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TRPC4 as a coincident detector of $G_{i/o}$ and $G_{q/11}$ signaling: mechanisms and pathophysiological implications

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Abstract

TRPC channels are Ca^{2+} -permeable nonselective cation channels activated downstream from phospholipase C (PLC). Although most TRPC channels can be activated by stimulating $G_{q/11}$ -coupled receptors, TRPC4 requires simultaneous stimulation of $G_{i/o}$ -coupled receptors, making it a perfect detector of coincident $G_{i/o}$ and $G_{q/11}$ signaling. Evidence shows that activated $G\alpha_{i/o}$ proteins work together with PLC $\delta 1$ to induce robust TRPC4 activation and the process is accelerated by stimulation of other PLC isozymes, such as PLC β through $G_{q/11}$ proteins. Mechanistically, $G_{q/11}$ -PLC β activation produces triggering proton and calcium signals to initiate self-propagating PLC $\delta 1$ activity, crucial for $G_{i/o}$ -mediated TRPC4 function. Thus, TRPC4-containing channels are activated under conditions not only when coincident $G_{i/o}$ and $G_{q/11}$ stimulation occurs, but also when $G_{i/o}$ stimulation coincides with proton and Ca^{2+} signals. The resulting cytosolic Ca^{2+} rise and membrane depolarization switch the inhibitory $G_{i/o}$ response to excitation. The conditions and implications of $G_{i/o}$ -mediated TRPC4 activation in physiology and pathophysiology warrant further investigation.

Keywords

heterotrimeric G proteins; GPCR; phospholipase C; TRP channels; acidosis; calcium signaling

1. Introduction

TRPC4 is a member of the canonical subfamily of Transient Receptor Potential (TRP) channels. TRP superfamily contains 28 mammalian members of related proteins that share homology with a *Drosophila* protein named as TRP due to the transient receptor potential phenotype found in the loss-of-function mutations that affected phototransduction of fruit

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Author contributions

J.J. and J.B.T. prepared illustrations. M.X.Z. wrote the manuscript. All authors proofread and approved the manuscript.

Conflict of Interest statement

The authors claim no conflict of interest.

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flies^{1,2}. All TRPs function as cation channels with variable selectivities and Ca²⁺ permeabilities and thus affecting membrane potential and Ca²⁺ signaling in different ways to contribute to physiology and pathophysiology.

Structurally, they share a common tetrameric assembly with each subunit containing 6 transmembrane (TM) segments (S1-S6) and the intervening pore loop between the S5 and S6 segments, as well as the cytoplasmically localized amino-terminus and carboxyl-terminus (Fig. 1A). The S5-pore-S6 domains from the four subunits form the ion conducting pore, encircled by the S1-S4 TM regulatory domains. This architecture resembles that of classical voltage-gated K⁺, Na⁺, and Ca²⁺ channels, to which TRP channels display identical membrane topology and some sequence homology³, except for the more extended lengths and functional motifs found in the cytoplasmic termini of TRP channels. Based on sequence homology, the mammalian TRPs are divided into six subfamilies, TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPP (polycystin), and TRPML (mucolipin), among which the seven TRPC members are closest to the original *Drosophila* TRP protein, sharing 30-35% sequence identity at the protein level².

TRPCs form Ca²⁺-permeable nonselective cation channels activated downstream of phospholipase C (PLC) signaling^{4,5}. Of the many mechanisms that activate PLC, the G_{q/11}-PLC β and receptor tyrosine kinase (RTK)-PLC γ pathways are the most commonly known, and both have been shown to induce robust TRPC channel activation, resulting in membrane depolarization and cytosolic Ca²⁺ concentration ([Ca²⁺]_c) elevation. Therefore, TRPC channels have been referred to as receptor-operated channels and because of their pronounced effects on [Ca²⁺]_c, they are also frequently studied in the context of receptor-operated Ca²⁺ entry and/or store-operated Ca²⁺ entry. The latter designation implies a mechanism of activation by Ca²⁺ depletion from the endoplasmic reticulum (ER) store. Whether or not TRPC channels are directly activated in response to ER Ca²⁺ store depletion in the absence of receptor activation is still a subject of debate. Evidence exists for TRPC channels or their mediation of Ca²⁺ influx to respond to store depletion, perhaps through interaction with STIM1⁶⁻⁸, an ER membrane protein that transmits store depletion signal to plasma membrane channels^{9,10}. However, the TRPC currents exhibit distinct Ca²⁺ selectivity, unitary conductance, and voltage dependence from the most commonly known store-operated current, namely Ca²⁺-release activated Ca²⁺ current¹¹. Here, we focus on receptor-operated TRPC activation, giving specific attention to channels formed by TRPC4 because of its unique requirement on pertussis toxin (PTX)-sensitive G_{i/o} proteins.

2. PLC dependence of TRPC channel activation

Nearly all constituents of PLC signaling have been implicated in the activation of TRPC channels⁵. These include diacylglycerols (DAGs), inositol 1,4,5-trisphosphate (IP₃) or IP₃ receptors, Ca²⁺, and phosphatidylinositol 4,5-bisphosphate (PIP₂). DAGs are thought to act as direct ligands of TRPC channels; however, the binding site on the channel remains undefined¹², although recent evidence suggests that DAGs may gain access to TRPC3 channels through a subunit-joining fenestration behind the selectivity filter¹³. Besides DAG, IP₃ receptors can bind to a conserved C-terminal motif, known as Calmodulin IP₃ Receptor Binding (CIRB) site (Fig. 1B), found in all TRPC proteins; whereas the binding by IP₃

receptors promoted, that by Ca^{2+} -calmodulin inhibited the channel activation¹⁴. Moreover, Ca^{2+} and PIP_2 exert both positive and negative effects on TRPC activities⁵. The dependence on PIP_2 may reflect the needs for the PIP_2 hydrolysis products, e.g. DAGs and protons, to support TRPC activation¹⁵⁻¹⁷, and in the case of TRPC4, it may also reflect the need for plasma membrane targeting of PLC δ 1, an isozyme specifically required for the activation of TRPC4 channels¹⁸ (see later). On the other hand, phosphoinositides, including PIP_2 , may exert a tonic inhibition on TRPC channels^{5,16,18}. The PIP_2 concentrations optimal for the activation and inhibition of different TRPC channels, including both homomeric and heteromeric ones¹⁹, and under different stimulation conditions still remain to be elucidated. Generally, TRPC channel activation is regulated by multiple factors associated with PLC activation. Additionally, there are other modulatory factors, such as nitric oxide, reactive oxygen species and thioredoxin^{20,22}, indicating complex mechanisms of regulation.

Most studies have relied on overexpression of TRPC proteins in heterologous cells, which enhances the chance of spontaneous channel activation likely due to high protein abundance and/or uncontrolled endogenous factors. As a result, it is not easy to distinguish modulation from true activation mechanisms. Examining TRPC4 activation through $\text{G}_{q/11}$ -PLC β signaling, Thakur et al¹⁸ discovered that while the muscarinic receptor agonist, carbachol, caused robust $[\text{Ca}^{2+}]_c$ rise in parental HEK293 cells, the same treatment only elicited very weak, if any, TRPC4 current, unless a $\text{G}_{q/11}$ -coupled muscarinic receptor, M1, M3, or M5, was also overexpressed in these cells. By contrast, there was no difficulty in inducing TRPC3, C5, C6, and C7 currents via carbachol stimulation of the endogenously expressed muscarinic receptors in the HEK293 cells^{18,23,24}. Since overexpressed receptors can promiscuously couple to multiple G protein subtypes²⁵, PTX was tested and shown to be able to abolish the carbachol-evoked TRPC4 currents no matter if M1, M3, or M5 receptors were overexpressed. Therefore, unlike other TRPCs, the receptor-operated activation of TRPC4 shows an absolute dependence on PTX-sensitive $\text{G}_{i/o}$ proteins. This is in line with the findings that stimulating $\text{G}_{i/o}$ -coupled receptors, such as M2 and M4 muscarinic receptors, μ opioid receptors (μOR), and receptors for sphingosine-1-phosphate (S1P) or oxidized phospholipids, also activate TRPC4 or TRPC5 channels^{18,26,29}. Notably, although TRPC5 is closely related to and shares many common features and regulatory mechanisms with TRPC4, PTX only inhibited ~50% of TRPC5 currents elicited by either endogenous or overexpressed M3 receptors, indicative of partial dependence on $\text{G}_{i/o}$ signaling¹⁸.

3. $\text{G}_{i/o}$ -mediated TRPC4 activation and its dependence on PLC δ 1

Receptor-mediated activation of $\text{G}_{i/o}$ proteins produces two transducers, the activated $\text{G}\alpha_{i/o}$ -GTP and free $\text{G}\beta\gamma$ dimer, between which the latter could directly activate certain PLC β subtypes³⁰ and thereby induce TRPC activation similarly as $\text{G}_{q/11}$. However, this mechanism does not seem to work in the HEK293 cells used, as stimulating the heterologously expressed μOR barely elicited any Ca^{2+} transients; nor it evoked detectable responses of the co-expressed TRPC3, C6, or C7¹⁸. Thus, at least in HEK293 cells, $\text{G}_{i/o}$ stimulation is not efficiently coupled to PLC signaling. On the other hand, in experiments where different G protein subunits were coexpressed with TRPC4 or TRPC5, the constitutively active $\text{G}\alpha_{i2}$, $\text{G}\alpha_{i3}$, and $\text{G}\alpha_o$, but not $\text{G}\alpha_{i1}$ or various combinations of $\text{G}\beta\gamma$, were found to support TRPC4/C5 activation in the absence of any stimulus³¹. Also, the

activated $G\alpha_{i2}$ physically interacts with TRPC4 carboxyl terminus and the CIRB motif of TRPC4 is important for $G\alpha_{i2}$ -mediated channel activation³¹.

Interestingly, the CIRB motif is located in an exposed region at the end of a long α -helix that extends all the way to the center core of the cytoplasmic assembly in recently reported high resolution TRPC4 cryogenic electron microscopy structures^{32,33}. A long stretch of unresolved structure of ~40 residues, including a short amino-terminal portion (~6 residues) of the CIRB motif, exists upstream from CIRB, outlining the widest area of the channel complex (Fig. 1B-E). This suggests flexibility of the pre-CIRB region for binding to different protein partners, whereby variable conformations may be adopted for specific functional outcomes by pulling or pushing the upstream TRP re-entrant loop connected to the TRP helix (Fig. 1A-C). The long α -helix initiated from the CIRB motif is also perfectly situated to transmit the protein-protein interaction signals allosterically to channel gating through its central access and perhaps interactions with TRP helix, S4-S5 linker, and/or the C-terminal end of the S6 TM segment (Fig. 1A-C).

Although the stimulation of $G_{i/o}$ -coupled μ OR did not cause obvious PLC activation in the absence of TRPC4, μ OR-mediated TRPC4 activation was strongly dependent on PLC, as it was completely prevented by the PLC inhibitor, U73122. Through a number of complementary approaches, RNA interference, dominant negative suppression and inhibition by RhoA, it was determined that a specific PLC isozyme, PLC δ 1, underlies this absolute dependence on PLC¹⁸. PLC δ 1 is likely the simplest and most ancient form of all thirteen mammalian PLC isoforms known to date³⁴. Unlike other PLC isoforms that have developed sensing mechanisms for various extra- and intracellular signals, it is only known to be activated by Ca^{2+} , with PIP₂ also required as both the substrate and membrane anchor. Therefore, PLC δ 1 may partially explain the Ca^{2+} and PIP₂ dependence of TRPC4 activation. Consistent with the functional coupling, the levels of both TRPC4 and PLC δ 1 were increased in surgical specimens of epilepsy patients with either cortical lesions of focal cortical dysplasia II or tuberous sclerosis complex as compared to controls³⁵.

Notably, although PLC β s are not absolutely required for $G_{i/o}$ -mediated TRPC4 activation, costimulating a $G_{q/11}$ -coupled receptor or an RTK (e.g. EGF receptors) facilitated TRPC4 current development. Because of the Ca^{2+} dependence of PLC δ 1, it was thought that $G_{q/11}$ -PLC β or RTK-PLC γ signaling probably provided the initial $[Ca^{2+}]_c$ elevation that was propagated to PLC δ 1, which then further amplified the signal to induce robust channel activation. However, substituting the receptor agonist with the Ca^{2+} ionophore, ionomycin, only increased the probability but not the rate of $G_{i/o}$ -mediated TRPC4 activation¹⁸. More recently, this missing component of $G_{q/11}$ -PLC β pathway is found to be protons¹⁷, a by-product of PIP₂ hydrolysis¹⁶. Indeed, intracellular acidification resulting from PIP₂ hydrolysis had been shown to facilitate activation of TRP and TRP-like channels in *Drosophila* photoreceptors¹⁶. It was shown that intracellular protons act at PLC δ 1, rather than TRPC4, to accelerate Ca^{2+} -dependent PLC δ 1 activation via a positive-feedback mechanism¹⁷. Such a self-propagating capability of PLC δ 1, supported by H^+ and Ca^{2+} signals generated from its own action on PIP₂ hydrolysis, likely also underpins its function in amplifying signaling initiated by $G_{q/11}$ -PLC β , RTK-PLC γ , and other PLC isozymes. Interestingly, for *Drosophila* TRP, intracellular protons also affected the channel through an

indirect effect³⁶. Thus, although $G_{i/o}$ signaling is crucial for receptor-operated TRPC4 activation, it requires coincident PLC δ 1 activity, which can be triggered through ligand stimulation of $G_{q/11}$ -PLC β or RTK-PLC γ , or intracellular acidification and $[Ca^{2+}]_c$ rise (Figs. 2, 3A).

4. Outstanding questions on $G_{i/o}$ -mediated TRPC4 activation

Mechanism of TRPC4 activation by $G_{i/o}$ remains undefined. Experimental evidence supporting the involvement of $G_{\alpha_{i/o}}$ but not $G\beta\gamma$ includes *a*) coexpression of constitutively active $G_{\alpha_{i2}}$, $G_{\alpha_{i3}}$, or G_{α_o} , but not $G_{\alpha_{i1}}$, G_{α_q} or $G\beta\gamma$ supported spontaneous TRPC4 activation³¹, *b*) selectively inhibiting $G_{\alpha_{i/o}}$ -GTP while enhancing free $G\beta\gamma$ production through GoLoco domain proteins suppressed TRPC4 activation³⁷, and *c*) intracellular pipette dialysis of antibodies for G_{α_o} , but not that for $G\beta$, suppressed the development of muscarinic cation current (m/CAT)³⁸, an endogenous current found in gastrointestinal smooth muscle cells mediated mainly by TRPC4³⁹. However, unequivocal evidence ruling out the involvement of $G\beta\gamma$ has not been obtained. Secondly, although a direct interaction between TRPC4 carboxyl-terminus (residues 621-890) and $G_{\alpha_{i2}}$ has been demonstrated by glutathione S-transferase pull-down using purified proteins, other experimental support of the interaction had come from co-immunoprecipitation of cell lysates and Forster resonance energy transfer (FRET) in intact cells^{31,40}. It remains to be elucidated whether $G_{\alpha_{i/o}}$ or other G_{α} or $G\beta\gamma$ also binds to TRPC4 and where exactly they bind or whether other proteins are involved in facilitating the interaction. More importantly, solving structures of TRPC4 in complex with $G_{i/o}$ subunit(s) will reveal molecular details on how $G_{i/o}$ proteins gate this channel.

Physiological relevance of $G_{i/o}$ -mediated TRPC4 activation is another important question. The best example is m/CAT being mostly mediated by TRPC4 and responsible for neurogenic cholinergic contraction of gastrointestinal smooth muscles³⁹. m/CAT is dependent on both M2 and M3 muscarinic receptors⁴¹, and is suppressed by PTX treatment⁴², suggesting a codependence on $G_{i/o}$ and $G_{q/11}$ pathways. However, in other systems where TRPC4 function has been implicated, the studies have only considered $G_{q/11}$ -PLC β signaling (Fig. 3C)⁴³⁻⁴⁵. It would be important to examine the unintended involvement of $G_{i/o}$ proteins in these functions. In some cases, for instance thrombin-induced endothelial permeation⁴⁶, this would be obvious as thrombin receptors are coupled to both $G_{q/11}$ and $G_{i/o}$. In other cases, the coregulation might involve a different transmitter or receptor subtype, which would require more careful examinations. Indeed, we found that PTX treatment completely eliminated the large depolarizing plateau in lateral septal neurons induced by (S)-3,5-dihydroxyphenylglycine (DHPG) (unpublished), a group 1 metabotropic glutamate receptor agonist that induces epileptiform burst firing through TRPC4-containing channels^{43,47}. Alternatively, the $G_{i/o}$ requirement might be abrogated by including TRPC1 in the heteromeric TRPC1-C4 channels (Fig. 3A-B)^{43,48,49}. However, since neither the native nor heterologously expressed TRPC1-C4 channels had been tested for PTX sensitivity, the involvement of $G_{i/o}$ proteins cannot be completely rule out. The likelihood of TRPC4 to function as a detector of coincident $G_{i/o}$ and $G_{q/11}$ signaling in various physiological settings should not be overlooked. Given that in the nervous system, the activation of $G_{q/11}$ is typically associated with excitation whereas that of $G_{i/o}$ is

accompanied with inhibition⁵⁰, the consequence and functional significance of $G_{i/o}$ and $G_{q/11}$ costimulation, which likely happens often and with $G_{i/o}$ and $G_{q/11}$ inputs having different strengths, warrant further investigation.

Although stimulation of $G_{i/o}$ -coupled receptors evoked TRPC4 currents in heterologous systems^{18,31}, no similar response has been demonstrated in native systems. Therefore, the physiological activation of TRPC4 most likely requires coincident inputs of $G_{i/o}$ and $G_{q/11}$, with the latter probably substitutable by RTK or other means that triggers PLC activation. The latest finding, however, suggests that tissue acidosis, which occurs commonly under pathological conditions and lowers not only extracellular but also intracellular pH, can facilitate $G_{i/o}$ -dependent TRPC4 activation through sensitizing PLC δ 1¹⁷. This could underlie the mechanism by which TRPC4 contributes to neurodegeneration under pathological conditions⁴³

In summary, as a nonselective cation channel codependent on $G_{i/o}$ and PLC δ 1 for activation, TRPC4 is perfectly suited in integrating metabotropic signaling through the $G_{i/o}$ and $G_{q/11}$ pathways. The $G_{q/11}$ pathway may be substituted by stimulating other PLC isozymes or intracellular acidification to give greater versatility of this coincident sensor for signaling. Although the physiological and pathological implications remain to be fully elucidated, the detailed regulatory mechanisms revealed thus far have tremendously enriched our knowledge on receptor-operated channels and their functional significance.

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Figure 1. Structural organization of TRPC4 channel.

A. Diagram of main structural features of TRPC4 protein single subunit. Cylinders highlight α -helices in the transmembrane domain (S1-S6), amino (N) and carboxyl (C) terminal domains. AR1-AR4 indicate ankyrin-like repeats and H1-H7 indicate helix-loop-helix domains in the N-terminus. TRP helix or TRP domain (TRP) is present in most TRP channels. The regulatory domain formed by S1-S4 and pore domain formed by S5-pore loop-S6 are common for all TRP channels and most voltage-gated cation channels³. **B.** Assembly of the TRPC4 channel with four subunits. For the cytoplasmic area, only two subunits are shown. Note the crossover of the C-terminal coiled-coil domain downstream from the connecting helix. The disordered region between TRP re-entrant (labeled in **A**) and the connecting helix is highlighted by the red chain. The calmodulin and IP₃ receptor binding (CIRB) motif that encompasses parts of the connecting helix and the disordered region is indicated by the oval shade. The CIRB motif has been implicated to be critical for TRPC4 interaction with and/or regulation by calcium calmodulin (Ca^{2+} -CaM), IP₃ receptors (IP₃R), SEC14 domain and spectrin repeat-containing protein 1 (SESTD1), and $\text{G}\alpha_{12}$ -GTP. **C-E.** Ribbon diagrams of closed mouse TRPC4 channel structure based on single particle cryogenic electron microscopy³², with side views for a single subunit (**C**) and all four protomers (**D**), as well as the top and bottom views of the whole channel (**E**). Adapted from ref. 28 with modifications. Key structural regions are labeled in (**C**). CIRB motif and the disordered region (missing in the resolved structures^{32,33}) are indicated as yellow ovals and red chains in (**C**) and (**D**). Arrowheads in (**E**) indicate that the CIRB motif is largely exposed in the periphery of the cytoplasmic area of the channel.

Figure 2. Mechanism of TRPC4 channel activation by coincident $G_{i/o}$ and $G_{q/11}$ signaling. Schematic drawing of factors critical for receptor-operated TRPC4 channel activation in response to agonists that simultaneously activate both $G_{i/o}$ -coupled and $G_{q/11}$ -coupled receptors. The light yellow and light pink circles highlight the propagating H^+ and Ca^{2+} signals, respectively, that support continued PLC $\delta 1$ activation via positive feedback mechanisms, which can be initiated by stimulating $G_{q/11}$ -PLC β with a $G_{q/11}$ -coupled receptor agonist ($G_{q/11}$ -agonist). The $G_{q/11}$ -PLC β requirement may be bypassed by intracellular acidification in combination with an increase in cytosolic Ca^{2+} level. The propagating PLC $\delta 1$ activation needs simultaneous $G_{i/o}$ activation, most likely $G\alpha_{i/o}$ -GTP although the contribution of $G\beta\gamma$ cannot be completely ruled out at this point (pink dashed arrow), in order to support robust TRPC4 activation. Red arrows, protein-based regulation; blue arrows, regulation by ions and small diffusible messengers. $\pm$ indicate that the effects (of PIP_2 and Ca^{2+}) on TRPC4 activation can be both positive and negative. Abbreviations are given in the text.

Figure 3. Physiological and pathophysiological functions mediated by TRPC4 in response to receptor-operated activation of $G_{q/11}$ and/or $G_{i/o}$ proteins. A & B.

Possible differences in the mechanisms of activation of homotetrameric TRPC4 channel (**A**) from TRPC1-TRPC4 heterotetrameric channels (**B**) in response to coincident $G_{q/11}$ and $G_{i/o}$ signaling. For homomeric TRPC4, the $G_{q/11}$ -PLC β pathway mainly serves to initiate the self-propagating and H^+ - and Ca^{2+} -dependent positive feedback activation of PLC $\delta 1$, and the channel opening requires coincident $G_{i/o}$ input (**A**). For heteromeric TRPC1-C4 channels, $G_{q/11}$ -GTP might exert a direct stimulatory effect through TRPC1⁴⁸, which might obviate the requirement for PLC $\delta 1$ and/or $G_{i/o}$ (**B**). However, these possibilities have not been directly tested. **C**. Physiological and pathophysiological functions demonstrated to require TRPC4 based on studies using *Trpc4* knockout mice. Among them, gastrointestinal motility and bladder detrusor contraction regulations are cholinergic and require M2 ($G_{i/o}$ -coupled) and M3 ($G_{q/11}$ -coupled) muscarinic receptors^{39,41,42}. Lung vascular permeability regulation⁴⁶ likely requires both $G_{q/11}$ and $G_{i/o}$ when it is induced via thrombin receptors. The depolarizing plateau that underlies epileptiform burst firing and neurodegeneration of lateral septal neurons⁴³, is triggered by stimulating group 1 metabotropic glutamate receptors^{43,47}, but the response is inhibited by pertussis toxin (unpublished). For fear related-behavior and serotonergic itch, only $G_{q/11}$ signaling has been suggested^{44,45}; however, no report has ruled out the involvement of $G_{i/o}$ signaling at this point.