Close spatio-association of the transient receptor potential canonical 4 (TRPC4) channel with Go-i in TRPC4 activation process

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Myeong J, Kwak M, Jeon JP, Hong C, Jeon JH, So I. Close spatio-association of the transient receptor potential canonical 4 (TRPC4) channel with Go-i in TRPC4 activation process. Am J Physiol Cell Physiol 308: C879–C889, 2015. First published March 18, 2015; doi:10.1152/ajpcell.00374.2014.—TRPC4 channels are Ca2+-permeable, nonselective cation channels that are activated by a wide variety of stimuli, including G protein-coupled receptors (GPCR). TRPC4 is commonly assumed to be activated by Gq/phospholipase C-coupled receptors. However, the other molecular mechanisms by which G proteins regulate TRPC4 remain unclear. Here, we found that Go-i regulates TRPC4 activation by direct binding. To investigate this mechanism, we used whole patch clamp and fluorescence resonance energy transfer (FRET). We tagged an isoform of mTRPC4 and G protein with CFP and YFP, respectively, and transiently transfected cells with the FRET pair. The FRET efficiency between TRPC4-CFP and the constitutively active mutant form of Go-i2 was nearly 15% and was greater than that observed with wild-type Go-i2 (nearly 5%). Gβγ and the TRPC4 channel showed a FRET efficiency lower than 6%. In HEK293 cells transfected with the M2 muscarinic receptor, the application of carbachol increased the FRET efficiency between TRPC4-CFP and Go-i2(WT)-YFP from 4.7 ± 0.4% (n = 7) to 12.6 ± 1.4% (n = 7). We also found that the TRPC4 channel directly interacts with Go-i2, but not with Goαq, when the channel is open. We analyzed the calcium levels in HEK293 cells expressing the channels and Go-i2 or Goαq using the calcium indicator YC6.1 (Yellow Cameleon 6.1). In response to the muscarinic agonist carbachol, M2-, Go-i2-, and TRPC4-expressing cells showed a prolonged Ca2+ influx compared with cells expressing only M2. Together, these data suggest that Go-i2 activates the TRPC4 channel by direct binding, which then induces Ca2+ entry.

TRPC4; G protein; FRET; calcium

THE SEVEN MAMMALIAN TRANSIENT RECEPTOR POTENTIAL CANONICAL PROTEINS (TRPC1–7) ARE CA2+-PERMEABLE, NONSELECTIVE CATION CHANNELS THAT ARE WIDELY EXPRESSED IN NUMEROUS CELL TYPES. THE TRANSMEMBRANE DOMAIN OF THESE PROTEINS IS FLANKED BY PLASMATIC NH2 AND COOH TERMINI (1). FUNCTIONAL TRPC CHANNELS PREVIOUSLY CONSIST OF HOMOTRAMETERS (4, 30). HETEROERIC TRPC CHANNELS HAVE ALSO BEEN CONFIRMED AMONG SUBFAMILIES AND CONSIST OF TRPC1/4/5 OR TRPC3/6/7 (27). SEVERAL ASSUMPTIONS ABOUT THE STRUCTURE AND FUNCTION OF TRPC CHANNELS WERE FORMED BASED ON ANALOGOUS REASONING. BASED ON SEQUENCE SIMILARITY, TRPCs CAN BE FURTHER SUBDIVIDED INTO FOUR GROUPS. GROUP 1 AND 2 EXCLUSIVELY CONTAIN THE ISOFORMS TRPC1 AND TRPC2, RESPECTIVELY. GROUP 3 INCLUDES TRPC3, TRPC6, AND TRPC7, WHETHER GROUP 4 INCLUDES TRPC4 AND TRPC5. GROUP 4 TRPCs ARE MOST CLOSELY RELATED TO GROUP 1. THE TRPC4 CHANNELS ARE PARTICULARLY HIGHLY EXPRESSED IN THE BRAIN AND SMOOTH MUSCLES. THESE CHANNELS HAVE BEEN REPORTED TO FUNCTION IN NEUROTRANSMITTER RELEASE, GROWTH CONE EXTENSION, AND SMOOTH MUSCLE CONTRACTION. IN INTESTINAL SMOOTH MUSCLE CELLS, TRPC4 FORMS A 55-pS CATION CHANNEL AND UNDERLIES MORE THAN 80% OF THE MUSCARINIC RECEPTOR-INDUCED CATION CURRENT (mCAT) (29). TWO OF THE MOST ABUNDANT TRPC4 VARIANTS ARE A “FULL-LENGTH” TRPC4α AND A SHORTER TRPC4β LACKING AN 84-AMINO ACID FRAGMENT (781–864 IN THE CASE OF MOUSE TRPC4) IN THE CYTOSOLIC COOH TERMINUS. HOWEVER, THE FUNCTION OF THE TRPC4 FRAGMENT DELETION REMAINS UNKNOWN.

TRPC4 CHANNELS ARE ASSUMED TO BE ACTIVATED BY THE STIMULATION OF Gq/phospholipase C-coupled receptors. STIMULATION IS FOLLOWED BY G PROTEIN-MEDIATED ACTIVATION OF PHOSPHOLIPASE C (PLC) AND RESULTS IN THE STIMULATION OF CURRENT THAT IS GENERATED BY TRP CHANNELS (17, 20, 23). IN MAMMALIAN CELLS, THE ACTIVATION OF PLCS BY EXTRACELLULAR SIGNALING MOLECULES LEADS TO THE PRODUCTION OF INOSITOL 1,4,5-TRIPHOSPHATE (InsP3) AND DIACYLGlycerol AND COUPLES WITH INTRACELLULAR SIGNALING CASCADES BY INCREASING THE CYTOSOLIC CA2+ CONCENTRATION ([Ca2+]i). THESE CHANGES IN [Ca2+]i RESULT FROM InsP3-MEDIATED CA2+ RELEASE FROM INTRACELLULAR STORES AND/OR CA2+ ENTRY FROM THE EXTRACELLULAR SPACE. CA2+ ENTRY CAN BE REGULATED BY DIFFERENT MECHANISMS. IN MANY CELL TYPES, THE DEPLETION OF INTRACELLULAR CA2+ STORES FOLLOWING CA2+ RELEASE REVERSES THE INHIBITION OF CA2+ UPTAKE AND LEADS TO CA2+ ENTRY FROM THE EXTRACELLULAR SPACE, A PROCESS OFTEN TERMED CAPACITATIVE CALCIUM ENTRY AND MEDIATED BY STORE-OPERATED CHANNELS (21, 22). HOWEVER, THE SIGNALING PROCESSES THAT ARE ACTIVATED DOWNSTREAM OF G PROTEIN-COUPLED RECEPTORS REMAIN HIGHLY CONTroversIAL.

IN OUR PREVIOUS STUDY (8), WE CONFIRMED THAT Go-i ACTIVATES TRPC4/5 CHANNELS, WHEREAS Goαq INHIBITS THEM, BY USING AN ELECTROPHYSIOLOGY TECHNIQUE AND COMMINOOCIpiATION (CO-IP). HOWEVER, WE ALSO FOUND THAT MANY OTHER G PROTEIN INTERACTIONS WITH TRPC4 WERE NOT CONstrained BY MEASUREMENTS. TO FURTHER INVESTIGATE DIRECT INTERACTIONS BETWEEN TRPC4 AND Go-i, WE USED A FLUORESCENCE (Förster) RESONANCE ENERGY TRANSFER (FRET)-BASED METHOD. FRET IS AN OPTICAL TECHNIQUE THAT DEPENDS ON THE CLOSE PROXIMITY, WITHIN 10 nm, OF TWO DYES TO MONITOR THEIR RELATIVE MOLECULAR DYNAMICS IN INTACT CELLS IN REAL TIME. IN THE PRESENT STUDY, WE CONFIRMED HOMO- AND HETEROERICAN CONFORMATION OF THE TRPC4α AND TRPC4β CHANNELS BY FRET. WE USED FRET TO MONITOR G PROTEIN ACTIVITY BY MEASURING BOTH THE INTERACTION OF Go WITH Gβγ, WHICH DECREASED IN THE PRESENCE OF ANTAGONIST, AND THE INTERACTION OF Go WITH TRPCB, AS WELL AS THE ACTIVITY OF TRPC4, WHICH INCREASED IN THE PRESENCE OF AGONIST. ELECTROPHYSIOLOGY WAS USED TO EXAMINE TRPC4 CHANNEL ACTIVATION. WE ALSO FOUND THAT THE TRPC4 CHANNEL DIRECTLY
interacts with Go12, but not Goi4, while the channel is opened. The opening of the TRPC4 channel also induced Ca^{2+} influx.

MATERIALS AND METHODS

Cell culture, transient transfection, and cDNA clones. Human embryonic kidney (HEK293) cells (ATCC, Manassas, VA) were maintained according to the supplier’s recommendations. HEK293 cells were incubated in DMEM supplemented with 10% heat-inactivated FBS and penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a 5% CO2-humidified incubator. The cells were seeded in confocal dishes for FRET recording or 12-well plates for whole cell patch clamp studies. The following day, transfection was performed with Fugen-6 according to the manufacturer’s instructions. TRPC4α-XFP (CFP or YFP), XFP-TRPC4β, TRPC4β-XFP, G protein, and/or muscarinic receptor were transfected. The next day, the electrophysiology experiment was performed, FRET or both studies.

Electrophysiology. The cells were transferred onto a solution chamber on the stage of an inverted microscope (IX70, Olympus, Japan). The whole cell configuration was used to measure TRPC channel current in HEK cells, as described previously (10–12, 26, 31). Cells were left for 10–15 min to attach to coverslips. Whole cell currents were recorded using an Axopatch 200B amplifier (Axon Instruments). Patch pipettes were made from boro-silicate glass and had resistances of 3–5 MΩ when filled with normal intracellular solutions. Bath

![Fig. 1. Characteristic of transient receptor potential canonical protein (TRPC4) 4. A: graphics of the tetrameric structure of TRPC4 channels. The TRPC4 channel encompasses 6 putative transmembrane helixes (S1–S6). Voltage changes are detected by a typical voltage sensor domain in S4. A tetramer of the S5 and S6 segments constitute a central ion-conducting pore. TRPC4β lacks 84 amino acids in the COOH terminus, which correspond to the last 2 calmodulin-binding sites of full-length TRPC4α. B: double rectifying I-V relationship of TRPC4α-CFP (red) and TRPC4β-CFP (black) channels. C: whole cell patch clamp recordings reveal that cells that express TRPC4β-XFP, XFP-mTRPC4β, and TRPC4α-XFP show robust GTPγS-induced current (n = 5–10). D: XY, YZ, and ZY plane images of channels and Goi protein. TRPC4α-CFP and TRPC4β-CFP channels were expressed at the plasma membrane (PM) with punctate distribution. YFP-tagged Go12(WT)-YFP was localized uniformly at the PM. The cyan box in the construct scheme indicates CFP. Error bars represent SE. *P < 0.05 by 2-sample t-test. Scale bar, 10 μm.](http://ajpcell.physiology.org/doi/10.220.32.246)
solution was changed from Normal Tyrode (NT) to Cs⁺-rich external solution after whole cell recording system was established. The NT contained 135 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES with a pH that was adjusted to 7.4 using NaOH. The Cs⁺-rich external solution contained equimolar CsCl rather than NaCl and KCl. The internal solution contained 140 mM CsCl, 10 mM HEPES, 0.2 mM Tris-guanosine 5′-triphosphate, 0.5 mM EGTA, and 3 mM Mg-adenosine 5′-triphosphate with a pH that was adjusted to 7.3 with CsOH. We used 0.2 mM guanosine 5′-O-[y-thio]triphosphate (GTP[S]) purchased from Sigma. Voltage ramp pulse was applied from +100 mV to −100 mV for 500 ms at −60 mV holding potential. Experiments were performed at room temperature (18–22°C). The recording chamber was continuously perfused at a flow rate of 1–2 ml/min.

Image quantification and FRET measurements. HEK293 cells were cultured in a 35-mm coverslip bottom dish to obtain images and to measure FRET efficiency. To obtain the image and FRET efficiency of a cell, we used an inverted microscope (IX70, Olympus, Japan) with a 60 oil objective lens and the 3-cube FRET calculation (3) controlled by MetaMorph 7.6 (Molecular Devices). We mainly used three-cube FRET; three-cube FRET efficiency (cube settings for CFP, YFP, and Raw FRET) were acquired from a pE-1 main unit to three-cube FRET (excitation, dichroic mirror, filter) through a fixed collimator: CFP (ET 435/20 nm, ET CFP/YFP/mCherry beam splitter, ET 470/24 nm, chroma); YFP (ET500/20 nm, ET CFP/YFP/mCherry beam splitter, ET535/30 nm, chroma); and Raw FRET (ET435/20 nm, ET CFP/YFP/mCherry beam splitter, ET535/30 nm, Chroma). The excitation LED and filter were sequentially rotated, the rotation period for each of the filter cubes was ~0.5 s, and all images (three for CFP/YFP/Raw FRET, respectively) were obtained within 1.5 s. Each of the images was acquired on a cooled 10-MHz (14 bit) CCD camera (DR-328G-C01-SIL; Clara, ANDOR Technology) with an exposure time of 100 ms with 2 × 2 or 3 × 3 binning under the control of MetaMorph 7.6 software. Our FRET recording of the fluorophores was restricted in a range of CFP/YFP ratio being 0.5 to 2.0.

FR and FRET efficiency computation. The three-cube FRET ratio (FR) is equal to the fractional increase in YFP emission due to FRET and was calculated as

\[
FR = \frac{F_{AD}}{F_A} = \frac{[S_{FRET}(DA) - R_{D1} \cdot S_{CFP}(DA)]}{R_{A1} \cdot [S_{YFP}(DA) - R_{D2} \cdot S_{CFP}(DA)]}
\]

SCUBE (SPECIMEN DA) denotes an intensity measurement, where CUBE indicates the filter cube (CFP, YFP, or FRET) and SPECIMEN.
The key prerequisites for our experiments on the interaction between TRPC4 and G proteins were the strategic attachment of CFP or YFP to the TRPC4 channel and the verification that the resulting fusion proteins exhibited preserved activation. After designing the GFP fusion variants of the TRPC4α and TRPC4β channels, we confirmed that the TRPC4 channels tagged with GFP variants functioned properly and formed tetramer complexes. With the patch clamp technique, functional TRPC4 channels typically show a double rectifying current-voltage (*I*-*V*) relationship and display reversal potentials close to 0 mV (8). TRPC4α (Fig. 1B, red) and TRPC4β (Fig. 1B, black) channels tagged with GFP variants at the COOH terminus both displayed a double rectifying *I*-*V* relationship. The TRPC4α and TRPC4β currents were induced by an intracellular infusion of GTPγS through the patch pipette. Fluorescence-tagged TRPC4 channels showed the typical TRPC4 current with a double rectifying shape (Fig. 1C). The current amplitude of the tagged TRPC4 channel varied based on the location of the tagging site (i.e., NH2 terminus or COOH terminus). The *I*-*V* curve for TRPC4 was obtained by subtracting the *I*-*V* curve in NT solution from the *I*-*V* curve recorded in Cs+-rich solution. The TRPC4α-CFP (Fig. 1D1) and TRPC4β-CFP (Fig. 1D2) channels were expressed at the plasma membrane with punctate distribution, as previously reported (8, 13).

Next, to study G protein subunit interactions in the cells, CFP or YFP was inserted into the αBC-loop within the α-belical domain of the Goα2, a domain that has been used previously to insert various sequences into Goα subunits (5, 6, 32). Goα2(WT)-YFP was expressed uniformly at the plasma membrane (Fig. 1D3).

**Sensitive and selective detection of FRET in living cells using three-cube FRET.** To investigate the interaction between the TRPC4 channel and G proteins, we first evaluated our three-cube FRET experiment system. The control experiment verified that three-cube FRET provided sensitive and selective detection of FRET (Fig. 2A). Cells coexpressing empty-CFP and empty-YFP showed no FRET (1.64 ± 0.62%, *n* = 19),
arguing against confounding concentration-dependent artifacts. Cells expressing a TRPC4α-CFP and empty-YFP pair (2.03 ± 1.11%, n = 8) and a TRPC4α-YFP and empty-CFP pair (0.46 ± 0.54%, n = 11) exhibited no FRET. In all FRET experiments, we performed a negative control experiment through the combination of empty-CFP or -YFP with the GFP variant-tagged proteins, which generated almost zero FRET efficiency. However, a significant increase in FRET efficiency was observed for cells expressing a CFP-YFP concatemer (52.74 ± 0.80%, n = 27), in which CFP and YFP are held within 10 nm of each other. CFP dequenching may represent the simplest and most practical alternative to three-cube FRET for our experiments and thus provided an important opportunity to independently verify the three-cube FRET measurements. We applied donor

Fig. 4. A: FRET efficiency between TRPC4α or TRPC4β and Gα proteins. Except for TRPC4β-CFP and Gα2(CA)-YFP, all tagged protein FRET pairs showed lower than 7% FRET efficiency. B: interaction of TRPC4α and TRPC4β with Gβ and Gγ protein did not generate any significant FRET efficiency. C: muscarinic receptors 2 and 3 tagged with YFP showed lower than 6% FRET efficiency with TRPC4α and Gα protein. D: Gα2(CA)-YFP was transfected in HEK293 cells. Line scan shows Gα2(CA)-YFP intensity followed by a white dashed line. E: TRPC4β-CFP and Gα2(CA)-YFP were expressed in HEK293 cells. F: TRPC4α-CFP and Gα2(CA)-YFP were expressed in HEK293 cells. Error bars represent SE. *P < 0.05 by 2-sample t-test. Scale bar, 10 μm.
Table 1. FRET efficiency of TRPC4 isoforms with G proteins

<table>
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<th>Expression Type</th>
<th>Goq2 (WT)-YFP</th>
<th>Goq2 (CA)-YFP</th>
<th>Goq4(WT)-YFP</th>
<th>Goq4(CA)-YFP</th>
<th>Gi2-YFP, Gi2</th>
<th>Gβγ-YFP</th>
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<td>CFP-mTRPC4β</td>
<td>8.1 ± 2.8</td>
<td>18.3 ± 4.0</td>
<td>3.5 ± 0.8</td>
<td>2.6 ± 1.0</td>
<td>2.2 ± 1.4</td>
<td>0.2 ± 0.7</td>
</tr>
<tr>
<td>mTRPC4β-CFP</td>
<td>4.9 ± 0.8</td>
<td>15.8 ± 1.2</td>
<td>6.4 ± 1.2</td>
<td>6.0 ± 1.3</td>
<td>5.9 ± 2.9</td>
<td>4.0 ± 1.7</td>
</tr>
<tr>
<td>mTRPC4α-CFP</td>
<td>4.2 ± 1.5</td>
<td>6.5 ± 1.2</td>
<td>3.0 ± 1.6</td>
<td>0.3 ± 0.5</td>
<td>2.8 ± 1.3</td>
<td>3.3 ± 1.5</td>
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Data are presented as means ± SE in %. TRPC, transient receptor potential canonical channel; FRET, fluorescence resonance energy transfer; WT, wild type; CA, constitutively active; CFP and YFP, fluorescent proteins.

dequenching to the CFP-YFP concatamer studied above. This approach yielded a FRET efficiency of 55.7 ± 0.8\% (n = 11), closely matching the value of 52.7 ± 0.8\% (n = 27) determined from three-cube FRET. When plotted together, the FRET efficiency values from the two approaches were indistinguishable (Fig. 2B). Previous studies and the preferential enrichment observed in this study encouraged us to undertake direct FRET-based tests of protein-protein interactions, as described below.

After validating the system, we tested the interactions within Ga\βγ heterotrimers. With YFP-tagged Gβ and Gγ proteins (25), we investigated whether the Ga\βγ heterotrimer was localized at the plasma membrane and its interactions based on the observed FRET signal (Fig. 2C). Under these conditions, we observed a FRET signal for YFP-Gβ (18.9 ± 2.0\%, n = 24) and YFP-Gγ (17.6 ± 3.0\%, n = 16), using Goq2(WT)-CFP as the donor molecule. Additionally, FRET signals were detected for Goq2 (CA)-CFP with YFP-Gβ (18.9 ± 2.0\%, n = 24) and YFP-Gγ (17.6 ± 3.0\%, n = 16).

Next, we tested the homotetrameric structure of TRPC4\α and TRPC4β (Fig. 3A). The coexpression of empty-YFP and TRPC4\α-CFP or TRPC4β-CFP resulted in a FRET efficiency of ~1\%. In striking contrast, the coexpression of TRPC4\α-CFP and TRPC4β-YFP channels showed robust FRET efficiencies (TRPC4\α, 23.8 ± 2.8\%, n = 11; TRPC4β, 22.1 ± 0.7\%, n = 73), indicating that the tagged channels formed a tetrameric structure. Moreover, we tested the heteromeric structure of the TRPC4 channel by coexpressing CFP- or YFP-tagged TRPC4\α and CFP- or YFP-tagged TRPC4\β channels (Fig. 3B). Interestingly, we observed that channels with GFP variants at the same termini showed greater proximity than the channels tagged at other termini. These results suggest that TRPC4\α and TRPC4\β channels could form homomeric and heteromeric structures with similar termini located closer to one another than to opposite termini.

Constitutively active Goq2 proteins activate TRPC4\β channels by direct binding. TRPC4 channels are commonly assumed to be activated by a Goq pathway-based mechanism. To confirm which subunit of the G protein interacts with the TRPC4 channels in living cells, we generated GFP variant-fused constructs of TRPC4 and G protein [Goq2(WT), Goq2(CA) (constitutively active), Goq2(WT), Goq2(CA), Gβ, Gγ] and measured the FRET efficiency among these proteins. The Goq(CA) lacks intrinsic GTPase activity. If local organization (within ~2–10 nm) exists at the plasma membrane, FRET between the donor and the acceptor molecule would be apparent. We collected fluorescent signals from the plasma membrane of HEK293 cells expressing various constructs. Under these conditions, we observed a FRET signal for Goq2(WT)-YFP (4.2 ± 1.5\%, n = 21), Goq2(CA)-YFP (6.5 ± 1.2\%, n = 9), Goq2(WT)-YFP (3.0 ± 1.6\%, n = 12), and Goq2(CA)-YFP (0.3 ± 0.6, n = 10), using TRPC4\α-CFP as the donor molecule (Fig. 4A). These results indicate that TRPC4\α did not exhibit any significant FRET signal with any Goq protein. FRET efficiencies were...
observed for Go12(WT)-YFP (4.9 ± 0.8%, n = 25), Go12(CA)-YFP (15.8 ± 1.2%, n = 15), Goq(WT)-YFP (6.4 ± 1.2, n = 8), and Goq(CA)-YFP (6.0 ± 1.3%, n = 10), using TRPC4β-CFP (Fig. 4A). These results indicate a close proximity between the COOH terminus of TRPC4 and Go12. To further confirm the interaction of TRPC4 and Go12, we observed the FRET efficiency between NH2-terminally tagged CFP-TRPC4 and Go12(WT)-YFP (8.1 ± 2.8%, n = 12), Go12(CA)-YFP (18.3 ± 4.0%, n = 11), Goq(WT)-YFP (3.5 ± 0.8%, n = 8), and Goq(CA)-YFP (2.6 ± 1.0%, n = 7). Results similar to those for COOH-terminally tagged TRPC4-CFP were obtained.

In addition, we tested the FRET efficiency of TRPC4-CFP channels with YFP-Gβ or YFP-Gγ protein (Fig. 4B) and CFP tagged TRPC4 channels with YFP tagged muscarinic receptor type 2 or type 3 (Fig. 4C). These proteins did not exhibit any significant FRET signals. We summarize FRET efficiency of TRPC4 channel with G proteins in Table 1.

To confirm the relationship between the FRET efficiency and channel activation, we used both whole cell voltage clamps and FRET in the same cells. In a previous study, we had found that TRPC4 channels were activated by Go12(CA) but not Goq(CA) (Fig. 5A; see also Ref. 8). To ensure that Go12 binding truly activates TRPC4β channels, we recorded whole cell current and FRET efficiency simultaneously (Fig. 5B). Cells expressing TRPC4β-CFP and Go12(WT)-YFP showed less than ~4% FRET efficiency and almost basal activity ~1.5 pA/pF (Fig. 5B, top). In contrast, with Go12(CA)-YFP, the observed FRET efficiency and channel activity at ~60 mV were 18.9% and ~45.8 pA/pF, respectively (Fig. 5B, bottom). Quantifying differences in FRET efficiency associated with activation. We tested the effects of the expressed Goα1-coupled M2 and Goq-coupled M3 muscarinic receptors on the activation of TRPC4-CFP by stimulation of 100 μM carbachol (CCh) and endogenous G protein in HEK cells (Fig. 6A). TRPC4-CFP channels displaying a typical doubly rectifying I-V relationship...
were stimulated by 100 μM CCh. Next, we investigated whether any changes in the FRET efficiency between TRPC4 and wild-type Goα2 or Goα4 were generated (Fig. 6B). Only cells expressing TRPC4-CFP, Goα2-YFP, and M2 showed a significant difference in the FRET efficiency between the control conditions (4.7 ± 0.4%, n = 7) and stimulation by 100 μM CCh (12.6 ± 1.4%, n = 7); however, no significant difference in the FRET efficiency at TRPCα was observed. Every cell was checked for FRET efficiency 10 s before and 30 s after CCh application. As expected, the FRET efficiency stimulated by CCh (12.6%) was almost similar to that between Goα2(CA)-YFP and TRPC4β-CFP (15.8%). We also tested the other possibility that the FRET efficiency might occur between the muscarinic receptor and TRPC4 channel (Fig. 6C). However, no significant changes in the FRET efficiency were observed with these proteins.

To investigate the timing of the observed interactions, we performed whole cell recording during FRET measurement of cells coexpressing TRPC4-CFP and Goα2(WT)-YFP (Fig. 7A, left). During the recording, an increase in both recorded values was observed 10 s after the application of CCh. We recorded half-maximum time of 100 mV (19.03 ± 1.58 s, n = 9), −60 mV (25.40 ± 3.85, n = 9), and FRET efficiency (12.33 ± 2.81 s, n = 9) after CCh stimulation. Furthermore, the TRPC4 current peaked according to a time course similar to that of the FRET efficiency values. Before CCh stimulation, the current densities at +100 mV, and −60 mV and FRET efficiency were 59.40 ± 12.04 pA/pF, −11.49 ± 2.23 pA/pF, and 7.23 ± 1.49%, respectively (red arrows, before 20 s). After CCh stimulation, the current densities and FRET efficiency were 163.55 ± 38.50 pA/pF, −28.25 ± 6.08 pA/pF and 14.83%, respectively (black arrows), after 40 s (Fig. 7A, right). We conducted this experiment nine times repeatedly in other cells. These results indicate that channel activation is accompanied by two proteins’ interaction. FRET efficiency also decayed with channel inactivation. This result suggests that the interaction of channels with Goα2 is important for channel activation and inactivation.

We evaluated the interactions within Goβγ protein heterotrimers by measuring changes in the FRET efficiency between Goα2(WT)-CFP and Gβ-YFP during channel current recordings (Fig. 7B). Jensen et al. (7) had observed that the amplitude of the FRET efficiency between Goα3 and Gβ1 proteins decreased with muscarinic stimulation. In our study, the resting...
FRET efficiency of Goα and Gβ was ~20%, and the FRET efficiency decreased to ~12% after stimulation with 100 μM CCh. Half-maximum time of FRET efficiency was 10.70 ± 1.64 s (n = 7) after CCh stimulation. Such a decrease indicates the binding/unbinding of Goβγ or conformational rearrangement within the Goβγ protein. We also found that any changes in FRET efficiency preceded channel activation. Before CCh stimulation, the current densities and FRET efficiency were 6.26 pA/pF, and 18.25% respectively (before 20 s). After CCh stimulation, the current densities and FRET efficiency were 136.48 ± 29.31 pA/pF, −24.52 ± 11.49 pA/pF, and 11.36 ± 1.58%, respectively (after 40 s) (Fig. 7B, right). We conducted this experiment seven times repeatedly in other cells.

Goα2 activation of TRPC4 channels induces an influx of Ca2+. Taken together, the results suggested that, after the interaction of Goα2 and the TRPC4 channel, constitutive Ca2+ influx leads to increased basal calcium levels. We used YC6.1 (Yellow Cameleon 6.1) to detect the cell calcium levels. YC6.1 is a fluorescent indicator for Ca2+ based on CFP, YFP, and calmodulin (CaM) and has been a useful tool for a long time for measuring free Ca2+ concentrations in living cells. YC6.1 changes the FRET efficiency within the physiologically significant range (0.05–1 μM) for cytoplasmic Ca2+ concentrations (28). Using YC6.1, we found that Goα4(CA) only increased the FRET efficiency, indicating that the Goα2-PLCβ pathway increases the cytosolic Ca2+ concentration, although constitutively active Goα4(CA) could not activate TRPC4 channels. Goαq-coupled muscarinic receptor activation stimulates phospholipase Cβ (PLCβ), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) to generate inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. In general, this cleaved IP3 induces endoplasmic reticulum calcium release. In contrast, Goα2(CA) did not alter the calcium concentration (n = 9–11; Fig. 8A).

Finally, we tested whether the Goα2-TRPC4 pathway contributes to changes in the cytosolic Ca2+ concentration (Fig. 8B). When only M2 muscarinic receptor was expressed, the cytosolic Ca2+ level change was monitored during the application of CCh. These calcium levels were increased as soon as CCh was applied and then decayed rapidly. Right width at half-maximum (RWHM) time of M2 expressed cell was 17.95 ± 4.78 s (n = 7). Next, when M2, Goα2, and TRPC4β were coexpressed, the calcium levels were raised soon after CCh application, as was observed in cells expressing M2 receptor only. However, the time required for calcium levels to decrease was greater compared with cells expressing only M2. RWHM time of M2, Goα2, and TRPC4β expressed cell was 93.24 ± 17.27 s (n = 9). We conducted this experiment repeatedly seven times for M2 expressed cells and nine times for M2, Goα2, and TRPC4β expressed cells, respectively. These data indicate that Goα2-coupled muscarinic receptor 2 activation stimulates TRPC4 channel opening and contributes to the maintenance of increased cytosolic calcium levels, whereas the M2-Goα2-TRPC4 activation pathway contributes only somewhat to the basal calcium level.

**DISCUSSION**

In this study, we found that 1) muscarinic stimulation increased with Goα2-TRPC4 interaction using FRET measure-
ments, 2) the FRET increase between Gα12 and TRPC4 occurs before the activation of the TRPC4 current by CCh stimulation, and 3) during CCh stimulation the increased intracellular Ca2+ levels were maintained.

Among the many methods to study protein-protein interactions, FRET methods have many advantages. FRET methods can record protein-protein interactions in real time. In this study, for the simultaneous recordings of FRET and current, muscarinic stimulation by CCh first increased the FRET efficiency and then increased the TRPC4 current (Fig. 7A). In support of the interactions between Gα12 and TRPC4, 1) no FRET was observed between the Gβγ subunit and TRPC4, and 2) the FRET between Gα12 and Gβγ decreased upon muscarinic stimulation by CCh (Fig. 7B).

The interactions between TRPC4α and TRPC4β, as well as homomeric interactions were tested with the FRET method. Interestingly, the interaction between the COOH terminus of TRPC4α and that of TRPC4β was greater than the interaction between the NH2 and COOH termini. The COOH termini of TRPC4 tetramers appear to be closer to one another than to the NH2 termini. For this interaction, we tested whether TRPC4 fluorescently tagged at the COOH or NH2 terminus is expressed well and acts as a functional channel with the patch clamp technique.

In GIRK channels, Gβ and Gγ dimers may undergo an orientation switch in relation to the channel’s cytosolic domains following receptor activation and directly bind with GIRK channel (24). Muscarinic stimulation induces the dissociation of the G protein trimer Gαβγ into Gα and Gβγ. The dissociated Gβγ binds with the COOH terminus of GIRK and activates GIRK channels. For TRPC4, the TRPC4 COOH terminus interacted with Gα but not with Gβγ. Contrary to GIRK activation by Gβγ, only Gα was able to bind with the cytosolic domains of TRPC4 and activate the TRPC4 channel. We also found that a decrease in FRET between Gα and Gβγ occurred during the muscarinic stimulation of TRPC4 channels (Fig. 7B).

Interestingly, an interaction between Gα12(CA) and TRPC4α is lower than that of other interactions, and a statistically significant difference was observed between TRPC4α and TRPC4β interactions, although both TRPC4β and TRPC4α were activated by Gα12(CA). In our previous study (9), we found the interaction of TRPC4α and Gαo using Co-IP. Interestingly, when HEK293 cells were transfected with Gα12(CA)-YFP, these proteins were expressed uniformly at the plasma membrane (Fig. 4D). However, when HEK293 cells were cotransfected with Gα12(CA)-YFP and CFP-tagged TRPC4α or -β, Gα12(CA)-YFP showed a punctate distribution following the punctate expression of the TRPC4α or -β channel (Fig. 4, E and F). We showed that line scan followed plasma membrane as well. These data showed that both TRPC4α and TRPC4β interact with Gα12, but the longer TRPC4α COOH terminus is thought to be responsible for low FRET signal. Thus, reduced FRET efficiency appears to be due to the additional 84 amino acids in the COOH terminus of TRPC4α.

Furthermore, Gαq(CA) increased the intracellular calcium levels for both TRPC4α and TRPC4β cotransfections (Fig. 8A). In a previous study, we had found that no interactions occurred between Gαq and TRPC4 (Fig. 4) (13). Given the FRET efficiency and the effects of Gαq on intracellular calcium, the increase in the basal calcium concentration generated by constitutive Gαq activation might have regulated the interaction between G protein and TRPC4 and unveiled the tendency for G protein and TRPC4 to interact. Alternatively, the constitutively active G proteins, either Gαq or Gαo, may have been able to bind TRPC4.

In conclusion, we applied a novel method to investigate the interaction between the TRPC channel and G proteins through FRET interaction and found the direct interaction between Gαq and TRPC4. The interaction precedes the activation of TRPC4 current by muscarinic stimulation and induces sustained increases in the intracellular calcium levels after current activation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


