1 mutant which cannot function to hydrolyze PIP\(_2\) (Fig. 5). We expected that the PLCζ1-reduced TRPC4β currents might recover to its own activity when the PLCζ1 has no effect to degrade PIP\(_2\). In electrophysiological experiments, these PLCζ1 mutants did not affect the channel currents. Therefore, it is likely that the PIP\(_2\) is the main source of negative feedback regulation by PLCζ1, and we stick to emphasize the PIP\(_2\) as a requisite component for TRPC4β activation in this negative feedback mechanism of PLC.

Until now, we only focused on PIP\(_2\) as an indispensable component for TRPC4 channel activity, and the channel seems to fall into desensitization when PIP\(_2\) level is decreased. It should be noted that other groups have proposed the inhibitory effect of PIP\(_2\) on TRPC4β21. Recent evidence suggests that PIP\(_2\) has a dual regulatory role, and yet it remains an open question as to how PIP\(_2\) decides their role. TRPV1 has also been proposed to be regulated by PIP\(_2\) with a duality of functioning. Initially, TRPV1 channel was suggested to be potentiated after PIP\(_2\) hydrolysis by releasing inhibitory PIP\(_2\) from the channel\(^{19,35-37}\). We hypothesized that each PIP\(_2\) interaction residue could have different effect by PIP\(_2\) binding, hence if a residue contributes to maintain the channel’s activity, the mutant would show decreased activity regardless of PLCζ1. And if, a residue contributes to the channel inhibition by PIP\(_2\) interaction, the mutant form would show increased channel activity and the presence of PLCζ1 would cause the robust current inhibition. We performed electrophysiological experiments with TRPC4β mutant which is previously identified as a PIP\(_2\) binding residues. Based on the precedent results and this study, the residues involved in maintaining TRPC4 are Lys\(_{419}\), Lys\(_{518}\) and His\(_{630}\), and the one involved in inhibiting TRPC4 is Lys\(_{664}\). According to the previous publication, the TRPC4β (K664A) did not show currents reduction, but only low FRET with PIP\(_2\)\(^3\). Unexpectedly, there was no significant difference in TRPC4β (R511A) compared to wildtype TRPC4β\(^6\). We suppose that Arg\(_{511}\) is an indecisive residue that is not involved in direct interaction with PIP\(_2\), but has a supportive role in this binding. In TRPV1, which the role of PIP\(_2\) showed duality, the regulatory role for PIP\(_2\) on TRPV1 has been proposed to be dependent on the degree of channel stimulation\(^{19,37}\). As EC50 of (-)-EA on TRPC4β is 10.4 nM\(^{38}\), the 100 nM concentration we used is strong enough to activate TRPC4β to the maximum. Thus, further experiments are required in agonistic stimulus strength to understand how the role of PIP\(_2\) on the channel is decided.

Previously, it is suggested that the activity of PLCζ1 does not exhibits receptor-specific activation\(^39\). In the electrophysiological experiments, we used channel-specific and strong agonist, hence we suggest that PLCζ1 is activated by the channel-induced influx. However, we have limitations in distinguishing signaling pathway with (-)-EA, because it is still obscure how (-)-EA activates TRPC4β and which signaling pathway is involved in. The other group specified that PLCζ1 is involved in the G\(_{\text{olig}}\)-mediated TRPC4 activation. It should be elucidated that whether the activation of PLCζ1 is confined to the specific pathway or the calcium through the channel itself is enough.

Collectively, our data reveals that TRPC4β-interacting PLCζ1 is sensitively activated by the calcium influx through TRPC4β and in turn, hydrolyzes TRPC4β-Pl(4,5)\(_2\), which maintains TRPC4β activity. Consequently, PLCζ1 is a negative regulator of TRPC4β. We identified key residues of TRPC4β for PIP\(_2\) binding have different role on channel regulation, maintains or inhibits TRPC4β by PIP\(_2\) binding.
Materials and Methods

Cell culture and transfection

Human embryonic kidney (HEK)293 cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco’s Modified Eagle’s Medium (HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (HyClone) at 37°C in a 5% CO₂ 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, Madison, WI, USA) and Lipofectamine 2000® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. All experiments were performed 20-30 h after transfection. The mutants of PLCδ1 were generated using a QuickChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer’s protocol. The sequence was verified by sequencing. The mutants of TRPC4β were generated as described3.

Solutions and drugs

The recording pipette containing standard intracellular solution; 140 mM CsCl, 10 mM HEPES, 10 mM BAPTA, variable CaCl₂, 3 mM Mg-ATP, 0.2 mM Tris-GTP, pH 7.3 with CsOH. The Free Ca²⁺ concentration was calculated using CaBuf software (G.Droogmans, Leuven, Belgium). External solution was perfused constantly as follows; 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, pH 7.4 with NaOH. Rapamycin was purchased from Sigma-Aldrich (St. Louis, MO, USA), and (-)-Englerin A was purchased from PhytoLab (Vestenbergsgreuth, Germany).

Electrophysiology

The cells were transfected onto a small chamber on the stage of an inverted microscope (IX70, OLYMPUS, Tokyo, Japan), 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, Holliston, MA, USA) using puller (PC-10, NARISHIGE, Tokyo, Japan). Whole-cell currents were recorded using Axopatch 200B amplifier (Molecular Devices, Foster City, CA, USA) and Digidata 1550B interface (Molecular Devices). Experiments were performed at room temperature (18-22°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 TRPC4β channel currents in HEK293 cells. Voltage ramps ranging from +100 to -100 mV over period of 500 ms were imposed every 10 s with a holding membrane potential of -60 mV. pCLAMP v.10.2 (OriginLab, Northampton, MA, USA) 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, Oberkochen, Germany) 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, Belfast, Northern Ireland) and the 440 nm and 500 nm wavelength were illuminated with LED light source (pE-2, CoolLED, Andover, England) under the control of MetaMorph 7.6 software (Molecular devices). Based on this imaging system, FRET measurements were made by the three-cube FRET method40 (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 s. 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., 20193. FRET Ratio (FR) is equal to the fractional increase in YFP emission due to FRET and calculated as:

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FR = \frac{F_{AD}}{F_A} = \frac{\left[S_{FRET}(DA) - R_{D1} \cdot S_{CFP}(DA)\right]}{R_{A1} \cdot \left[S_{YFP}(DA) - R_{D2} \cdot S_{CFP}(DA)\right]}
\]
Here, $S_{\text{CUBE}}(\text{SPECIMEN})$ 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; CFP), acceptor (A; YFP), or both (DA). $R_{D1} = S_{\text{FRET}}(D) / S_{\text{CFP}}(D)$, $R_{D2} = S_{\text{YFP}}(D) / S_{\text{CFP}}(D)$, and $R_{A1} = S_{\text{FRET}}(A) / S_{\text{YFP}}(A)$ 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 ($E_{\text{EFF}}$) was determined as:

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

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\textsuperscript{41}. We determined this ratio to be 0.094 based on maximal extinction coefficients for ECFP and EYFP and excitation spectra\textsuperscript{42}.

**Western blotting, Co-immunoprecipitation analysis**

We did western blotting following methods in Kwak et al., 2018, except the antibodies\textsuperscript{43}. 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 MgCl$_2$, 2 mM EDTA, pH 7.5) by being passed through a 26-gauge needle ten to twenty times. Lysates were centrifuged at 13,000 $\times$ 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-PLC$\delta$, 500 μl of cell lysates (500-1000 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-11222, Thermo Fisher Scientific, Waltham, MA, USA); 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; *$p<0.05$, **$p<0.01$, ***$p<0.001$. 

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

**Additional information:** The authors declare that they have no conflicts of interest with the contents of this article.
Figure Legends

Figure 1. TRPC4β and PLCδ1 colocalize together

(a, c) Localizations of TRPC channel and PLCδ enzyme. Upper panel: Channel with PLCδ1; Lower panel: Channel with PLCδ3. ECFP-tagged channel and EYFP-tagged PLCδ were co-expressed in HEK293 cells. The line scanned position is indicated by arrow in overlay images. Line scan graph shows TRPC4β colocalize with PLCδ1, but not with PLCδ3. (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 PLCδ. Proteins from each condition were subjected to immunoprecipitation using anti-GFP antibody and probed with anti-Flag antibody. TRPC4β interacts with PLCδ1 directly but not with another subtype. TRPC5 interacts with neither. Full-length blots are presented in Supplementary Figure S2.

Figure 2. PLCδ1 inhibits TRPC4β currents

(a) Rapamycin-induced translocation of CFP-FKBP-PLCδ1 to the plasma membrane. CFP-FKBP-PLCδ1 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 μm. (b, c) Representative whole-cell current recordings of HEK293 cells co-expressed with TRPC4β, FKBP-PLCδ1 in the absence (b) or presence of Lyn-FRB (c). Left panel: Time course of currents at ± 100 mV every 10 s; Right panel: I-V relationship for selected time points. Stippled lines indicate zero currents. Application of 50 nM rapamycin and 100 nM (-)Englerin A (EA) is indicated. The pipette solution contained 100 nM free Ca²⁺. (d) Summaries of peak current densities at -60 mV induced by rapamycin and (-)-EA. Rapamycin-induced PLCδ1 translocation to plasma membrane significantly reduced TRPC4β currents. *p < 0.05 by t-test. The numbers in parentheses refer to cell numbers.

Figure 3. Ca²⁺-dependent activation of PLCδ1 occurs in physiological intracellular calcium range.

(a, c, e) Representative whole-cell current recordings of HEK293 cells co-expressed with TRPC4β in the absence or presence of PLCδ1 using 50 nM (a), 100 nM (c), and 500 nM (e) free Ca²⁺ recording pipette solutions. Left panel: Time course of currents at ± 100 mV every 10 s; Left panel: I-V relationship for selected time points. Stippled lines indicate zero currents. Application of 100 nM (-)-Englerin A (EA) are indicated. (b, d, f) Summaries of peak current densities at -60 mV induced by (-)-EA. The PLCδ1 inhibited TRPC4 currents when using 100 nM and 500 nM [Ca²⁺] recording solutions. **p < 0.01, ***p < 0.001 by t-test. The numbers in parentheses refer to cell numbers.

Figure 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 images. The line scanned regions are indicated by dashed line. Scale bars, 10 μm.

Figure 5. Nonfunctional PLCδ1 mutant 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 s; 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. **p < 0.01, ***p < 0.001 by t-test. The numbers in parentheses refer to cell numbers.

Figure 6. PIP₂ binding residues of TRPC4β have distinct effect on channel activity by PIP₂
Representative whole cell current recordings of HEK293 cells co-expressed with TRPC4β mutant in the absence and the presence of PLCδ1. (a) TRPC4β (K419A); (c) TRPC4β (R511A); (e) TRPC4β (K518A); (g) TRPC4β (H630A); (i) TRPC4β (K664A). Left panel: Time course of currents at ±100 mV every 10 s; 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⁺. (b, d, f, h, j) Summaries of peak current densities at -60 mV induced by (-)-EA. Presence of PLCδ1 unaffected the currents of TRPC4β (K419A) (b), TRPC4β (K518A) (f), and TRPC4β (H630A) (g) mutants. TRPC4β (K664A) showed exceptional increased current, and the presence of PLCδ1 extremely inhibited channel activity (j). *p < 0.05, **p < 0.01 by t-test. The numbers in parentheses refer to cell numbers.
1 Kim, B. J., Kim, M. T., Jeon, J. H., Kim, S. J. & So, I. Involvement of phosphatidylinositol 4,5-bisphosphate in the desensitization of canonical transient receptor potential S. Biol Pharm Bull 31, 1733-1738, doi:10.1248/bpb.31.1733 (2008).
2 Kim, H. et al. An essential role of PI(4,5)P2 for maintaining the activity of the transient receptor potential canonical (TRPC)β. Pflug Arch Eur J Phy 465, 1011-1021, doi:10.1007/s00424-013-1236-x (2013).
3 Ko, J., Myeong, J., Shin, Y. C. & So, I. Differential PI(4,5)P2 sensitivities of TRPC4, C5 homomeric and TRPC1/4, C1/5 heteromeric channels. Sci Rep 9, 1849, doi:10.1038/s41598-018-38443-0 (2019).
4 Otsuguro, K. et al. Isoform-specific inhibition of TRPC4 channel by phosphatidylinositol 4,5-bisphosphate. J Biol Chem 283, 10026-10036, doi:10.1074/jbc.M707306200 (2008).
5 Mederos, Y. S. M., Gudermann, T. & Storch, U. Emerging roles of diacylglycerol-sensitive TRPC4/5 channels. Cells 7, doi:10.3390/cells7110218 (2018).
6 Storch, U. et al. Dynamic NHERF interaction with TRPC4/5 proteins is required for channel gating by diacylglycerol. Proc Natl Acad Sci USA 114, E37-E46, doi:10.1073/pnas.1612263114 (2017).
7 Zhu, M. H. et al. Desensitization of canonical transient receptor potential channel 5 by protein kinase C. Am J Physiol Cell Physiol 289, C591-600, doi:10.1152/ajpcell.00440.2004 (2005).
8 Myeong, J. et al. Dual action of the Gaq-PLCβ-PI(4,5)P2 pathway on TRPC1/4 and TRPC1/5 heterotramers. Sci Rep 8, 12117, doi:10.1038/s41598-018-30625-0 (2018).
9 Ko, J., Myeong, J., Kwak, M., Jeon, J. H. & So, I. Identification of phospholipase C beta downstream effect on transient receptor potential canonical 1/4, transient receptor potential canonical 1/5 channels. Korean J Physiol Pharmacol 23, 357-366, doi:10.4196/kjpp.2019.23.5.357 (2019).
10 Berridge, M. J. Inositol trisphosphate and calcium signalling. Nature 361, 315-325, doi:10.1038/361315a0 (1993).
11 Noh, D. Y., Shin, S. H. & Rhee, S. G. Phosphoinositide-specific phospholipase C and mitogenic signaling. Biochim Biophys Acta 1242, 99-113 (1995).
12 Rhee, S. G. & Choi, K. D. Regulation of inositol phospholipid-specific phospholipase C isozymes. J Biol Chem 267, 12393-12396 (1992).
13 Lee, S. B. & Rhee, S. G. Significance of PIP2 hydrolysis and regulation of phospholipase C isozymes. Curr Opin Cell Biol 7, 183-189 (1995).
14 Kim, Y. H. et al. Phospholipase C-δ1 is activated by capacitative calcium entry that follows phospholipase C-β activation upon bradykinin stimulation. J Biol Chem 274, 26127-26134 (1999).
15 Allen, V., Swigart, P., Cheung, R., Cockcroft, S. & Katan, M. Regulation of inositol lipid-specific phospholipase Cδ by changes in Ca2+ ion concentrations. Biochem J 327 (Pt 2), 545-552 (1997).
16 Lee, W. K. et al. Molecular cloning and expression analysis of a mouse phospholipase C-δ1. Biochem Biophys Res Commun 261, 393-399, doi:10.1006/bbrc.1999.1035 (1999).
17 Rebecchi, M. J. & Pentyala, S. N. Structure, function, and control of phosphoinositide-specific phospholipase C. Physiol Rev 80, 1291-1335, doi:10.1152/physrev.2000.80.4.1291 (2000).
18 Cifuentes, M. E., Delaney, T. & Rebecchi, M. J. β- myo-inositol 1,4,5-trisphosphate inhibits binding of phospholipase C-δ1 to bilayer membranes. J Biol Chem 269, 1945-1948 (1994).
19 Lukacs, V. et al. Dual regulation of TRPV1 by phosphoinositides. J Neurosci 27, 7070-7080, doi:10.1523/JNEUROSCI.1866-07.2007 (2007).
20 Rohacs, T., Lopes, C. M., Michailidis, I. & Logothetis, D. E. PI(4,5)P2 regulates the activation and desensitization of TRPM8 channels through the TRP domain. Nat Neurosci 8, 626-634, doi:10.1038/nn1451 (2005).
21 Thakur, D. P. et al. Critical roles of Gβs proteins and phospholipase C-δ1 in the activation of receptor-operated TRPC4 channels. Proc Natl Acad Sci USA 113, 1092-1097, doi:10.1073/pnas.1522294113 (2016).
22 Blair, N. T., Kaczmarek, J. S. & Clapham, D. E. Intracellular calcium strongly potentiate agonist-activated TRPC5 channels. J Gen Physiol 133, 525-546, doi:10.1085/jgp.200810153 (2009).
23 Garcia, P. et al. The pleckstrin homology domain of phospholipase C-δ1 binds with high affinity to phosphatidylinositol 4,5-bisphosphate in bilayer membranes. Biochemistry 34, 16228-16234, doi:10.1021/bi00049a039 (1995).
24 Ellis, M. V., U. S. & Katan, M. Mutations within a highly conserved sequence present in the X region of phosphoinositide-specific phospholipase C-delta 1. Biochem J 307 (Pt 1), 69-75, doi:10.1042/bj3070069 (1995).
Zhang, Z., Okawa, H., Wang, Y. & Liman, E. R. Phosphatidylinositol 4,5-bisphosphate rescues TRPM4 channels from desensitization. J Biol Chem 280, 39185-39192, doi:10.1074/jbc.M506965200 (2005).

Liu, D. & Liman, E. R. Intracellular Ca\(^{2+}\) and the phospholipid PIP\(_2\) regulate the taste transduction ion channel TRPMS. Proc Natl Acad Sci U S A 100, 15160-15165, doi:10.1073/pnas.2334159100 (2003).

Liu, B. & Qin, F. Functional control of cold- and menthol-sensitive TRPM8 ion channels by phosphatidylinositol 4,5-bisphosphate. J Neurosci 25, 1674-1681, doi:10.1523/JNEUROSCI.3632-04.2005 (2005).

Daniels, R. L., Takashima, Y. & McKemy, D. D. Activity of the neuronal cold sensor TRPM8 is regulated by phospholipase C via the phospholipid phosphoinositol 4,5-bisphosphate. Proc Natl Acad Sci U S A 110, 940-945, doi:10.1073/pnas.1215690110 (2013).

Lee, K. P. et al. TRPC4 is an essential component of the nonselective cation channel activated by muscarinic stimulation in mouse visceral smooth muscle cells. Mol Cells 20, 435-441 (2005).

Klein, R. M., Ufret-Vincenty, C. A., Hua, L. & Gordon, S. E. Determinants of molecular specificity in phosphoinositide regulation. Phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P2) is the endogenous lipid regulating TRPV1. J Biol Chem 283, 26208-26216, doi:10.1074/jbc.M801912200 (2008).

Banno, Y., Okano, Y. & Nozawa, Y. Thrombin-mediated phosphoinositide hydrolysis in Chinese hamster ovary cells overexpressing phospholipase C-δ1. J Biol Chem 269, 15846-15852 (1994).

Liu, B., Zhang, C. & Qin, F. Functional control of cold-sensitive TRPM8 ion channels by phosphatidylinositol 4,5-bisphosphate. J Neurosci 25, 1674-1681, doi:10.1523/JNEUROSCI.3632-04.2005 (2005).

Daniels, R. L., Takashima, Y. & McKemy, D. D. Activity of the neuronal cold sensor TRPM8 is regulated by phospholipase C via the phospholipid phosphoinositol 4,5-bisphosphate. Proc Natl Acad Sci U S A 110, 940-945, doi:10.1073/pnas.1215690110 (2013).

Lee, K. P. et al. TRPC4 is an essential component of the nonselective cation channel activated by muscarinic stimulation in mouse visceral smooth muscle cells. Mol Cells 20, 435-441 (2005).

Klein, R. M., Ufret-Vincenty, C. A., Hua, L. & Gordon, S. E. Determinants of molecular specificity in phosphoinositide regulation. Phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P2) is the endogenous lipid regulating TRPV1. J Biol Chem 283, 26208-26216, doi:10.1074/jbc.M801912200 (2008).

Banno, Y., Okano, Y. & Nozawa, Y. Thrombin-mediated phosphoinositide hydrolysis in Chinese hamster ovary cells overexpressing phospholipase C-δ1. J Biol Chem 269, 15846-15852 (1994).

Liu, B., Zhang, C. & Qin, F. Functional control of cold-sensitive TRPM8 ion channels by phosphatidylinositol 4,5-bisphosphate. J Neurosci 25, 1674-1681, doi:10.1523/JNEUROSCI.3632-04.2005 (2005).

Daniels, R. L., Takashima, Y. & McKemy, D. D. Activity of the neuronal cold sensor TRPM8 is regulated by phospholipase C via the phospholipid phosphoinositol 4,5-bisphosphate. Proc Natl Acad Sci U S A 110, 940-945, doi:10.1073/pnas.1215690110 (2013).

Lee, K. P. et al. TRPC4 is an essential component of the nonselective cation channel activated by muscarinic stimulation in mouse visceral smooth muscle cells. Mol Cells 20, 435-441 (2005).

Klein, R. M., Ufret-Vincenty, C. A., Hua, L. & Gordon, S. E. Determinants of molecular specificity in phosphoinositide regulation. Phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P2) is the endogenous lipid regulating TRPV1. J Biol Chem 283, 26208-26216, doi:10.1074/jbc.M801912200 (2008).

Banno, Y., Okano, Y. & Nozawa, Y. Thrombin-mediated phosphoinositide hydrolysis in Chinese hamster ovary cells overexpressing phospholipase C-δ1. J Biol Chem 269, 15846-15852 (1994).

Liu, B., Zhang, C. & Qin, F. Functional control of cold-sensitive TRPM8 ion channels by phosphatidylinositol 4,5-bisphosphate. J Neurosci 25, 1674-1681, doi:10.1523/JNEUROSCI.3632-04.2005 (2005).

Daniels, R. L., Takashima, Y. & McKemy, D. D. Activity of the neuronal cold sensor TRPM8 is regulated by phospholipase C via the phospholipid phosphoinositol 4,5-bisphosphate. Proc Natl Acad Sci U S A 110, 940-945, doi:10.1073/pnas.1215690110 (2013).

Lee, K. P. et al. TRPC4 is an essential component of the nonselective cation channel activated by muscarinic stimulation in mouse visceral smooth muscle cells. Mol Cells 20, 435-441 (2005).

Klein, R. M., Ufret-Vincenty, C. A., Hua, L. & Gordon, S. E. Determinants of molecular specificity in phosphoinositide regulation. Phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P2) is the endogenous lipid regulating TRPV1. J Biol Chem 283, 26208-26216, doi:10.1074/jbc.M801912200 (2008).