Selective Goi Subunits as Novel Direct Activators of Transient Receptor Potential Canonical (TRPC)4 and TRPC5 Channels

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The ubiquitous transient receptor potential canonical (TRPC) channels function as non-selective, Ca2+-permeable channels and mediate numerous cellular functions. It is commonly assumed that TRPC channels are activated by stimulation of Goi-PLC-coupled receptors. However, whether the Goi-PLC pathway is the main regulator of TRPC4/5 channels and how other Goi proteins may regulate these channels are poorly understood. We previously reported that TRPC4/TRPC5 can be activated by Goi. In the current work, we found that Goi subunits, rather than Goi, are the primary and direct activators of TRPC4 and TRPC5. We report a novel molecular mechanism in which TRPC4 is activated by several Goi subunits, most prominently by Goi, and TRPC5 is activated primarily by Goi. Activation of Goi by the muscarinic M2 receptors or expression of the constitutively active Goi mutants equally and fully activates the channels. Moreover, both TRPC4 and TRPC5 are activated by direct interaction of their conserved C-terminal SESTD (SEC14-like and spectrin-type domains) with the Goi subunits. Two amino acids (lysine 715 and arginine 716) of the TRPC4 C terminus were identified by structural modeling as mediating the interaction with Goi. These findings indicate an essential role of Goi proteins as novel activators for TRPC4/5 and reveal the molecular mechanism by which G-proteins activate the channels.

Transient receptor potential canonical (TRPC) channels are considered the molecular candidates for receptor-operated Ca2+-permeable cation channels. The G-protein-coupled receptor (GPCR)-Goi-PLC is assumed to be the primary pathway for activation of all TRPC channels, even though the exact mechanism by which the channels are activated remains unknown (1). Several mediators have been proposed to mediate channel activation by stimulation of GPCR. Among them are SESTD1 (2), intracellular Ca2+ (3, 4), lipid metabolites (5, 6), PIP3 (7–9), calmodulin (10, 11), CaM kinase (12), MLCK (13–15), and channel exocytosis (16). In addition, TRPC4 and TRPC5 can be activated by thioredoxin (17) and NO (18).

The physiological role of these channels was established recently, demonstrating that TRPC4 and TRPC6 are the molecular candidates for the non-selective cation channels activated by muscarinic receptor stimulation (mLCAT) in visceral smooth muscle cells. mLCAT mediates the physiological action of acetylcholine in evoking smooth muscle contraction (19). Activation of muscarinic receptors causes the opening of non-selective cationic channels in smooth muscle cells of the gastrointestinal tract (20, 21). In these cells, PTX-sensitive G-proteins but not Gβγ were suggested to mediate channel activation (22, 23). At the level of M2 or M3 muscarinic receptors, Sakamoto et al. (24) showed three distinct signaling pathways that activate cationic channels in murine gut smooth muscle cells. The three pathways include the M2, M3, and M2/M3 pathways and were demonstrated using M2 KO, M3 KO, and M2/M3 double KO mice, respectively. In addition, the M2/M3 pathway but not the M2 or M3 pathways involves processes in which Ca2+ has a potentiating effect on channel activation, suggesting that the M3 pathway may facilitate the function of the M2/M3 pathway.

The abbreviations used are: TRPC, transient receptor potential canonical; GPCR, G-protein-coupled receptor; EGFP, enhanced GFP; GTPγS, guanosine 5′-3′-(thio)triphosphate; PTX, pertussis toxin; BAPTA, 1,2-bis(o-tetraacetic acid; PIP3, phosphatidylinositol 4,5-bisphosphate; CaMK, Ca2+-/calmodulin-dependent protein kinase; PLC, phospholipase C; GI, gastrointestinal; pS, picosiemens; n.s., not significant; N.B., non-buffered.

Background: Activation of TRPC4/5 channels is mediated by GPCR activation.

Results: TRPC4/5 was activated by the Goi-coupled receptor and the Goi protein, which interacted directly with each other.

Conclusion: Goi proteins play an essential role as novel activators of TRPC4/5.

Significance: Our findings provide new insights into the activation mechanism of inhibitory Goi proteins.

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through inositol 1,4,5-trisphosphate-induced Ca\(^{2+}\) release (24). Similarly, activation of mICAT requires the simultaneous activation of both the M2 and M3 muscarinic receptors (20), further suggesting involvement of Go\(_i\) in channel activation.

Several studies have also suggested that PTX-sensitive G-proteins play an important role in the activation process of TRPC4 and TRPC5 by GPCR (6, 8, 25). In our previous study, we showed that Go\(_i\) activates TRPC4 and that PTX inhibits the activation of TRPC4 by stimulation of M2 muscarinic receptors (25). However, the specificity for Go\(_i\) subunits and how the PTX-sensitive G-proteins activate the channels are not yet known; it is assumed that the PTX-sensitive G-proteins activate the channels by an indirect mechanism that involves the generation of second messengers. The well-known second messenger of PTX-sensitive G-proteins is cyclic AMP (cAMP), found downstream of adenylate cyclase. PTX-sensitive G-proteins inhibit adenylate cyclase, which in turn decreases cAMP concentration. In our recent study, we showed that cAMP inhibits TRPC4 and TRPC5 currents by activating PKA and phosphorylating TRPC4 and TRPC5 channels (26). Thus, it cannot be the mechanism by which Go\(_i\) activates the channels.

These findings prompted us to ask whether TRPC4 and TRPC5 are activated by other PTX-sensitive Go\(_i\), subunits and whether the activation is direct. We were also interested in identifying the TRPC4/5 domain that mediates the interaction with the channels and the activation by Go\(_i\), subunits, as well as the roles Go\(_q\) plays in modulating TRPC4/5. In the present study, we focused on the role of Go\(_i\) proteins in regulating TRPC4/5 and report that Go\(_i\) subunits specifically activate TRPC4 and TRPC5 by direct interaction with the channels. Moreover, the regulation is specific to Go\(_i\) subunits. TRPC4 is mainly activated by Go\(_i2\), whereas TRPC5 is primarily activated by Go\(_i3\). These findings explain how TRPC4 is activated to regulate G\(_i\) motility. Strategies can now be developed to understand the functional consequences of activation of TRPC4/5 in the central and peripheral nervous systems.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transient Transfection, cDNA Clones**—Human embryonic kidney (HEK293) cells (ATCC, Manassas, VA) were maintained according to the supplier’s recommendations. For transient transfection, cells were seeded in 12-well plates. The following day, 0.5 μg/well of pcDNA3 vector containing the cDNA for mouse TRPC4β was mixed with 50–100 ng/well of pEGFP-N1 (Clontech) and transfected using the transfection reagent FuGENE 6 (Roche Molecular Biochemicals), as detailed in the manufacturer’s protocol. Human TRPC5-EGFP cDNA and mouse TRPC4β-EGFP cDNA were also transfected in the same way. Coexpression of TRPC channels with G-proteins or receptors was achieved through a channel to G-protein transfection ratio of 1:1. After 30–40 h, the cells were trypsinized and transferred to a small recording chamber (RC-11, Warner Instruments) for whole-cell recording. HEK293 cells stably expressing mouse TRPC4β were established by G418 selection. The cells were cultured as for the transient transfection, except that the medium was supplemented with G418 (400 μg/ml). Human Go\(_i1\), Go\(_i2\), Go\(_i3\), G\(_{q12}\), G\(_{q13}\), rat Go\(_i2\), and human Go\(_i3\) were cloned into pcDNA3.1+. Human G\(_\beta\), G\(_\beta\), and bovine G\(_\gamma\) were cloned into pcDNA3.1+ (Invitrogen). Human Go\(_i\)Q205L, M2 receptor (the Missouri S&T cDNA Resource Center), and M3 receptor were cloned into pcDNA3.1+. Human G\(_\beta\) was used to insert the G\(_\beta\)^W99A and G\(_\beta\)^180A mutations using the QuikChange site-directed mutagenesis kit (Stratagene).

**Western Blotting and Co-immunoprecipitation**—Transfected cells were collected and lysed using 300 μl of binding buffer (50 mM HEPES, pH 7.4, 120 mM NaCl, 2 mM EDTA, 2 mM MgCl\(_2\), complete protease inhibitor mixture tablet, phosphatase inhibitor mixture tablet (Roche Applied Science), and 0.5% Triton X-100). The lysates were sonicated, and any insoluble material was removed by centrifugation at 13,300 x g for 10 min. For co-immunoprecipitation of TRPC4β-GFP and TRPC5-GFP with Go\(_i2\) and Go\(_i3\), anti-GFP antibody (1 μg, Invitrogen, A11122) was added to 100 μl cell extract and incubated for 12 h at 4 °C. Then, 50 μl of a 1:1 slurry of protein G-Sepharose 4B beads was added to the antibody-extract mix and incubated for 12 h at 4 °C. Beads were washed three times with binding buffer; proteins were released from the beads with 50 μl of 2 x SDS-loading buffer and analyzed with 10% or 8% SDS-PAGE. Go\(_i2\) and Go\(_i3\) were co-precipitated with GFP antibody and probed by mouse monoclonal anti-Go\(_i2\) antibody (2 μg, Santa Cruz Biotechnology, sc-13534) and mouse monoclonal anti-EE antibody for Go\(_i3\) (2 μg, Covance, MMS-115P). The mouse monoclonal anti-Go\(_i3\) antibody was used for a reciprocal co-immunoprecipitation with GFP antibody in a sequential experiment. Co-immunoprecipitation of TRPC4β-GFP with Go\(_i\) was achieved using the same procedures. Go\(_i\) was probed by mouse monoclonal anti-Go\(_i\) antibody (Santa Cruz Biotechnology, sc-136181).

Rat brain from day 15 was homogenized on ice using a Dounce homogenizer. The homogenate buffer had the same composition as the binding buffer. Homogenates were centrifuged at 13,000 rpm for 30 min at 4 °C. Supernatants were re-centrifuged at 13,000 rpm at 4 °C for 20 min. Supernatants were pre-cleared with protein G-Sepharose beads for 12 h at 4 °C. Fifty microliters of a 1:1 slurry of protein G-Sepharose beads for 1 h at 4 °C and centrifuged at 2000 rpm for 2 min at 4 °C. Fifty microliters of a 1:1 slurry of protein G-Sepharose beads was added to the rabbit polyclonal antibody (anti-Go\(_i2\), Santa Cruz Biotechnology, sc-7276 and anti-Go\(_i3\), Santa Cruz Biotechnology, sc-262) extract mix and incubated for 12 h at 4 °C. The omission of primary antibody was used as a control. Beads were washed three times with binding buffer. Immunoprecipitated proteins were probed with anti-TRPC4 (NeuroMab, 75–119) and anti-TRPC5 (NeuroMab, 75–104) on an 8% SDS-PAGE gel. Anti-TRPC4 and Anti-TRPC5 were used for reciprocal pulldowns. Controls were omission of the primary antibody and substitution of normal anti-mouse IgG with non-immune serum (Santa Cruz Biotechnology, sc-2025). Rabbit polyclonal anti-Go\(_i2\) (2 μg, Santa Cruz Biotechnology) and rabbit polyclonal anti-Go\(_i3\) (2 μg, Santa Cruz Biotechnology) antibodies were used to probe co-immunoprecipitation samples on a 10% SDS-PAGE gel.

**Surface Biotinylation**—Cells were washed with and suspended in PBS. Suspended cells were incubated in 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce) in PBS for 30 min on ice. Free biotin was quenched by the addition of 100 mM glycine in PBS. Lysates were prepared in lysis buffer by being passed 7–10 times.
through a 26-gauge needle after sonication. Lysates were centrifuged at 13,300 × g for 10 min at 4 °C, and protein concentrations of the supernatants were determined. Forty microliters of a 50% slurry of avidin beads (Pierce) was added to cell lysates equivalent to 400 μg of protein. After incubation for 1 h at room temperature, beads were washed three times with 0.5% Triton X-100 in PBS, and proteins were extracted in sample buffer. Collected proteins were then analyzed by 8% SDS-PAGE gel and probed by anti-GFP antibody (Invitrogen, A11122).

GST Pulldown Assays—The C-terminal domain of TRPC4 (621–890) was cloned into BamHI-Sall restriction sites of pGEX4T-1 (Amersham Biosciences Pharmacia) by PCR. The GST fusion constructs were expressed and purified from Escherichia coli (BL21(DE3)). Briefly, E. coli were grown in liquid cultures containing 0.1 mM isopropyl 1-thio-β-D-galactopyranoside with vigorous agitation for 18 h at 20 °C to an A600 of 0.6. Channel protein was purified from the soluble extract using glutathione-agarose beads (Amersham Biosciences). GST fusion proteins appeared to be sensitive to degradation and carefully utilized in subsequent binding assays conducted within 24 h following purification. Histidine-tagged Ga12 Q205L protein was expressed in E. coli (BL21(DE3)) from the pET15b plasmid containing full-length human Ga12 Q205L cDNA. Histidine-tagged forms of Ga12 Q205L were purified using immobilized Ni2+–nitrilotriacetic acid affinity chromatography. Binding between Ga12 Q205L and the GST fusion C-terminal domain of TRPC4 was allowed to occur for 1 h at room temperature on a plate rotator. Each reaction sample was subsequently centrifuged at 500 × g for 5 min. After three washes with 500 μl of PBS with 0.1% Triton X-100, the GST protein-G-protein complexes were eluted with 15 μl of 2x SDS sample buffer, and the entire sample was run on a 10% polyacrylamide-SDS gel. Mouse monoclonal anti-Ga12 (Santa Cruz Biotechnology) and anti-GST antibodies (Santa Cruz Biotechnology, sc-138) were used for immunoblot analyses. Unless otherwise stated, all pulldown assays were repeated three times for each condition.

Whole-cell Patch Clamp Experiment—The whole-cell configuration was used to measure TRPC channel current in HEK cells as described previously (13, 19, 23, 27). Cells were transferred to a small chamber on the stage of an inverted microscope (TE2000S, Nikon Japan), and attached to coverslips in the small chamber for 10 min prior to patch recording. Currents were recorded using an Axopatch 200B patch clamp amplifier (Axon Instrument). Bath solutions were constantly perfused with a physiological salt solution at a rate of 1–2 ml/min. Glass microelectrodes with 2–4 meqohm resistance were used to obtain gigahm seals. After establishing the whole-cell configuration, the external solution was changed from Normal Tyrode to Cs+–rich external solution. The current was recorded in 500-ms duration RAMPs from +100 to −100 mV and from a holding membrane potential of −60 mV. pCLAMP software (version 10.2) and Digidata 1440A (Axon Instruments) were used for data acquisition and application of command pulses. Data were filtered at 5 kHz and displayed on a computer monitor. Data were analyzed using pCLAMP (version 10.2) and Origin software (Microcal origin, version 7.5).

Solutions and Drugs—For all TRPC channel recordings, physiological salt solution containing 135 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.4 using NaOH. Cs+–rich external solution was prepared by replacing NaCl and KCl with equimolar CsCl. The pipette solution contained 140 mM CsCl, 10 mM HEPES, 0.2 mM Tris-GTP, 0.5 mM EGTA, and 3 mM Mg-ATP. The pH was adjusted to 7.3 with CsOH. Pertussis toxin was purchased from Calbiochem (La Jolla, CA), and carbachol, HEPES, and GTPγS were purchased from Sigma.

**RESULTS**

Expression of TRPC4 alone results in a minimal spontaneous current (2.1 ± 1.1 pA/pF; see Fig. 2B, mock, open column, n = 5), compared with TRPC5, which showed a significant basal current (Fig. 2C, 36.6 ± 9.2 pA/pF, n = 14; see also Ref. 26). Although HEK cells endogenously express muscarinic receptors, most likely the M3 subtype (31), the endogenous receptors do not activate TRPC4 (Fig. 1B, mock) (32). In the case of TRPC5, the endogenous muscarinic receptor elicited only small transient TRPC5 activation. Therefore, we analyzed the TRPC4 and TRPC5 currents induced by heterologously expressed muscarinic receptors or by intracellular infusion of GTPγS through the patch pipette. The TRPC maximal inward currents (in Cs+–rich solution) at negative membrane potentials (~−60 mV) are represented as a current density (pA/pF).

Muscarinic Receptor Stimulation Activates TRPC4 and TRPC5 Through Endogenous Ga12—First, we tested the effects of the expressed Ga12–coupled M2 and the Ga12–coupled M3 muscarinic receptors on the activation of TRPC4 or TRPC5 by endogenous G-proteins in HEK cells (Fig. 1). Activation of the
M2 receptors with carbachol elicited 2–3-fold higher TRPC4 and TRPC5 currents than activation of the M3 receptors. The TRPC4 and TRPC5 currents showed a typical doubly rectifying current-voltage relationship (Fig. 1, A and E). The stimulation of the M2 receptor increased both TRPC4 and TRPC5 currents in a dose-dependent manner (Fig. 1, B and F, open column, 1, 10, and 100 μM; n = 5, n = 4, n = 12, and n = 4, n = 4, n = 10), whereas the stimulation of the M3 receptor increased the TRPC4 current dose-dependently and the TRPC5 current dose-independently at the concentration range 1–100 μM carbachol (Fig. 1, B and F, closed column, 1, 10, and 100 μM; n = 5, n = 8, n = 16, and n = 5, n = 7, n = 11). Treatment with PTX markedly inhibited the TRPC4 and TRPC5 currents activated by M2 receptor stimulation and GTPγS (Fig. 1, C, D, G, and H; mock/PTX; n = 8/n = 8, n = 5/n = 5, n = 7/n = 4, and n = 6/n = 3). Thus, the M2 receptor-Gαq/Q11 pathway was more effective than the M3 receptor-Gαq/k pathway in channel activation by engaging the endogenous PTX-sensitive Gα proteins.

**Specific Gα Isoforms Increase TRPC4 and TRPC5 Activity**—To determine which Gα isoform is involved in the activation of TRPC4 and TRPC5, we used constitutively active forms of the Gα subunits (GαQL mutants). Intracellular application of GTPγS through the patch pipette increased the TRPC4 current to 45.0 ± 7.2 pA/pF (Fig. 2B, mock, closed column, n = 13). The TRPC4 channel was activated to a different extent by all constitutively active GαQ11, subunits, even in the absence of GTPγS. Constitutively active GαQ3 and Gα13 mimicked the activation of TRPC4 by GTPγS (open/closed column, n = 10/n = 8 and n = 9/n = 11). Constitutively active Gα13 activated the TRPC4 channel, whereas application of GTPγS significantly inhibited the TRPC4 current (open/closed column, n = 13/n = 11). Constitutively active GαQ12 (GαQ12Q205L) was the most effective activator among the Gα subunits tested (Fig. 2B, open columns, and supplemental Fig. S1A, n = 6). Notably, the application of GTPγS had no further effect on the TRPC4 current, indicating that GαQ12 fully activates TRPC4 (closed column, n = 8). Furthermore, stimulation of the M2 receptors (Fig. 1) and GαQ12 (Fig. 2) activated TRPC4 to the same extent. Of particular significance, constitutively active Gαq was unable to activate TRPC4 (Fig. 2B, open/closed column, n = 4/n = 3). Moreover, constitutively active Gαq inhibited the stimulatory effect of GTPγS. This was addressed further below.

Again, TRPC5 showed significant basal activity (36.6 ± 9.2 pA/pF, Fig. 2C, mock, open column). As with TRPC4, application of GTPγS further increased the TRPC5 current to 130.7 ± 16.7 pA/pF (Fig. 2C, mock, closed column, n = 14). GαQ3Q205L was the most effective activator of TRPC5, as all other Gα isoforms tested actually reduced the spontaneous current and GTPγS-induced currents, likely by competing with endogenous Gα13 (Fig. 2C and supplemental Fig. SIB, open/closed column; Gα13, GαQ12, GαQ3, and GαQ3Q205L, n = 12/n = 9, n = 6/n = 4, n = 7/n = 5, and n = 8/n = 9). GTPγS did not increase the TRPC5

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**FIGURE 1. Activation of endogenous Gαq/k by the muscarinic M2 receptor activates TRPC4 and TRPC5.** A, carbachol (CCh) activated-TRPC4 current was recorded in HEK cells expressing TRPC4 and either M2 and M3 receptors. The representative I-V relationship and current densities of M2- and M3-activated TRPC4 currents activated by 100 μM carbachol were recorded by voltage RAMPS of ±100 mV during 500-ms durations, whereas the cells were held at −60 mV. B summarizes the amplitude of M2- and M3-activated TRPC4 currents activated by 1–100 μM carbachol. Current density is represented by maximal current peaks (subtracted Cs+ basal current) at −60 mV in Cs+ solution (changed from Normal Tyrode, Na+ 1) and is indicated by means ± S.E. Statistical significance was denoted by an asterisk (open column, 1 μM versus 10 μM) and double asterisk (open column, 10 μM versus 100 μM), single dagger (closed column, 1 μM versus 10 μM) and double dagger (closed column, 10 μM versus 10 μM) at p < 0.05. C and D, I-V relationship and current densities of M2- and GTPγS-activated TRPC4 currents show inhibition by PTX pretreatment (100 ng/ml for 16 h). Statistical significance was denoted by an asterisk (p < 0.05). E, the representative I-V relationship of M2 and M3-activated TRPC5 currents were measured in cells stimulated with 100 μM carbachol. F, summary of the M2-activated TRPC5 current at 1–100 μM carbachol. Current density was obtained by the methods described above. Statistical significance was denoted by an asterisk (open column, 1 μM versus 10 μM) and double asterisk (open column, 10 μM versus 100 μM) at p < 0.05. G and H, the representative I-V relationships and current densities of M2- and GTPγS-activated TRPC5 currents show inhibition by PTX pretreatment (as described under “Experimental Procedures”). Statistical significance was denoted by an asterisk (p < 0.05). n.s., not significant.
current activated by Gaq33,205L. As was found with TRPC4, constitutively active Gaq1 did not activate TRPC5 and instead inhibited the TRPC5 current (open/closed column, n = 3/n = 3).

Gβγ Isoforms Are Not Required for TRPC4 and TRPC5 Activation—Although Gaq1α subunits are key activators of TRPC4 or TRPC5, Gβγ subunits may also be involved in the activation of the channels, as is the case with GIRQ channels, or in the regulation of the channels by altering the availability of activated Gaq. To address these questions, we tested the effects of various Gβγ combinations on TRPC4 and TRPC5 activity. Figs. 3, A–C show that none of the Gβγ combinations tested activated the channels or reduced the activation by GTPγS. The exception is Gβ1,2γ2, which slightly inhibited the activation of TRPC5, likely by sequestering some of the Gaq3 even in the presence of GTPγS. Moreover, even the free form Gβ1180A mutant (18, 20, 33, 34) did not activate TRPC4 or TRPC5 (Fig. 3B; open/closed column; n = 4/n = 5, and Fig. 3C; open/closed column; n = 3/n = 14). These results indicate that the PTX-sensitive Gaq12/3 subunits are the activators of TRPC4 and TRPC5.

As is shown with the use of the Gβ mutant, Gβ1,W99A, the major role of Gβ1γ1 in the activation of TRPC4 and TRPC5 is the sequestering of Gaq1α subunits. Gβ1,W99A keeps the G-protein as the heterotrimer Gaqβγ because Gβ1,W99A is unable to support nucleotide exchange on Gaq (34). Gβ1,W99A inhibited activation of TRPC4 by M2 receptor stimulation to 61.4% ± 7.0% (p = 0.03, without/with Gβ1,W99A, n = 10/n = 12), whereas Gβ1,W99A did not inhibit the modest M3-induced TRPC4 current (without/with Gβ1,W99A, n = 8/n = 10). With TRPC5, Gβ1,W99A inhibited M2-induced TRPC5 current to 53.4% ± 16.7% (p = 0.02, without/with Gβ1,W99A, n = 11/n = 8), without affecting the minimal M3-induced TRPC5 current (without/with Gβ1,W99A, n = 4/n = 6, Fig. 3D). Importantly, Gβ1,W99A inhibited GTPγS-induced TRPC4 current to 46.2% ± 4.4% (p = 0.00079, without/with GTPγS, n = 9/n = 8) and GTPγS-induced TRPC5 current to 28.7% ± 7.7% (p = 0.00075, without/with GTPγS, n = 6/n = 6, Fig. 3E).

TRPC4 Is Inhibited by Increased Gaq Activity—Activation of the Gaq2-PLC pathway has been shown to modestly activate TRPC4 and TRPC5 (Fig. 1) by an unknown mechanism (1, 7, 8). Hence, the inhibition of TRPC4/5 by the constitutively active Gaq (Fig. 2) was completely unexpected. These results imply that intense overstimulation of a Gaq3-activated pathway inhibits TRPC4 and TRPC5. We considered several potential mechanisms, including increased or decreased cytoplasmic Ca2+, interference of channel interaction with Gaq, reduced surface expression of TRPC4, modified cellular PIP2, and channel phosphorylation by PKC, known to induce desensitization of TRPC5 with phosphorylation of residue Thr-972 at the C terminus (35).

Loading the cells with BAPTA-AM recovered only 15 ± 5.6% (mock, 56.7 ± 11.2 pA/pF; n = 6); recovery by BAPTA, 9.8 ± 3.1 pA/pF; n = 6, BAPTA with GTPγS; n = 12, Gaq QL; n = 3, Fig. 4A), and inhibition of PKC with Goeb6976 recovered only
**Goα Subunits versus TRPC4 and TRPC5 Channels**

**FIGURE 3. Lack of effect of Gβγ isomers on TRPC4 and TRPC5 activity.** A shows representative I-V curves of TRPC4 and TRPC5 co-expressed with or without the indicated Gβγ isomers. B and C summarize the current density recorded in cells transfected with the indicated Gβγ subunits and infused with GTPγS (closed columns; mock, Gβγi, GβγQ209L, GβγW99A, GβγQ209L/W99A, Gβγ2γ3, and Gβγ2γ3 with TRPC4; n = 24, n = 5, n = 3, n = 7, and n = 7; mock, Gβγi, GβγQ209L, and GβγQ209L/W99A with TRPC5; n = 10, n = 8, and n = 6) or without (open columns; mock, Gβγi, GβγQ209L, GβγW99A, GβγQ209L/W99A, Gβγ2γ3, and Gβγ2γ3 with TRPC5; n = 3, n = 5, and n = 3). D shows the effects of GβγQ209L/W99A on activation of TRPC4 and TRPC5 by muscarinic receptors. The activation of TRPC4 and TRPC5 by the M2 receptor was inhibited by GβγW99A whereas no current was activated by the M3 receptors. E, inhibition by GβγW99A of TRPC4 and TRPC5 activated by GTPγS. All current densities represent maximal current peaks (subtracted Cs− basal current) at −60 mV in Cs− solution and are indicated by means ± S.E. Statistical significance was denoted by an asterisk (p < 0.05).

**FIGURE 4. Inhibition of TRPC4 by Goαq is rescued by PIP2.** A–C show current densities in HEK293 cells stably expressing mTRPC4β and its inhibition by Goαq. Loading the cells with BAPTA-AM and inhibition of PKC with Goe6976 did not effectively reverse channel inhibition by Goαq. High intracellular Ca2+ (5 μM) reversed channel inhibition by Goαq. D, TRPC4 and C-terminal truncation TRPC4 mutants did not co-IP with Goαq. E, intracellular application of diC8-PIP2 (50 μM) almost recovered the inhibition of TRPC4 by Goαq. All current densities represent subtracted maximal current peaks at −60 mV in Cs− solution and are indicated by means ± S.E. Statistical significance was denoted by an asterisk (p < 0.05). All whole-cell currents were recorded in the intracellular application of GTPγS. F, surface expression of TRPC4 and Goαq co-expressed TRPC4 in HEK cells. Surface expression of TRPC4 was not altered by Goαq QL as determined by co-expression and surface biotinylation. Immunoblots of surface and total were detected by anti-GFP antibody (upper panel). Expression of endogenous Goαq and transfected Goαq QL were detected by Goαq antibody (bottom panel). IB, immunoblot. N. B., non buffered.

13 ± 8.7% (mock, 67.6 ± 12.6 pA/pF (n = 5); recovery by PKC inhibitor, 10.1 ± 5.8 pA/pF (n = 8); Goαq QL (n = 3) Fig. 4B) of the TRPC4 current inhibited by Goαq. The Goαq−TRPC4-inhibited current was recovered to only 27.7 ± 9.2% by 5 μM intracellular Ca2+ (without GoαqQ209L, non-buffered/5 μM, n = 7/n = 3; with GoαqQ209L, non-buffered/5 μM, n = 3/n = 3; Fig. 4C). Co-IP experiments showed that TRPC4 does not interact directly with Goαq (Fig. 4D). Finally, activated Goαq did not change the surface expression of TRPC4 (Fig. 4F, upper blots).

These findings rule out the effects of changes in cytosolic Ca2+, PKC, and altered interaction with Goαq in channel inhibition by Goαq, as the major inhibitors of the current induced by activated Goαq. Moreover, they point to an indirect effect of Goαq on channel function.

Goαq activates PLC to hydrolyze PIP2, reported to have an effect of TRPC4 and TRPC5 channel activity (7, 8). When diC8-PIP2 (50 μM) was applied via the patch pipette together with GTPγS, it recovered Goαq-induced inhibition of TRPC4 up to
51.83 ± 12.83% of the PIP2 control (open/closed column, mock, PIP2 alone, Goα₃ Q205L, and PIP2 with Goα₃ Q205L; n = 18/26, n = 4/15, n = 3/18, and n = 3/14, Fig. 4E). PIP2 was reported to inhibit TRPC4α but not the TRPC4β isoform (8), the isoform used in the present work. In addition, we reported that PIP2 slows TRPC5 desensitization (7). Thus, the combined results in Fig. 4 indicate that PIP2 and perhaps increased cytoplasmic Ca²⁺ are required for TRPC4 and TRPC5 activation.

Mechanisms Associated with Interaction between Goα₁₂ with C Terminus of TRPC4—Together, the results in Figs. 1–4 indicate that activation of Goα subunits by GPCRs is the primary mechanism for activating TRPC4 and TRPC5. This raised the question of whether activation of the channels requires direct interaction with the Goα subunits, as was shown for other channels regulated by Go (36) and GBY (33, 37) subunits. To address this question, we identified the TRPC4 and TRPC5 domain that might interact with the Goα subunits. To characterize the association between TRPC4β with Goα₁₂ in vivo, HEK cells were transfected with TRPC4β-GFP and Goα₁₂, and their association was analyzed by co-immunoprecipitation. Immunoprecipitation of Goα₁₂ pulled down TRPC4β-GFP (Fig. 5A, upper panel). Likewise, Goα₁₂ was present in TRPC4β-GFP immunoprecipitates (Fig. 5A, lower panel). Similar co-immunoprecipitation occurred between TRPC5 and Goα₁₂ (supplemental Fig. S2A). Pulldown assays were utilized to examine the binding of purified Goα₁₂ Q205L to GST fusion protein containing the C-terminal domain of TRPC4. Goα₁₂ Q205L bound to the C-terminal domain of TRPC4 (Fig. 5A, right panel).

To map the Goα₁₂ binding domain in TRPC4β, a series of TRPC4β-GFP truncation or deletion mutants were generated (Fig. 5B). Binding domains for regulatory molecules are clustered in the C-terminal region of TRPC4 and TRPC5. Because considerable evidence suggests that modulation of TRPC4 and TRPC5 function are directed by elements present in this region, we focused on the C-terminal region. We made deletion mutants based on well known binding domains: the CIRB (calmodulin and inositol 1,4,5-trisphosphate receptor binding region) (amino acids 695–724), the SESTD1 (SEC14-like and spectrin-type domain 1) (amino acids 700–728), and the α-spectrin binding domain (amino acids 730–758). Of the truncations shown in Fig. 5C, Δ759–870 and Δ730–758 retained activation by Goα₁₂ Q205L with peak current amplitude similar to that of WT-TRPC4, although TRPC4 (Δ730–758) lost activation by GTPγS (open/closed column, mock, Δ695–724, Δ700–728, Δ730–758, and Δ759–870; n = 8/7, n = 3/5, n = 3/4, and n = 9/6; Fig. 5C). These results implicate amino acids upstream of 730 in channel activation by Goα₁. To further narrow the functional site, we examined the function of the 11 deletion or truncation TRPC4 mutants listed in Fig. 5D. Goα₁₂ Q205L did not activate TRPC4β-GFP 1–700, Δ700–710, Δ710–720, Δ700–740, Δ700–870, and Δ720–870, but fully or partially activated the other mutants with the typically doubly rectifying I–V curve (open/closed column, mock, Δ700–710, Δ710–720, Δ700–740, Δ720–740, and Δ720–870; Fig. 5E and supplemental Fig. S3). Similarly, Goα₁ Q205L did not activate TRPC5-GFP Δ701–733, Δ707–717, Δ707–727, Δ707–735, Δ707–747, and Δ707–954, but fully or partially activated Δ737–765 and Δ764–954 (open/closed column, mock, Δ701–733, Δ707–747, Δ707–954, Δ737–765, and Δ764–954; n = 12/12, n = 3/5, n = 3/3, n = 3/3, n = 3/3, n = 3/3, n = 3/3, n = 3/3, n = 7/3, n = 3/3, n = 3/3, and n = 11/7; supplemental Fig. S2).

The TRPC4β-GFP constructs were also co-expressed with Goα₁₂ in HEK cells, and their interaction was monitored by co-immunoprecipitation. C-terminal (TRPC4β(1–720) and (1–740) truncations and TRPC4 (Δ721–740 and Δ759–870) deletions did not affect binding to Goα₁₂, whereas deletion of the SESTD1 binding domain (Δ700–728) or of Δ700–870 eliminated association with Goα₁₂ (Fig. 5F). Thus, the Goα₁₂ binding domain maps to amino acids 700–728, the SESTD1 domain of TRPC4.

To further analyze the interaction between the TRPC4 C terminus and Goα₁, we prepared C-terminal fragments of TRPC4 and examined their interactions with Goα₁. TRPC4β(621–700) did not interact with Goα₁, but TRPC4 fragments 621–720, 621–740, and 621–760 did (Fig. 5G), strengthening the conclusion that TRPC4(701–720) mediates the interaction with Goα₁₂. The TRPC4(701–720) encompass the CaM and IP₃R binding region (10) (supplemental Fig. S4). The modeling in Fig. 6A of the interaction between Goα₁₂ and TRPC4 suggested that the 711RLVVK₇¹₆ region is important for binding, and thus, we prepared the mutants R711A, N712R, K715A, and R716A (Fig. 6 and supplemental Fig. S5). Of these mutants, K715A and R716A retained activation by Goα₁₂ Q205L with peak current amplitude similar to that of WT-TRPC4, whereas R711A and N712R lost partial or complete activation by both Goα₁₂ Q205L and GTPγS. However, the double mutant K715A/R716A did not respond to Goα₁₂ Q205L but maintained responsivity to GTPγS (open/closed column, mock, R711K, R711A, N712R, K715A, R716A, and K715A/R716A; n = 6/11, n = 3/3, n = 15/3, n = 3/3, n = 3/3, n = 7/8, and n = 6/6, Fig. 6B). These results implicate amino acids Lys-715 and Arg-716 in channel activation by Goα₁. Ordaz et al. (11) showed that a similar sequence in mTRPC5 (7¹⁸RLVVK₇²₃) is involved in the activation process by calmodulin (11). Thus, we used the mTRPC5 CIRBm1 (R718A/K722A/R723A), CIRBm2 (I717D/L720E/V721A), and CBII mutants (ΔPro-828–Asn-854) (11) to test properties of TRPC5 activation by Goα₁ (open/closed column, mock, CIRBm1, CIRBm2, and CBII; n = 7/6, n = 4/6, n = 4/5, and n = 4/3 in absence of Goα₁; supplemental Fig. S6). The constitutively active Goα₁₂ mutant could not activate the CIRBm1 and CIRBm2 deletion mutants but did activate the CBII mutant (open/closed column, mock, CIRBm1, CIRBm2, and CBII; n = 6/6, n = 5/3, n = 4/4, and n = 6/4; supplemental Fig. S6).

Interaction between Goα₁ and the TRPC channels can also be demonstrated in vivo, as revealed by co-IP of TRPC4 with Goα₁₂ and of TRPC5 with Goα₃ in brain extract (Fig. 5H).

DISCUSSION

We report here that the primary mechanism for the activation of TRPC4 and TRPC5 in gastric smooth muscle in vivo is through activation of GPCRs. TRPC4 and TRPC5 activation appears to require both M2-G/o and M3-G/q₁₁ muscarinic receptors (20). The mechanism involves specific activation of
**TrpC4 and TrpC5 Channels**

**A**

| IP: Anti-Gαi3 | IB: Anti-GFP (C4) | Lysate | IB: Anti-Gi2 | IB: Anti-Gi2 |
|---------------|-------------------|--------|--------------|--------------|
| TRPC4         | 250               |        | 75           | 75           |
| Gαi3Q205L     | 250               | 75     | 25           | 25           |

**B**

| Current density (pA/pf) | Gαi3QL (+) | GTP/βS (+) |
|-------------------------|------------|-----------|
| 1695-724                | 150        | 150       |
| 1700-724                | 150        | 150       |
| 1705-725                | 150        | 150       |
| 1709-876                | 150        | 150       |

**C**

| Current density (pA/pf) | Gαi3QL (+) | GTP/βS (+) |
|-------------------------|------------|-----------|
| 1695-724                | 150        | 150       |
| 1700-724                | 150        | 150       |
| 1705-725                | 150        | 150       |
| 1709-876                | 150        | 150       |

**D**

| Current density (pA/pf) | Gαi3QL (+) | GTP/βS (+) |
|-------------------------|------------|-----------|
| 1695-724                | 150        | 150       |
| 1700-724                | 150        | 150       |
| 1705-725                | 150        | 150       |
| 1709-876                | 150        | 150       |

**E**

| Current density (pA/pf) | Gαi3QL (+) | GTP/βS (+) |
|-------------------------|------------|-----------|
| 1695-724                | 150        | 150       |
| 1700-724                | 150        | 150       |
| 1705-725                | 150        | 150       |
| 1709-876                | 150        | 150       |

**F**

| Current density (pA/pf) | Gαi3QL (+) | GTP/βS (+) |
|-------------------------|------------|-----------|
| 1695-724                | 150        | 150       |
| 1700-724                | 150        | 150       |
| 1705-725                | 150        | 150       |
| 1709-876                | 150        | 150       |

**G**

| Current density (pA/pf) | Gαi3QL (+) | GTP/βS (+) |
|-------------------------|------------|-----------|
| 1695-724                | 150        | 150       |
| 1700-724                | 150        | 150       |
| 1705-725                | 150        | 150       |
| 1709-876                | 150        | 150       |

**H**

| Current density (pA/pf) | Gαi3QL (+) | GTP/βS (+) |
|-------------------------|------------|-----------|
| 1695-724                | 150        | 150       |
| 1700-724                | 150        | 150       |
| 1705-725                | 150        | 150       |
| 1709-876                | 150        | 150       |
in murine gut smooth muscle cells involves three separate pathways (24). 1) The M3-Gαq PLCβ system, which transiently activates the 70-pS and 120-pS cationic channels concurrently with inositol 1,4,5-trisphosphate-induced Ca\(^{2+}\) release. 2) The M2 pathway, which transduces signals from M2 receptors via Gαq, to the 70-pS cationic channel and shifts the transient activation toward a longer open mode. 3) The M2/M3 pathway, which transmits M2 signals via Gαs and M3 signals via a Gαq-independent PLC, to the 70-pS cationic channel, resulting in a much longer open mode. The latter pathway does not work well when either the M2 or the M3 receptors are lacking or when either Gαs or PLC are inactivated. In addition, the M2/M3 pathway, but not the M2 or M3 pathway, involves processes in which Ca\(^{2+}\) has a potentiating effect on channel activation, suggesting that the M3 pathway may facilitate the function of the M2/M3 pathway through inositol 1,4,5-trisphosphate-induced Ca\(^{2+}\) release (38). We argue that if the 70-pS channel is mediated by TRPC4, then the pathways involving the M2 receptors use Gα12 to directly activate the channel.

In addition to TRPC4/5, activation by the Gα12/13 subunits have been reported to regulate several other TRP channels. For example, in Gq-coupled mGluR6, Gαq closes a downstream nonselective cation channel in ON bipolar cells that is mediated by TRPM1-L (39). Pheromone sensing in the vomeronasal organ is mediated by V1R-Gq and V2R-Gq complexes that activate TRPC2 (40). Whether activation of these channels is by direct interaction with Gα12/13 subunits as shown here for TRPC4/5 remains to be determined.

Our findings indicate that regulation of TRPC4 and TRPC5 by G-proteins is more complex than assumed previously. The newly discovered mechanism for activation of TRPC4 and TRPC5 suggests that Ca\(^{2+}\) influx through these channels can be activated by several mediators depending on receptor stimulation. As reported before and shown in Fig. 1, TRPC4 and TRPC5 can clearly be activated by Gαq-coupled receptors and mediated by several of the mechanisms reported before. However, activation by Gαq-coupled receptors appears to be modest (Fig. 1). More significant activation of TRPC4/5 is elicited by stimulation of Gαq-coupled receptors that is mediated by direct activation of the channels by Gαi subunits. The Gαi binding domain in TRPC4 and TRPC5 shares binding motifs with other regulatory molecules (CIRB, SESTD1) in the C-terminal region of TRPC4 and TRPC5. Modeling and mutation analyses indicate that the 711RLNLVKR716 region is important for the interaction between Gαi and TRPC4 (supplemental Fig. S6). By contrast with GIRK channels, G\(\gamma\) subunits do not appear to be involved in the direct activation of TRPC4 or TRPC5 by Gαi.

**FIGURE 5. Interaction of Gα12 with the C Terminus of TRPC4. A**, TRPC4 co-immunoprecipitates with Gα12. HEK cells were transfected with empty vector, Gα12 alone, and Gα12 with TRPC4-GFP and were used to test reciprocal co-IP of TRPC4 and Gα12. TRPC4 C-terminal GST fusion protein was used to probe direct binding between Gα12 and TRPC4. Binding of the TRPC4 C-terminal GST fusion protein with recombinant human Gα12(GD203) protein was shown by Gα12 antibody in vitro binding assay. B, a schematic of GFP-fused TRPC4. C, summary of the effects of Gα12 on TRPC4 C-terminal truncation mutants in the presence and absence of GTPγS stimulation. Current densities are represented by subtracted maximal current peaks at −60 mV in Cs\(^+\) solution and are indicated by means ± S.E. D, schematic of GFP-fused TRPC4 deletion and truncation mutants used (upper panel). Wild-type TRPC4 and mutants were probed using the GFP antibody in immunoblotting (bottom). E, summary of the effects of Gα12 on current by TRPC4 deletion and truncation mutants in the presence and absence of GTPγS stimulation. Current density was obtained by the methods described above. F, interaction between Gα12, TRPC4, and mutants was tested by co-IP, and the interaction site was mapped to the 700–728 region (the SESTD1 domain of TRPC4). G, a schematic of GFP-fused C-terminal fragments of TRPC4 (upper panel) and their co-IP with Gα12, H, the association between Gα12 with TRPC4/5 in vivo. Gα12 and Gα12 were immunoprecipitated from rat brain extract and were probed for TRPC4 and TRPC5 to show co-IP in vivo (upper panel). TRPC4 and TRPC5 were co-immunoprecipitated reciprocally. Lanes of IgG and control (−) did not show Gα12 binding. Input was indicated as 10% input of brain extract. IB, immunoblot.
is likely that the major role of GBY is regulating channel function by sequestering Gα12/13 subunits in the resting state. The mode of TRPC4/TRPC5 regulation by Gα12 subunits reported here likely constitutes a major pathway in smooth muscle. It will be interesting to explore the full potential of this form of regulation in other cell types, particularly when the regulation by Gα12/13-coupled receptors involves changes in cellular Ca2+. Examples include acetylcholine-induced vasoregulation, lung microvascular permeability (41, 42) and the increase of 5-hydroxytryptamine 2 receptor-coupled GABA release in thalamic interneurons (43). In summary, the present findings expand knowledge regarding signal transduction by cytoplasmic Ca2+ beyond the GBY to the Gα subunits of Gαi/o coupled receptors. Moreover, our findings point to a signaling function that is activated rather than inhibited by Gαi/o proteins and add an important function to the repertoire of functions activated by Gαi/o subunits.

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