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Expression of the C-Terminal Domain of Phospholipase C β 3 Inhibits Signaling via G α q-Coupled Receptors and Transient Receptor Potential Channels

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Abstract: Transient receptor potential (TRP) channels are cation channels that play a regulatory role in pain and thermosensation, insulin secretion, and neurotransmission. It has been proposed that activation of TRP channels requires phosphatidylinositol 4,5-bisphosphate, the major substrate for phospholipase C (PLC). We investigated whether inhibition of PLC β has an impact on TRP channel signaling. A genetic approach was used to avoid off-target effects observed when using a pharmacological PLC β inhibitor. In this study, we show that expression of PLC β 1ct and PLC β 3ct, truncated forms of PLC β 1 or PLC β 3 that contain the C-terminal membrane binding domains, almost completely blocked the signal transduction of a G α q-coupled designer receptor, including the phosphorylation of ERK1/2. In contrast, expression of the helix-turn-helix motif (H α 1—H α 2) of the proximal C-terminal domain of PLC β 3 did not affect G α q-coupled receptor signaling. PLC β 3ct expression impaired signaling of the TRP channels TRPM3 and TRPM8, stimulated with either progrenolone sulfate or icilin. Thus, the C-terminal domain of PLC β 3 interacts with plasma membrane targets, most likely phosphatidylinositol 4,5-bisphosphate, and in this way blocks the biological activation of TRPM3 and TRPM8, which require interaction with this phospholipid. PLC β thus regulates TRPM3 and TRPM8 channels by masking phosphatidylinositol 4,5-bisphosphate with its C-terminal domain.

Keywords: G α q-coupled designer receptor; ERK1/2; *m*-3M3FBS; phospholipase C β ; TRPM3; TRPM8



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1. Introduction

TRP (transient receptor potential) channels are cation channels that have a similar modular structure, but differ substantially in their primary structures. TRP channels have six transmembrane regions and the ion pore has been located between transmembrane regions 5 and 6. Ca²⁺ ions and/or Na⁺ ions can flow into the cells from the extracellular environment through this pore, dependent on the TRP channel. TRP channels play an important role in the regulation of sensory pathways, thermosensation, pain sensation, mechanoperception, and perception of pungent chemicals. In addition, TRP channels have been described as being involved in the regulation of tumorigenesis, cell proliferation, insulin secretion, and neurotransmission [1]. TRP channels are embedded into the plasma membrane, and it has been proposed that channel activity is modulated by the plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate, which acts as a necessary cofactor for other ion channels [2]. Phosphatidylinositol 4,5-bisphosphate is a negatively charged phospholipid located at the cytoplasmic side of the plasma membrane and binds to ion channels via electrostatic interactions.

It has been reported that the biological activities of TRP channels TRPM3 and TRPM8 are regulated by phosphatidylinositol 4,5-bisphosphate [3–7]. TRPM3 channels are cation channels that respond to heat and the steroid pregnenolone sulfate [8,9]. Activation of TRPM3 has been linked to temperature and pain sensation, vascular smooth muscle contraction, gene transcription, insulin secretion, and tumorigenesis [10]. TRPM3 functions as a chemo- and

thermosensor in the somatosensory system and, together with the TRPA1 and TRPV1 channels, regulates heat sensation [11,12]. TRPM3 activity is enhanced by phosphoinositides in cell-free inside-out patches, and inhibited by the expression of ci-VSP, a voltage-sensing phosphatase that catalyzes the removal of 5-phosphate from phosphoinositides [3,4].

TRPM8 channels mediate the influx of Ca^{2+} ions into the cells after stimulation with cooling agents (menthol, icilin, eucalyptol) and by reduced temperature [13]. TRPM8 channels are present in a subset of temperature-sensing dorsal root and trigeminal and ganglion neurons and function as cold nociceptors in these neurons, mediating nocifensive responses to noxious cold [14,15]. TRPM8 channel activity has been linked to a number of diseases, including migraines and cancer [13]. Expression of the phosphatase ci-VSP inhibited TRPM8-induced current, suggesting that the plasma membrane phosphatidylinositol 4,5-bisphosphate concentration is important for TRPM8 channel activity [4,5].

Phospholipase C (PLC) catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate, generating 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Thus, activation of PLC reduces the concentration of phosphatidylinositol 4,5-bisphosphate. Assuming that phosphatidylinositol 4,5-bisphosphate is required for TRPM3 and TRPM8 activity, PLC activation should negatively influence TRPM3 and TRPM8 channel activity. Accordingly, $\text{G}\alpha\text{q}$ -coupled receptor activation of $\text{PLC}\beta$ has been shown to inhibit TRPM3 and TRPM8 activities [3,4,6]. Furthermore, activation of TrkA receptors or PDGF receptors, which trigger an activation of $\text{PLC}\gamma$, resulted in inhibition of TRPM8 current [6,7].

Previous studies have measured the reduction of membrane-bound phosphatidylinositol 4,5-bisphosphate via an indirect assay based on the translocation of a fluorescently labeled $\text{PLC}\gamma$ -derived PH domain from the plasma membrane to the cytoplasm. The use of this translocation assay has some pitfalls, as this PH domain does not bind specifically to phosphatidylinositol 4,5-bisphosphate but binds 20-fold more strongly to IP_3 [2,16] and may act as an IP_3 sponge that attenuates IP_3 downstream signaling events. In addition, $\text{PLC}\gamma$ -PH binding to the membrane can be disrupted by increased intracellular Ca^{2+} concentrations [17], as is the case after stimulation of $\text{G}\alpha\text{q}$ -coupled receptors or TRP channels. Furthermore, by decorating the plasma membrane the GFP-tagged PH domain fusion proteins sequester their target and may interfere with the binding of other proteins to phosphatidylinositol 4,5-bisphosphate, disrupting intracellular signaling pathways downstream of TRP channels and producing off-target effects.

In this study, we did not attempt to affect the level of phosphatidylinositol 4,5-bisphosphate at the plasma membrane. Rather, we used a genetic tool to inhibit $\text{PLC}\beta$ activity. Expression of the C-terminal domain of either $\text{PLC}\beta 1$ or $\text{PLC}\beta 3$ almost completely inhibited $\text{G}\alpha\text{q}$ -coupled receptor signaling. Under these circumstances, TRPM3 and TRPM8 intracellular signaling was impaired as well. The C-terminal domain of $\text{PLC}\beta$ harbors the primary membrane tagging site of the enzyme. We conclude that $\text{PLC}\beta$ and TRPM channels compete for the same target(s) at the plasma membrane, suggesting that membrane-bound $\text{PLC}\beta$ negatively affects TRPM3 and TRPM8 activities without altering the concentration of phosphatidylinositol 4,5-bisphosphate. Furthermore, we show that the compound *m*-3M3FBS, described as a pharmacological activator of PLC, does not trigger a signaling cascade comparable to that initiated by stimulating $\text{G}\alpha\text{q}$ -coupled receptors. Nevertheless, treatment of the cells with *m*-3M3FBS effectively impaired TRPM3 and TRPM8 channel signaling.

2. Results

2.1. Expression of the C-Terminal Domain of Phospholipase C $\beta 3$ Blocks Signaling Induced by Stimulation of a $\text{G}\alpha\text{q}$ -Coupled Designer Receptor

The aim of this study was to block PLC activity to determine whether this inhibition has an effect on TRP channel signaling. The aminosteroid U73122 has been widely used to inhibit $\text{PLC}\beta$ enzymatic activity. However, the specificity of this compound is questionable and several PLC-independent effects have been observed [18–22]. Instead, we decided to use genetic inhibition of PLC. The substrate of PLC is a phospholipid embedded in the plasma membrane. PLC must, therefore, bind to the membrane to exert its enzymatic activ-

ity. Figure 1A shows the modular structure of the enzyme, including the enzymatic core and the C-terminal regulatory region, as well as the modular structure of a truncated mutant, PLC β 3ct. The C-terminal domain (CTD), encompassing approximately 400 amino acids, is thought to be responsible for maintaining the enzyme in a spatial structure required for enzymatic activation [23]. We expressed the C-terminal domain of PLC β 3, termed PLC β 3ct, together with a FLAG epitope, as depicted in Figure 1B. PLC β is the major effector enzyme of G α q-coupled receptors. We tested the activity of the PLC β 3ct mutant in HEK293 cells expressing R α q (Figure 1C), a designer receptor that specifically couples to G α q [24]. The designer receptor R α q is a mutated M3 muscarinic receptor activated with the artificial ligand clozapine-*N*-oxide (CNO) [25,26]. G α q-coupled receptor signaling can be measured by analyzing the intracellular Ca²⁺ or IP₃ concentrations, by analyzing the phosphorylation status of ERK1/2, or by analyzing transcriptional activation. We chose to determine transcriptional activation after receptor stimulation, because in this case, a signaling cascade has occurred from the plasma membrane via the cytoplasm into the nucleus. R α q stimulation has been shown to trigger the activation of the transcription factor AP-1 [24]. We used a collagenase promoter/luciferase reporter gene (Coll.luc, Figure 1D) integrated into the chromatin as a sensor for AP-1 [27] (Figure 1D). Integration into the genome ensured that the reporter gene was embedded into a nucleosomal structure. HEK293 cells were infected with a lentivirus containing the Coll.luc reporter gene and with a lentivirus encoding the designer receptor R α q. In addition, cells were infected with a lentivirus encoding either PLC β 3ct or β -galactosidase (mock). Figure 1E shows that expression of PLC β 3ct almost completely inhibited R α q-mediated activation of AP-1. AP-1 activity was reduced by 95.5%. These results indicate that expression of the C-terminal domain of PLC β 3 acts in a dominant-negative manner and prevents signal transduction through G α q-coupled receptors.

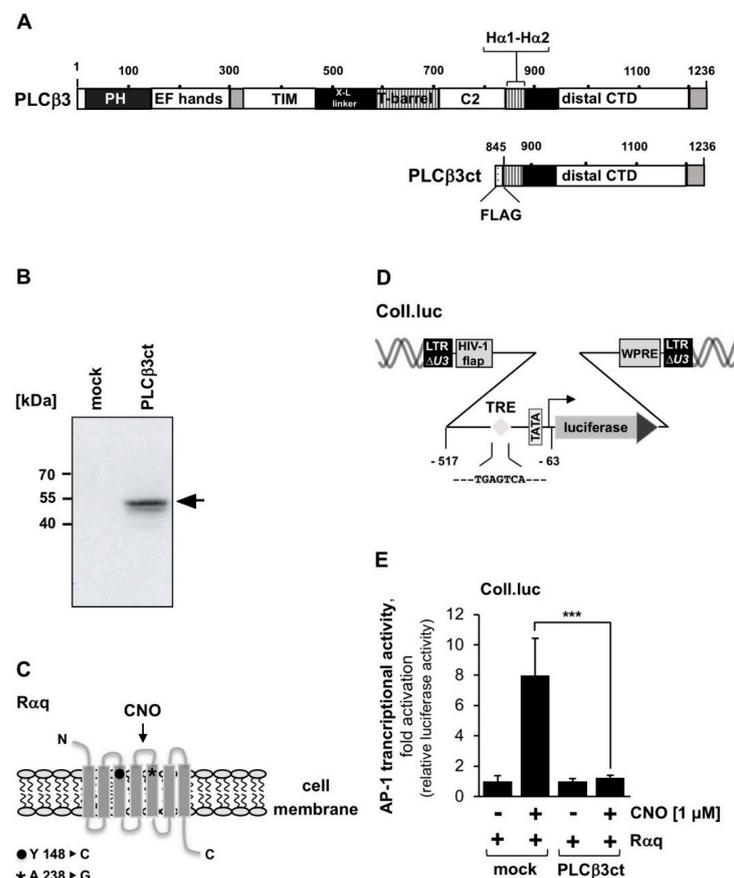


Figure 1. Expression of the C-terminal domain of phospholipase C β 3 blocks signaling of R α q, a G α q-coupled designer receptor. (A) Multi-domain structure of phospholipase C β 3 and the truncated

mutant PLC β 3ct. PLC β 3 contains a pleckstrin homology (PH) domain, four tandem EF-hand repeats, the catalytic triose phosphate isomerase (TIM) barrel domain, the C2 domain, the helix-turn-helix motif (H α 1—H α 2), and a C-terminal domain (CTD). (B) Expression of PLC β 3ct mutant in HEK293 cells following infection of the cells with an PLC β 3ct-encoding lentivirus. Cells were infected with a lentivirus encoding either PLC β 3ct or β -galactosidase (mock). The Western blot was developed with an antibody against the FLAG-tag. kDa, molecular mass markers. (C) Modular structure of R α q. The two-point mutations essential to change the M3 muscarinic acetylcholine receptor into a G α q-coupled, CNO-sensitive designer receptor, are indicated. (D) Provirus, depicting the Coll.luc reporter gene, used as a sensor to measure AP-1 activity. (E) HEK293 cells containing a chromatin-integrated Coll.luc reporter gene were infected with a lentivirus encoding the designer receptor R α q. In addition, cells were infected with a lentivirus encoding either PLC β 3ct or β -galactosidase (mock). Serum-starved cells were stimulated with CNO (1 μ M) for 24 h. Cell extracts were prepared, and luciferase activities and protein concentrations were determined. Luciferase activity was normalized to the protein concentration. Data shown are means \pm SD of four experiments performed in quadruplicate (** $p < 0.001$).

2.2. Expression of the C-Terminal Domain of Phospholipase C β 3 Blocks Phosphorylation of Extracellular Signal-Regulated Protein Kinase (ERK1/2) after Stimulation of a G α q-Coupled Designer Receptor

Stimulation of a G α q-coupled receptor leads to the phosphorylation and activation of the protein kinase extracellular signal-regulated protein kinase (ERK1/2) [24,28]. Accordingly, the phosphorylation status of ERK1/2 was used to measure G α q-coupled receptor signaling [29,30]. Figure 2 shows that stimulation of the designer receptor triggered the phosphorylation of ERK1/2 in HEK293 cells. Expression of the PLC β 3ct mutant of PLC β 3 efficiently blocked ERK1/2 phosphorylation and, thus, ERK1/2 activation.

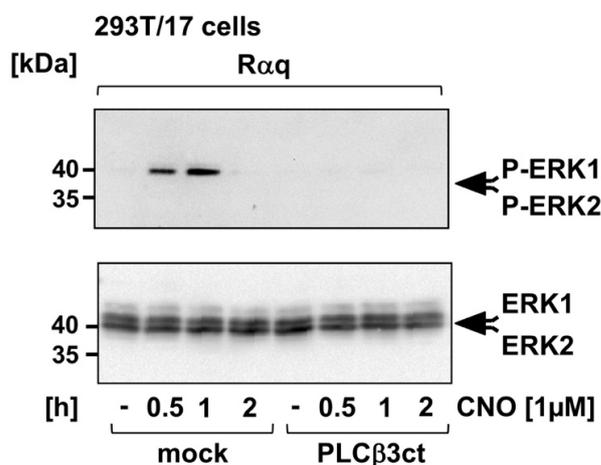


Figure 2. Expression of the C-terminal domain of phospholipase C β 3 blocks the phosphorylation of extracellular signal-induced protein kinase following stimulation of the R α q receptor. HEK293 cells expressing the R α q designer receptor were infected with a lentivirus encoding either PLC β 3ct or β -galactosidase (mock). Cells were serum-starved for 24 h and then stimulated with CNO (1 μ M). Cells were harvested at different times. Whole cell extracts were prepared and subjected to Western blot analysis. The blots were incubated with a monoclonal antibody directed against the phosphorylated active form of ERK1/2. As loading control, an antibody detecting ERK1/2 was used.

2.3. Expression of Membrane-Tagged Cyan Fluorescence Protein Containing the Helix-Turn-Helix Motif (H α 1—H α 2) of PLC β 3 Has No Effect on Downstream G α q Signaling

PLC β enzymes have a helix-turn-helix (HTH) domain C-terminal of the C2 domain, which has been shown to be an important binding site for activated G α q [31]. A peptide comprising this HTH motif has been shown to inhibit G α q-induced activation of PLC β 3 [32], suggesting that it acts as a dominant negative for PLC β 3. We expressed a FLAG-

tagged fusion protein, HTH-CFP-CaaX, in HEK293 cells consisting of the HTH domain, a cyan fluorescence protein (CFP), and the C-terminal membrane anchoring domain of Rac1 (Figure 3A). The FLAG-tag was used to show that the fusion protein was expressed as expected (Figure 3B). Next, we tested whether expression of the HTH-CFP-CaaX fusion protein affected G α q-coupled receptor signaling in HEK293 cells. The results show that there was no significant impairment of G α q-coupled receptor signal transduction in the presence of the HTH-CFP-CaaX protein, suggesting that expression of the HTH domain of PLC β 3 alone does not inhibit PLC β signaling.

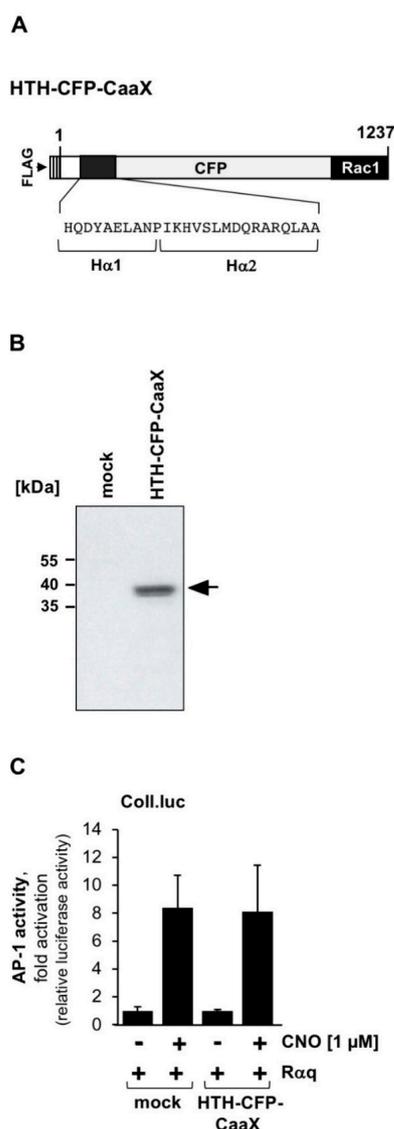


Figure 3. A fusion protein containing the helix-turn-helix (H α 1—H α 2) motif of PLC β 3 does not function as a dominant negative of G α q-coupled receptor signaling. (A) Modular structure of HTH-CFP-CaaX. The FLAG-tagged fusion protein consists of the H α 1—H α 2 domain of PLC β 3, CFP, and the C-terminal domain of Rac1. (B) Expression of HTH-CFP-CaaX in HEK293 cells following infection with lentivirus encoding HTH-CFP-CaaX. As a control, a virus encoding β -galactosidase (mock) was used for infection. The Western blot was developed with an antibody against the FLAG-tag. kDa, molecular mass markers. (C) HEK293 cells containing a chromatin-integrated Coll.luc reporter gene were infected with a lentivirus encoding the designer receptor R α q. In addition, cells were infected with a lentivirus encoding either HTH-CFP-CaaX or β -galactosidase (mock). Cells were stimulated, harvested, and analyzed as described in the legend to Figure 1 (n = 4). Expression of HTH-CFP-CaaX did not significantly impair G α q-coupled receptor signaling.

2.4. Expression of the C-Terminal Domain of Phospholipase C β 1 Blocks Signaling Induced by Stimulation of a G α q-Coupled Designer Receptor

The PLC β isoenzymes PLC β 1 and PLC β 4 have been found to be associated with the plasma membrane, whereas the isoenzymes PLC β 2 and PLC β 3 have been found in the cytoplasm [33]. This difference in cellular expression pattern is attributed to sequence differences within the C-terminal domain, which has only 30–35% identical amino acids between the different isoforms [34,35]. Subcellular analysis of GFP-tagged C-terminal domains of PLC β 1 and PLC β 3 revealed that the fusion protein of GFP together with the C-terminal domain of PLC β 1 was clearly visible at the plasma membrane. The fusion protein of GFP together with C-terminal domain of PLC β 3 was also located at the plasma membrane as well as in the cytosol [33]. We, therefore, tested whether the C-terminal domain of the membrane-associated PLC β 1 had similar or even greater activity in inhibiting G α q-coupled receptor signaling. Figure 4A shows the domain structure of PLC β 1 and the truncated mutant PLC β 1ct. The expression of the mutant in HEK293 cells is depicted in Figure 4B. Cells containing the Coll.luc reporter gene were infected with a lentivirus encoding R α q. In addition, we infected the cells with a lentivirus encoding either PLC β 1ct or β -galactosidase (mock). The results show that expression of PLC β 1ct was very effective in blocking G α q-coupled receptor signaling, as underscored by the fact that AP-1 transcriptional activity was reduced by 90.5% in the presence of PLC β 1ct. Thus, no major differences were obtained after expression of the C-terminal domains of PLC β 1 and PLC β 3.

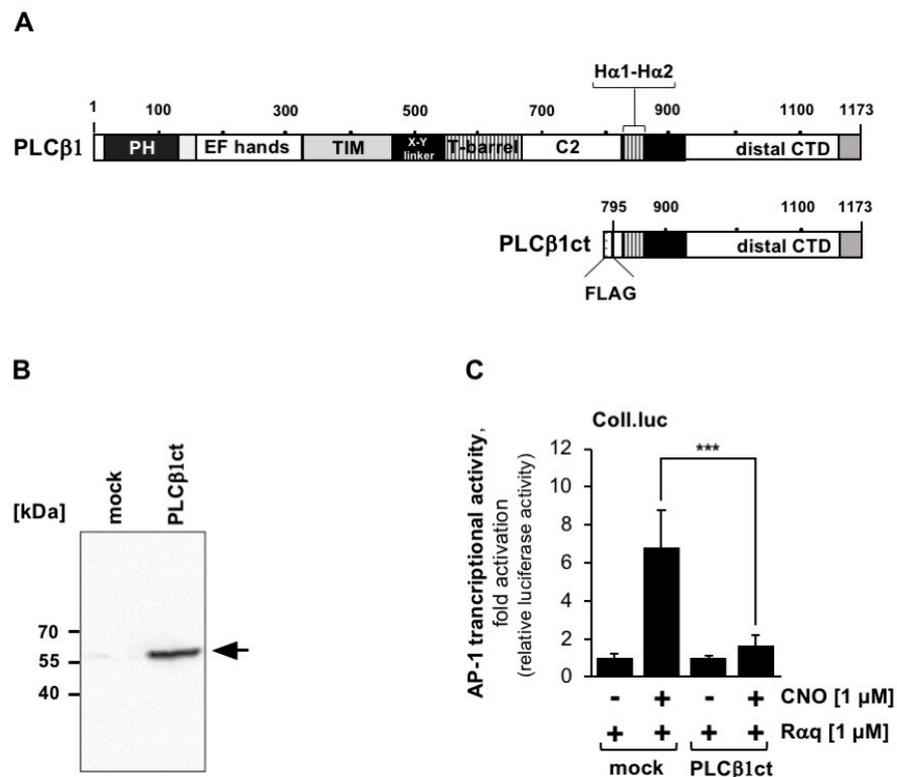


Figure 4. Expression of the C-terminal domain of phospholipase C β 1 blocks signaling R α q, a G α q-coupled designer receptor. (A) Multi-domain structure of phospholipase C β 1 and the truncated mutant PLC β 1ct. PLC β 1 has a similar domain structure as depicted for PLC β 3 in Figure 1. (B) Expression verification of PLC β 1ct in HEK293 cells following infection with an PLC β 1ct-encoding lentivirus. Cells were infected with a lentivirus encoding either PLC β 3ct or β -galactosidase (mock). The Western blot was developed with an antibody against the FLAG-tag. kDa, molecular mass markers. (C) HEK293 cells containing a chromatin-integrated Coll.luc reporter gene were infected with a lentivirus encoding the designer receptor R α q. Cells were infected with a lentivirus encoding either PLC β 1ct or β -galactosidase (mock). Serum-starved cells were stimulated with CNO (1 μ M) for 24 h. Cells were harvested and analyzed as described in the legend to Figure 1 (n = 3; *** p < 0.001).

2.5. Expression of the C-Terminal Domain of Phospholipase C β 3 Blocks Intracellular Signaling Triggered by Transient Receptor Potential (TRP) M8 Channel Stimulation

Given the hypothesis that many TRP channels “are either activated downstream of the PLC pathway, or modulated by it” [36], we tested whether inhibition of PLC β 3 affects intracellular signaling mediated by TRPM8 channels. These channels are activated by cold temperatures and chemically by cooling agents such as menthol and icilin. Figure 5A shows the modular structure of TRPM8, including the ion pore. Stimulation of TRPM8 channels has been shown to trigger activation of the transcription factor AP-1 [37]. Therefore, we determined the activity of AP-1 as a measure of TRPM8 signaling. HEK293-M8 cells containing an integrated Coll.luc reporter gene were infected with a lentivirus encoding either PLC β 3ct or β -galactosidase. Cells were stimulated with the super-cooling compound icilin to activate TRPM8. Figure 5B shows that TRPM8 signaling was significantly impaired in the presence of PLC β 3ct. Quantification revealed that inhibition of PLC β 3 resulted in 73% inhibition of TRPM8 signaling.

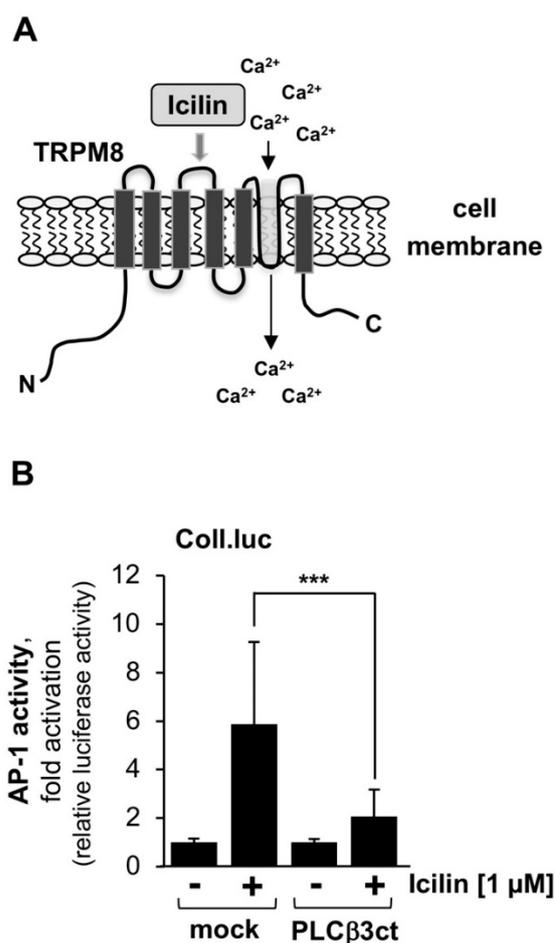


Figure 5. Expression of the C-terminal domain of phospholipase C β 3 blocks icilin-induced signaling via TRPM8. (A) Modular structure of TRPM8. (B) HEK293-TRPM8 cells containing a chromatin-integrated Coll.luc reporter gene were infected with a lentivirus encoding either PLC β 3ct or β -galactosidase (mock). Serum-starved cells were stimulated with icilin (1 μ M) for 24 h. Cells were harvested and analyzed as described in the legend to Figure 1 (n = 3; *** p < 0.001).

2.6. Expression of the C-Terminal Domain of Phospholipase C β 3 Blocks Intracellular Signaling Induced by Stimulation of TRPM3 Channels

TRPM3 channels are involved in thermoregulation and several other activities, including regulation of insulin secretion. Figure 6A shows the modular structure of TRPM3 channels that can be stimulated with the steroid pregnenolone sulfate. We examined the

effect of inhibition of PLC β 3 on TRPM3 signaling. Stimulation of TRPM3 channels triggers the activation of the transcription factor AP-1 [9,10]. Therefore, we determined the activity of AP-1 as a measure of TRPM3 signaling. Figure 6B shows that inhibition of PLC β 3 caused a 46% inhibition of TRPM3 signaling. From these data, we conclude that both TRPM3 and TRPM8 activities are regulated by PLC β .

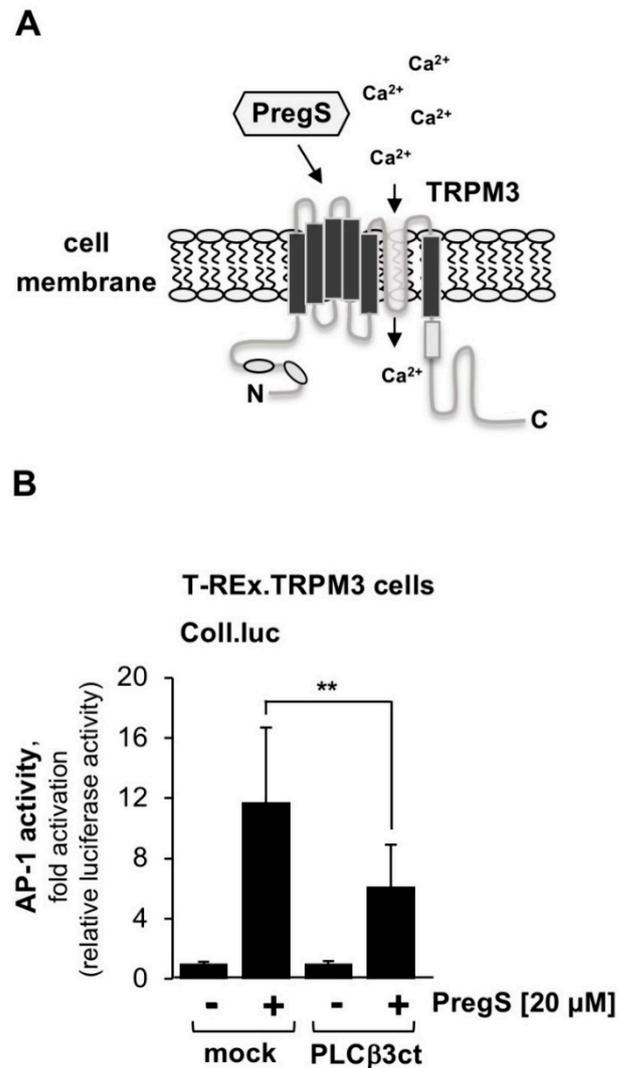


Figure 6. Expression of the C-terminal domain of phospholipase C β 3 blocks pregnenolone sulfate-induced signaling via TRPM3. **(A)** Modular structure of TRPM3. **(B)** T-REx-TRPM3 cells containing a chromatin-integrated Coll.luc reporter gene were infected with a lentivirus encoding either PLC β 3ct or β -galactosidase (mock). Cells were serum-starved for 24 h in the presence of tetracycline (1 μ g/mL) to induce TRPM3 expression. Serum-starved cells were stimulated with icilin (1 μ M) for 24 h. Cells were harvested and analyzed as described in the legend to Figure 1 (n = 4; ** p < 0.01).

2.7. The Benzenesulfonamide *m*-3M3FBS Blocks TRPM3 and TRPM8 Signaling

Next, we investigated whether activation of PLC β affects TRP channel signaling. We used the compound *m*-3M3FBS, which has been described as an activator of PLC [38]. The chemical structure of *m*-3M3FBS is shown in Figure 7A. We hypothesized that pharmacological activation of PLC β would directly trigger synthesis of IP $_3$, release of Ca $^{2+}$ from the endoplasmic reticulum, activation of protein kinase C and ERK1/2, and, subsequently, activation of AP-1 in the nucleus. We tested different concentrations of *m*-3M3FBS for their activity in triggering a signaling cascade leading to AP-1 activation. Figure 7B shows that a concentration of 1 μ M marginally activated AP-1 activity, whereas higher concen-

trations decreased AP-1 activity. On the basis on these experiments, *m*-3M3FBS cannot be considered a PLC activator. Nevertheless, we tested whether *m*-3M3FBS had an effect on TRP channel signaling. Figure 7C,D show that this compound strongly affected TRPM8 (Figure 7C) and TRPM3 (Figure 7D) signaling. Quantification of these results showed that *m*-3M3FBS caused nearly 90% inhibition of TRPM8 signaling and nearly 98% inhibition of TRPM3 signaling. Thus, we conclude that *m*-3M3FBS does not activate PLC but acts as an inhibitor of both TRPM8 and TRPM3 signaling.

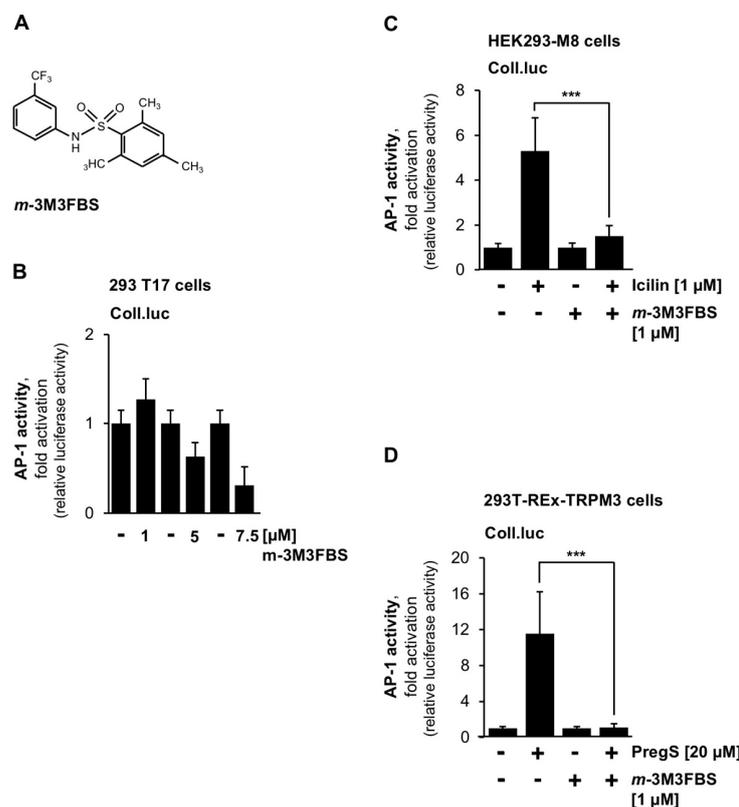


Figure 7. The compound *m*-3M3FBS blocks intracellular signaling mediated by TRPM8 and TRPM3. (A) Chemical structure of *m*-3M3FBS. (B) HEK293 cells containing the Coll.luc reporter gene were incubated with different concentrations of *m*-3M3FBS for 24 h. Cells were harvested and analyzed as described in the legend to Figure 1 (n = 3). (C) HEK293-M8 cells were infected with a recombinant lentivirus containing the Coll.luc reporter gene. Cells were serum-starved for 24 h, preincubated for 3 h with *m*-3M3FBS (5 μM), and then stimulated with icilin (1 μM). Cells were harvested and analyzed as described in the legend to Figure 1 (n = 3; *** *p* < 0.001). (D) T-REx-TRPM3 cells were infected with a recombinant lentivirus containing the collagenase promoter/luciferase reporter gene (Coll.luc). Cells were serum-starved for 24 h in the presence of tetracycline (1 μg/mL) to induce TRPM3 expression. Cells were preincubated for 3 h with *m*-3M3FBS (5 μM) and then stimulated with pregnenolone sulfate (PregS, 20 μM) for 24 h in the presence of the inhibitor. Cells were harvested and analyzed as described in the legend to Figure 1 (n = 3; *** *p* < 0.001).

2.8. The Compound *m*-3M3FBS Has No Effect on B-Raf-Induced Signal Transduction

To locate the target of *m*-3M3FBS, we examined signal transduction of a constitutively active B-Raf protein kinase expressed as a fusion protein together with the estrogen receptor ligand binding domain (Figure 8A). Activation of this B-Raf estrogen receptor fusion protein with the compound 4-hydroxytamoxifen (4OHT) triggers the activation of the ERK1/2 signaling pathway, which ultimately leads to activation of AP-1 in the cells (Figure 8B). Figure 8C shows that the B-Raf-induced activation of AP-1 was not affected in the presence of *m*-3M3FBS, suggesting that this compound alters the activities of targets upstream of B-Raf protein kinase.

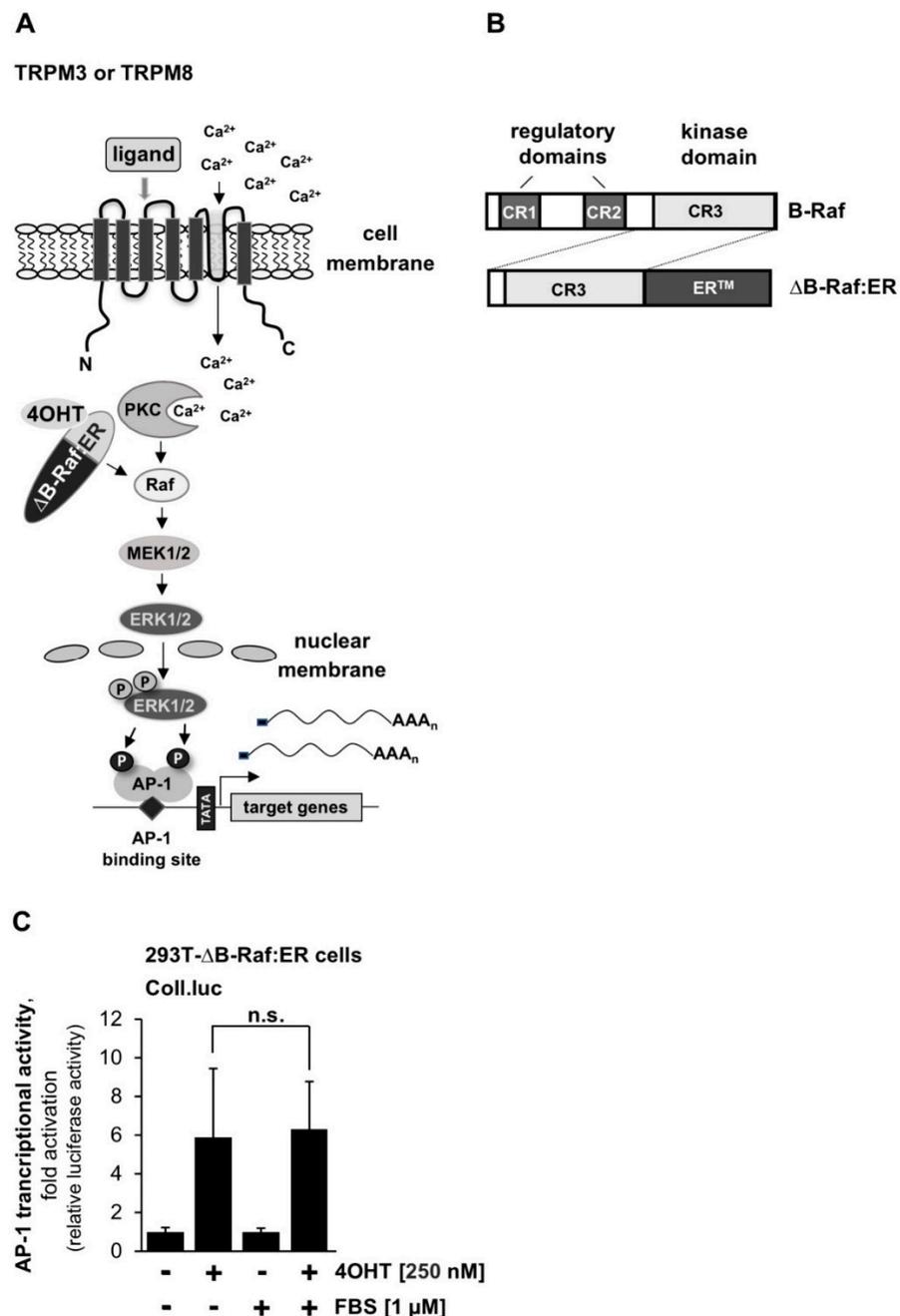


Figure 8. The compound *m*-3M3FBS fails to block B-Raf signaling. **(A)** Signaling pathway connecting TRPM8 and TRPM3 stimulation with AP-1-induced gene transcription. The activation point of the fusion protein Δ B-Raf:ER is indicated. **(B)** Signaling pathway connecting TRPM8 and TRPM3 stimulation with AP-1-induced gene transcription. The activation point of the fusion protein Δ B-Raf:ER is indicated. **(C)** HEK293- Δ B-Raf:ER cells were infected with a recombinant lentivirus containing Coll.luc reporter gene. The cells were incubated in medium containing 0.05% serum for 24 h. Stimulation was performed with 4OHT (100 nM) for 24 h. Cells were harvested and analyzed as described in the legend to Figure 1 (n = 3; n.s., not significant).

3. Discussion

The background of this study was the hypothesis that manipulation of PLC β activity affects TRP channel signaling based on the hypothesis that many TRP channels “are either activated downstream of the PLC pathway, or modulated by it” [36]. Many studies have used pharmacological inhibition of PLC with the compound U73122. However, numerous off-target effects of U73122 have been reported, including activation of TRPM4 channels,

inhibition of K^+ channels, and a PLC-independent release of Ca^{2+} ions from internal stores [16–22]. U73122 has been reported to induce alkylation of cysteine residues, which may explain its pleiotropic effects. We, therefore, decided to use genetics to inhibit PLC β .

The C-terminal domain of PLC β enzymes, comprising approximately 400 amino acids, consists of a proximal and a distal C-terminal domain. The proximal domain, located at the C-terminal of the C2 domain, contains a helix-turn-helix motif (H α 1—H α 2), the primary binding site for G α q [31], followed by the H α 2' helix, which has an autoinhibitory activity produced by docking to a cleft near the active site of PLC β in the absence of G α q [39]. It has been proposed that the H α 2' helix prevents the membrane interaction of the PLC β catalytic core [40]. PLC β enzymes have to interact with the membrane to efficiently hydrolyze their substrate, phosphatidylinositol 4,5-bisphosphate. The C-terminal domain of PLC β has been described as a major regulator of membrane binding of the enzymes, as membrane association and PLC β activity are reduced when this domain is deleted or mutated [41–43]. In particular, a coiled-coil structure within the C-terminal domain with clusters of lysine residues is thought to be involved in binding to lipids and/or proteins [44]. Experimentally, it has been shown that the C-terminal domain of PLC β 3 is required to target the enzyme to the lipid monolayer containing phosphatidylinositol 4,5-bisphosphate [23]. Overexpression of the C-terminal domains of PLC β enzymes impaired the G α q-mediated increase in intracellular Ca^{2+} concentration and additionally inhibited the hydrolysis of phosphatidylinositol 4,5-bisphosphate [33]. The authors proposed that the C-terminal domain of PLC β binds to activated G α q and, in this way, interferes with the interaction of G α q with wild-type PLC β enzymes. Expression of the C-terminal domain of PLC β 1 has been shown to block M1 muscarinic acetylcholine receptor-induced activation of α 1E Ca^{2+} channels [45]. In this study, we have shown that expression of the C-terminal domain of either PLC β 1 or PLC β 3 efficiently blocked intracellular signaling triggered by stimulation of a G α q-coupled designer receptor. The expressed C-terminal domains included the proximal C-terminal domain, including the helix-turn-helix motif (H α 1—H α 2), and the extended C-terminal domain, which is thought to be important for the membrane interaction. The H α 1—H α 2 motif has been identified as the major binding site for G α q, and experiments have shown that a mutated form of PLC γ containing an engineered H α 1—H α 2 domain, becomes subject to regulation by G α q [31]. We expressed the H α 1—H α 2 domain as a fusion protein together with a fluorescent protein and a membrane anchor and found that this protein was unable to inhibit designer receptor signaling. On the basis of these data, we propose that the expressed C-terminal domains of PLC β 1 and PLC β 3 interfere with plasma membrane components that are necessary for PLC β biological activity. The most likely candidate, of course, would be phosphatidylinositol 4,5-bisphosphate.

The biological activity of TRP channel activity, including TRPM3 and TRPM8, is regulated by phosphatidylinositol 4,5-bisphosphate. Sophisticated electrogenetic and chemical genetic tools used to reduce plasma membrane phosphatidylinositol 4,5-bisphosphate levels have shown that this phospholipid is required for TRPM3 and TRPM8 channel activities [3,4,6,46]. In addition, it has been suggested that activation of G α q-coupled receptors, which stimulates PLC β , inhibits TRPM3 and TRM8 activities [4,6]. Activation of TrkA receptors, which stimulates PLC γ , has been shown to inhibit TRPM8 current [6]. However, a direct relationship between the G α q-coupled receptor, TrkA or PDGF receptor-induced phosphatidylinositol 4,5-bisphosphate depletion, and impaired TRPM3 and TRPM8 activities was not been presented in this study because the decrease in phosphatidylinositol 4,5-bisphosphate level was estimated only in an indirect manner using the GST-PLC γ -PH biosensor. Due to possible off-target effects, results showing a loss of membrane localization of this biosensor after PLC activation should be taken with caution [16].

In our study, we did not aim to alter the concentration of phosphatidylinositol 4,5-bisphosphate. Rather, we focused on genetically induced inhibition of PLC β signaling. This study showed that expression of the C-terminal domain of PLC β 3, which contains no enzymatic activity, massively impaired cellular signaling induced by TRPM3 and TRPM8

channels. We hypothesize that the PLC β 3 C-terminal domain masks phosphatidylinositol 4,5-bisphosphate, which is then unavailable for regulation of TRP channel activity.

To complement these data obtained by inhibition of PLC β , we performed experiments with the compound *m*-3M3FBS, which has been described as an activator of PLC [38]. Several studies have been published directly linking the effects of *m*-3M3FBS to PLC activation and TRP channel activity [47,48]. The results presented in this study show that we were able to archive only a marginal increase in G α q signaling at a concentration of 1 μ M *m*-3M3FBS. Higher concentrations caused inhibition of G α q signaling and showed increased toxicity. Incubation of the cells with a concentration of 7.5 μ M already resulted in detachment of 50% of the cells from their support and we could not investigate whether *m*-3M3FBS acts as an activator of PLC at higher concentrations. Therefore, the results of experiments performed with an *m*-3M3FBS concentration of 25 to 30 μ M [47–49] are questionable. The classification of *m*-3M3FBS as a PLC activator has also been questioned by others. A study showed that *m*-3M3FBS affects Ca²⁺ homeostasis in a PLC-independent manner [50]. Most importantly, in this study, PLC activity was measured biochemically in the presence and absence of *m*-3M3FBS, in contrast to other reports that used an indirect assay based on translocation of a fluorescently labeled PLC γ -derived PH domain from the plasma membrane to the cytoplasm [47]. This suggests that conclusions based on the use of *m*-3M3FBS as a pharmacological PLC activator should be taken with caution [47–49]. In fact, we demonstrated that incubation of the cells with *m*-3M3FBS efficiently inhibited both TRPM3 and TRPM8 signaling. However, the direct target of this compound remains to be identified, and no direct correlation between PLC activity and the effects of *m*-3M3FBS can be postulated.

4. Materials and Methods

4.1. Cell Culture and Reagents

HEK293T/17 cells were infected with a lentivirus to express of R α q, a G α q-coupled designer receptor, as described [24]. HEK293 cells expressing TRP channels TRPM3 (T-REx-TRPM3 cells) and TRPM8 (HEK293-M8 cells) have been described elsewhere [4,51]. HEK293- Δ B-Raf:ER cells [52] express a conditionally active B-Raf protein kinase mutant that could be activated with 4-hydroxytamoxifen (4OHT, Sigma # H7904, dissolved in ethanol) for 24 h in medium containing 0.05% fetal calf serum. HEK293 cells, T-REx-TRPM3 cells, HEK293-M8 cells, and HEK293- Δ B-Raf:ER were incubated in DMEM containing 0.05% fetal bovine serum for 24 h prior to stimulation. Stimulation was performed with clozapine-*N*-oxide (1 μ M CNO, dissolved in ethanol, Enzo Life Sciences, Lörrach, Germany, # NS-105-0005), pregnenolone sulfate (PregS, 20 μ M, dissolved in DMSO, Sigma-Aldrich GmbH, Taufkirchen, Germany, # P162), icilin (1 μ M, Santa Cruz Biotechnology, Heidelberg, Germany, # sc-201557), or 4-hydroxytamoxifen (4OHT) (100 nM, Sigma # H7904, with ethanol as solvent) for 24 h in medium containing 0.05% fetal bovine serum. Cells were preincubated for 3 h with the compound 2,4,6-Trimethyl-*N*-[3-(trifluoromethyl)phenyl]benzenesulfonamide (*m*-3M3FBS) (Tocris, Bristol, UK, Cat.No. 1941, dissolved in DMSO) at a concentration of 5 μ M. Cells were stimulated for 24 h in the presence of *m*-3M3FBS.

4.2. Lentiviral Gene Transfer

The lentiviral transfer vector pFUW-R α q has been described elsewhere [24]. Plasmids pSYFP1-CTbeta1a and pSYFP1-CTbeta3, containing the C-terminal domains of PLC β 1 and PLC β 3 fused to YFP, were kindly provided by Joachim Goedhart and Theodorus Gadella, University of Amsterdam, The Netherlands [33]. Plasmid pSYFP1-CTbeta1a was cut with XhoI, filled in with the Klenow fragment of DNA polymerase I, and recut with BamHI. The fragment was cloned into the plasmid 3xFLAG-CMV that had been cut with HindIII, filled in with the Klenow fragment, and recut with BamHI, generating plasmid pCMV-FLAG-PLC β 1ct. This plasmid was cut with Sp1 and EcoRI and filled in with the Klenow fragment of DNA polymerase I. The insert was cloned into a lentiviral transfer vector, generating

plasmid pFCW-FLAG-PLC β 1ct. Plasmid pSYFP-CTbeta3 was cut with HindIII, filled in with the Klenow fragment, and recut with BamHI. The fragment was cloned into plasmid 3xFLAG-CMV. This plasmid (pCMV-FLAG-PLC β 3ct) was cut Sp1 and EcoRI, and filled in with the Klenow fragment of DNA polymerase I. The insert was cloned into a lentiviral transfer vector, resulting in plasmid pFCW-FLAG-PLC β 3ct. The FLAG-tagged C-terminal domains of PLC β 1 and PLC β 3 were expressed under the control of the cytomegalovirus IE promoter/enhancer. Plasmid YFP-HTH-CFP-Rac1-CaaX was a kind gift of John Sondek, University of North Carolina, Chapel Hill, NC, USA [32]. The plasmid was cut with BamHI and EcoRI and cloned into the filled in BglIII site of plasmid 3xFLAG-CMV, resulting in plasmid pCMV-FLAG-HTH-CFP-Rac1-CaaX. This plasmid was cut with BamHI, filled in with the Klenow fragment of DNA polymerase I, and recut with Ecl136II. The fragment was cloned into HpaI-cut plasmid pFUW, resulting in lentiviral transfer plasmid pFUW-FLAG-HTH-CFP-Rac1CaaX. Viral particles were produced by triple transfection of HEK293-TN cells with the gag-pol-rev packaging plasmid, the pCMVG plasmid that encodes the glycoprotein of vesicular stomatitis virus, and the lentiviral transfer vector [53].

4.3. Reporter Gene Assay

The lentiviral transfer vector pFWColl.luc has been described elsewhere [27]. Infected cells were maintained in medium containing 0.05% fetal bovine serum for 24 h and then stimulated with the appropriate ligands for 24 h. Cell extracts were prepared using reporter lysis buffer (Promega, Mannheim, Germany) and assayed for luciferase activities. Luciferase activity was normalized to the protein concentration. Luciferase activities of the extracts were measured using a luminometer (Berthold Detection Systems, Pforzheim, Germany). The light units were normalized to the protein concentration of the extracts, which was determined using a BCA protein assay kit.

4.4. Western Blots

Thirty micrograms of proteins were separated by SDS-PAGE. Blots were incubated with an antibody directed against either the extracellular signal-regulated protein kinase ERK1/2 (Santa Cruz Biotechnology, Heidelberg, Germany, # sc-153) or the phosphorylated form of ERK2 (Santa Cruz Biotechnology, Heidelberg, Germany, # sc-7383). The anti-ERK2 antibody reacted to a lesser extent with ERK1. FLAG-tagged proteins were detected in Western blot experiments using monoclonal antibody M2 (Sigma-Aldrich, Steinheim, Germany, # F1804) at a dilution of 1:3000 in TBS. Immunoreactive bands were detected with enhanced chemiluminescence, using a 1:1 solution of solution 1 (100 mM Tris-HCl, pH 8.5, 5.4 mM H₂O₂) and solution 2 (2.5 mM Luminol, 400 μ M p-coumaric acid, 100 mM Tris-HCl, pH 8.5).

4.5. Statistics

Data shown are means \pm SD of at least three independent experiments performed in quadruplicate. The two-tailed Student's *t*-test was used for the statistical analyses. Statistical probability is expressed as *** $p < 0.001$; ** $p < 0.01$, and * $p < 0.05$. We considered values significant when $p < 0.05$.

5. Conclusions

Expression of PLC β 1ct and PLC β 3ct, truncated forms of PLC β 1 and PLC β 3 comprising the C-terminal membrane association domains of the enzymes, almost completely blocked the signal transduction of a G α q-coupled designer receptor. Moreover, expression of PLC β 3ct significantly impaired signal transduction of TRP channels TRPM3 and TRPM8. The C-terminal domain of PLC β 3 interacts with plasma membrane targets, most likely phosphatidylinositol 4,5-bisphosphate, and, in this way, blocks the biological activation of TRPM3 and TRPM8, which require interaction with this phospholipid. Thus, PLC β regulates TRPM channels by masking phosphatidylinositol 4,5-bisphosphate with its C-terminal domain.

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