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Regulator of G-protein signalling and GoLoco proteins suppress TRPC4 channel function via acting at $G\alpha_{i/o}$

Jae-Pyo Jeon[*], **Dhananjay P. Thakur**[*,†], **Jin-bin Tian**[*], **Insuk So**[‡], and **Michael X. Zhu**[*,†,1]

[*]Department of Integrative Biology and Pharmacology, The University of Texas Health Science Center at Houston, Houston, TX 77030, U.S.A.

[†]Graduate Program in Cell and Regulatory Biology, The University of Texas Health Science Center at Houston, Houston, TX 77030, U.S.A.

[‡]Department of Physiology, Seoul National University, College of Medicine, Seoul 110-799, South Korea

Abstract

Transient receptor potential canonical 4 (TRPC4) forms nonselective cation channels implicated in the regulation of diverse physiological functions. Previously, TRPC4 was shown to be activated by the $G_{i/o}$ subgroup of heterotrimeric G-proteins involving $G\alpha_{i/o}$, rather than $G\beta\gamma$, subunits. Because the lifetime and availability of Gα-GTP are regulated by regulators of G-protein signalling (RGS) and $G\alpha_{i/o}$-Loco (GoLoco) domain-containing proteins via their GTPase-activating protein (GAP) and guanine-nucleotide-dissociation inhibitor (GDI) functions respectively, we tested how RGS and GoLoco domain proteins affect TRPC4 currents activated via $G_{i/o}$-coupled receptors. Using whole-cell patch-clamp recordings, we show that both RGS and GoLoco proteins [RGS4, RGS6, RGS12, RGS14, LGN or activator of G-protein signalling 3 (AGS3)] suppress receptor-mediated TRPC4 activation without causing detectable basal current or altering surface expression of the channel protein. The inhibitory effects are dependent on the GAP and GoLoco domains and facilitated by enhancing membrane targeting of the GoLoco protein AGS3. In addition, RGS, but not GoLoco, proteins accelerate desensitization of receptor-activation evoked TRPC4 currents. The inhibitory effects of RGS and GoLoco domains are additive and are most prominent with RGS12 and RGS14, which contain both RGS and GoLoco domains. Our data support the notion that the Gα, but not $G\beta\gamma$, arm of the $G_{i/o}$ signalling is involved in TRPC4 activation and unveil new roles for RGS and GoLoco domain proteins in fine-tuning TRPC4 activities. The versatile and diverse functions of RGS and GoLoco proteins in regulating G-protein signalling may underlie the complexity of receptor-operated TRPC4 activation in various cell types under different conditions.

Keywords

G-proteins; GTPase-activating protein (GAP); guanine-nucleotide-dissociation inhibitor (GDI); TRP channels

[1]To whom correspondence should be addressed (michael.x.zhu@uth.tmc.edu).

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INTRODUCTION

Transient receptor potential canonical (TRPC) channels are receptor-operated Ca^{2+} -permeable cation channels involved in many physiological processes (see reviews in [1,2]). Among the seven TRPCs (TRPC1–TRPC7), TRPC4 has been implicated to function in neurons, smooth muscles, endothelium and cancer. These include contributions to epileptiform burst firing in brain neurons and seizure-induced neurodegeneration [3,4], synaptic transmission [5,6], contractility regulation of intestinal smooth muscle [7,8], microvascular permeability [9] and renal cancer proliferation [10,11]. These activities are believed to be related to Ca^{2+} and Na^{+} influx mediated by TRPC4 channels, which triggers Ca^{2+} signalling and membrane depolarization. To achieve strong control of the cellular function, the TRPC4 channels are tightly regulated through multiple levels of cross-talk among signalling networks [12].

Generally, the activation of TRPC channels is thought to be triggered by the stimulation of the phospholipase C (PLC) pathway via either the $G_{q/11}$ subgroup of heterotrimeric G-proteins or receptor tyrosine kinases (RTKs) [13,14]. However, for TRPC4 and TRPC5, the $G_{i/o}$ subgroup of G-proteins also plays an important role in channel activation [15–18]. In particular, the activation of TRPC4 depends on activated $G\alpha_{i/o}$ subunits [18], which are usually produced through stimulation of a subset of G-protein-coupled receptors (GPCRs), known as $G_{i/o}$-coupled receptors. To fully activate TRPC4, the stimulation by $G_{i/o}$ proteins also needs to coincide with Ca^{2+} -dependent activation of PLCδ1, which forms a positive-feedback loop, allowing sustained TRPC4 activity [12].

However, it was not clear to what extent the relative activities of $G_{i/o}$ proteins and their sustainability affect TRPC4 channel function during continued GPCR stimulation. GPCRs may be considered as guanine-nucleotide-exchange factors (GEFs) that promote the release of GDP from heterotrimeric G-proteins in exchange for binding of GTP. This causes the dissociation of the heterotrimer into GTP-bound Gα and free Gβγ subunits. Each GPCR type has its own subset of preferred G-proteins with specificity set typically by the Gα subunits. For example, M_2 muscarinic receptor (M_2R) and μ-opioid receptor (μOR) are coupled to $G_{i/o}$, whereas M_3 muscarinic receptor (M_3R) is coupled to $G_{q/11}$. Both Gα-GTP and free Gβγ dimers act as signal transducers in cell signalling through effector coupling. The termination of G-protein signalling is determined by the intrinsic GTPase activity of the Gα subunit, which hydrolyses GTP into GDP, allowing the Gα to reassociate with the Gβγ subunits.

The intrinsic GTPase activity of Gα can be accelerated by GTPase-activating proteins (GAPs), such as regulator of G-protein signalling (RGS) proteins. A family of more than 30 genes encoding RGS proteins has been identified [19]. Through GAP activities, the RGS proteins help to switch off G-protein signalling and would therefore be expected to accelerate the deactivation kinetics of downstream effectors and decrease their activities.

$G\alpha_{i/o}$-Loco (GoLoco) motif containing proteins, on the other hand, act as guanine-nucleotide-dissociation inhibitors (GDIs) of Gα subunits [20], which interrupt the GDP

dissociation from Gα and in turn prevent G-protein activation by GPCRs or GEFs. The GoLoco motifs specifically act at $G_{i/o}$ proteins, locking $G\alpha_{i/o}$ in the inactive GDP-bound form but releasing Gβγ at the same time. This results in an inhibition of $G\alpha_{i/o}$-mediated signalling but an enhancement of Gβγ-mediated functions [21,22]. The mammalian GoLoco motif-containing proteins consist of a diverse group of distantly related members sharing one or more 19-amino-acid GoLoco motifs, including in group 1 the R12 subfamily of RGS proteins (RGS12 and RGS14), in group 2 G-protein signalling modulators 1 and 2 (GPSM1 and GPSM2), formerly known as activator of G-protein signalling 3 (AGS3) and LGN respectively, and in group 3, GPSM3 and GPSM4, also known as G18 and Purkinje cell protein-2 (Pcp-2) respectively [21,22].

Previously, G-protein-activated inwardly rectifying K^+ (GIRK) channels have been shown to be modulated by RGS and GoLoco proteins [23–26]. These studies confirmed some predictions expected from regulation by the Gβγ dimer, such that, although the basal GIRK current was increased by LGN due to enhanced free Gβγ release, deactivation of the stimulated GIRK current was accelerated by RGS4 because of its GAP function. However, the studies also uncovered some unexpected effects. For example, RGS4 also accelerated the agonist-induced GIRK channel activation and increased the current amplitude [23,24]; the GoLoco motifs progressively reduced the responses of the channel to repeated agonist stimulation [26].

Because TRPC4 channels are activated by $G_{i/o}$ signalling, the modulation of these G-proteins by RGS and GoLoco proteins will probably affect the activation process of TRPC4 channels. In the present study, we examined how several RGS and GoLoco domain proteins affect $G_{i/o}$-mediated activation of TRPC4 heterologously expressed in human embryonic kidney (HEK)293 cells. We show that TRPC4 currents activated through stimulation of $G_{i/o}$-coupled receptors are suppressed by the expression of $G\alpha_{i/o}$-coupled RGS and GoLoco proteins. The GAP and GoLoco domains are responsible for the inhibitory actions of these proteins. On the other hand, these proteins do not alter the surface expression of TRPC4 proteins or TRPC4 currents elicited by its direct agonist, englerin A [10,11], indicating that RGS and GoLoco proteins specifically regulate $G_{i/o}$-mediated TRPC4 function.

MATERIALS AND METHODS

cDNA constructs and mutagenesis

cDNAs for human RGS4, RGS6, RGS12, RGS14, LGN, AGS3 and 3x-HA–M_2R (HA is haemagglutinin) were purchased from Missouri S&T cDNA Resource Center (http://www.cdna.org) and were placed in pcDNA3.1+ (Invitrogen). Point mutations of human RGS4 and RGS14 were introduced using the QuikChange site-directed mutagenesis kit (Stratagene). The mutations were verified by DNA sequencing. The AGS3-short ($AGS3^{sh}$) and myristoylatable AGS3 short form (Myr-$AGS3^{sh}$) expression constructs were provided by Dr J.B. Blumer and Dr S. Lanier (Medical University of South Carolina, Charleston, SC, U.S.A.). cDNAs for mouse TRPC4β in pEGFPN1 (Clontech) and rat M_2R or μOR in pIREShyg2 (Clontech) were as described previously [16,18].

Cell culture and transient transfection

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FBS and 2 mM L-glutamine at 37°C in a humidity-controlled incubator with 5% CO_2. The stable HEK293 cell line expressing μOR [16] was maintained in the above medium supplemented with 100 μg/ml hygromycin B (Calbiochem). All cell culture reagents were purchased from Invitrogen and Sigma–Aldrich. For transient transfection, cells were seeded in wells of a 12-well plate and allowed to grow overnight. The following day, the transfection was carried out using polyethyleneimine (PEI) and a total of 0.5 μg/well cDNA as recently described [27]. For co-expression with μOR, the TRPC4β/RGS (or GoLoco) cDNA ratio was 1:1.5 and the transfection was performed on the stable μOR-expressing cells. For co-expression with M_2R, the TRPC4β/M_2R/RGS (or GoLoco) cDNA ratio was 1:1:1 and transfection was performed on wild-type HEK293 cells. Electrophysiological recordings were performed between 24 h and 36 h after transfection.

Electrophysiology

After trypsinization, cells were transferred to a recording chamber on the stage of an inverted fluorescence microscope (TE200, Nikon) and allowed to attach to the glass coverslip at the bottom of the chamber for at least 10 min prior to patch-clamp recording. Transfected cells were identified by the green fluorescence of TRPC4β–GFP. Whole-cell voltage clamp recordings were made using pipettes pulled from standard wall borosilicate tubing with filament (Sutter Instrument) to a tip resistance of 3–6 MΩ when filled with the intracellular solution containing 140 mM CsCl, 0.5 mM EGTA, 0.2 mM Tris/GTP, 3 mM Mg-ATP and 10 mM HEPES, with the pH adjusted to 7.3 using CsOH. The standard or physiologically relevant external solution [PSS (physiologically relevant external solution) or normal tyrode's] contained 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, with the pH adjusted to 7.4 using NaOH. The Cs^+ -rich external solution was prepared by replacing NaCl and KCl of the PSS with equimolar CsCl and the pH was adjusted to 7.4 also using CsOH.

Voltage commands were given and currents were recorded using a MultiClamp 700A amplifier, coupled to Digidata 1350A, and operated using the pCLAMP software (v.9) (all from Molecular Devices). Currents were continuously recorded at 5 kHz with the cell held at −60 mV. Voltage ramps from +100 to −100 mV over a period of 500 ms were applied from the holding potential of −60 mV every 10 s to examine the current–voltage (*I–V*) relationship of the currents. Carbamoylcholine (carbachol; CCh) was purchased from Sigma–Aldrich, [D-Ala^2, *N*-MePhe^4, Glyol]-enkephalin (DAMGO) was from Bachem Chemicals and englerin A was from Cerilliant. The drugs were diluted to the final desired concentrations in the Cs^+ -rich external solution and applied using a gravity-fed continuous whole-chamber perfusion system. All electrophysiological recordings were performed at room temperature (22–24°C). Data analyses were made using pCLAMP v.10.3 and Origin software v.75 (Microcal).

Western blotting and surface biotinylation

Transfected cells were washed with PBS and then incubated in 0.5 mg/ml sulfo-succinimidyl-6-(biotinamido) hexanoate (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, after which cell lysates were prepared by passing the cell suspension in a lysis buffer [50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM $MgCl_2$, Complete™ protease inhibitor mixture tablet (Roche Applied Science) and 1% Triton X-100] through a 26-gauge needle 10–20 times. The lysates were centrifuged at 13 300 ***g*** for 15 min at 4°C to remove any insoluble material, and protein concentrations of the supernatants were estimated by the absorbance at 280 nm determined using a NanoDrop-1000 instrument (Thermo Scientific). To isolate biotinylated (surface-expressed) proteins, 40 μl of a 50% slurry of NeutrAvidin beads (Pierce) was added to cell lysates containing 500 μg of proteins. After incubation for 1 h at room temperature with continuous rotation, the mixtures were centrifuged at 325 ***g*** at room temperature for 2 min in a microcentrifuge and supernatants were discarded. The beads were then washed three times with ice-cold 0.5% Triton X-100 in PBS. For TRPC4–GFP, the washed beads were extracted in 4× SDS/PAGE sample buffer (1× contains 62.5 mM Tris/HCl, 2.1% SDS, 5% 2-mercaptoethanol and 13.1% glycerol, pH 6.8) with heating at 60°C for 2 min. For HA–M_2R, the washed beads were incubated in 2× SDS/PAGE sample buffer, supplemented with 50 mM DTT, at room temperature for 90 min. Aliquots of total cell lysates were also treated in similar fashion for determination of total TRPC4–GFP and HA–M_2R respectively. The treated samples were then analysed by SDS/PAGE (8% gel) and probed by anti-GFP (Invitrogen, A11122; 1:10000 dilution) and anti-HA (1:100 dilution, Roche, 11867423001) antibodies for TRPC4–GFP and HA–M_2R respectively for Western blotting.

Data presentation and statistical analysis

All data are expressed as means ± S.E.M. Statistical significance was determined using unpaired Student's *t* tests or ANOVA. *P* values of less than 0.05 are considered statistically significant.

RESULTS

Co-expression of RGS proteins inhibits TRPC4 currents

To test how RGS proteins affect TRPC4 currents, we co-expressed TRPC4–GFP with M_2R together with a selected RGS protein in HEK293 cells. TRPC4 currents in response to activation of M_2R using a muscarinic receptor agonist, CCh, were recorded by the whole-cell voltage clamp technique. In these cells, an endogenous $G_{q/11}$-coupled muscarinic receptor type, probably M_3R, is also present and has been shown to facilitate $G_{i/o}$-mediated TRPC4 activation [12]. To further help the development of TRPC4 currents, a Cs^+ -based internal solution was used throughout and the bath was replaced with a Cs^+ -rich external solution soon after the establishment of whole-cell configuration in the Na^+ -based normal Tyrode's solution before agonist application. Under these conditions, CCh (100 μM) evoked a large inward current at −60 mV, which typically reached a peak in less than 20 s and then slowly desensitized (Figure 1A). The *I–V* relationship exhibited an `S-shaped' curve (Figure 1C) with inward currents at negative potentials typically larger than the outward currents at positive potentials and a `flat' or `negative slope' region between 5 and 40 mV, which was probably caused by Mg^{2+} block as shown for TRPC5 channels [28]. This *I–V* relationship is

typical for homomeric TRPC4 and TRPC5 when these channels are maximally activated [13,29].

As expected from the enhanced GAP activity, which shortens the lifespan of Gα-GTP, co-expression of RGS4 reduced CCh-evoked current density of TRPC4 by ~70% (Figures 1B and 1D) and accelerated rate of current decline (desensitization) in the continued presence of CCh (Figures 1B and 1E). On the other hand, the co-expression of RGS4 did not significantly alter the surface or total expression of TRPC4 or M_2R (Figures 1F–1I), indicating that the decreased current was due to a change in functional coupling. Furthermore, the functional effects were dependent on the type of RGS. RGS proteins have different preferences towards G-protein subtypes. For example, RGS2 prefers $G\alpha_q$, whereas RGS4 mainly acts at $G\alpha_{i/o}$ subunits [30,31]. Consistent with the idea that TRPC4 activation is dependent on $G_{i/o}$ rather than $G_{q/11}$ signalling, the co-expression of RGS2 did not significantly alter TRPC4 currents (Figure 1D).

To examine whether the GAP activity of RGS4 was critical for attenuating TRPC4 currents, we used two RGS4 mutants, N88S and L159F, which had been shown previously not to bind to $G\alpha_{i1}$ and exhibit only 15% and 17% GAP activity respectively as compared with the wild-type RGS4 [32]. We found that co-expression of $RGS4^{N88S}$ or $RGS4^{L159F}$ with TRPC4 did not alter the amplitude of TRPC4 currents (Figure 1D); however, both mutations appeared to slow down desensitization (Figure 1E), suggesting that they may also be dominant-negative, at least in the context of $G\alpha_{i/o}$-mediated TRPC4 activation. These results support the idea that RGS proteins negatively affect TRPC4 channel activities through enhancing the GTPase activity of $G\alpha_{i/o}$ and switching off their signalling.

To ensure that the observed inhibitory effect was not due to the specific RGS protein (RGS4) or the muscarinic receptor, we also tested the effect of another $G_{i/o}$-selective RGS protein, RGS6, on TRPC4 currents evoked through stimulation of the $G_{i/o}$-coupled μOR. The use of μOR also allowed for $G_{i/o}$ stimulation without a concomitant $G_{q/11}$ activation when using a μOR-specific agonist, DAMGO. This differs from the use of M_2R because the muscarinic agonist CCh could also act at the endogenous $G_{q/11}$-coupled muscarinic receptors to cause simultaneous stimulation of both $G_{i/o}$ and $G_{q/11}$ pathways in the M_2R-expressing cells [12]. Application of DAMGO (0.5 μM) in the Cs^+ -rich external solution to HEK293 cells that stably expressed μOR did not induce appreciable current (results not shown). However, transient expression of TRPC4–GFP in these cells allowed for robust current development in response to DAMGO (Figure 2A), with an *I–V* relationship similar to that seen in M_2R/TRPC4 co-expressing cells (Figure 2C). The co-expression of RGS6 in these cells reduced DAMGO-evoked TRPC4 current by ~80% (Figures 2B and 2D) and accelerated its desensitization in the continued presence of DAMGO (Figures 2B and 2E). Taken together, the above data demonstrate that $G\alpha_{i/o}$-selective RGS proteins negatively modulate TRPC4 channel function by terminating $G\alpha_{i/o}$ signalling through their GAP activities and this may be a general effect unrelated to the receptors involved in $G_{i/o}$ activation.

Co-expression of GoLoco domain-containing proteins inhibits TRPC4 currents

To test how GoLoco proteins affect TRPC4 currents, we co-expressed LGN or AGS3 with TRPC4 and M_2R in HEK293 cells. Both LGN and AGS3 contain four GoLoco motifs,

which act as GDI on $G\alpha_{i/o}$ [22]. The co-expression of LGN led to ~56% reduction in CCh-induced TRPC4 currents via M_2R (Figures 3A, 3B, 3D and 3E). Unlike RGS proteins, LGN did not significantly alter the rate of current desensitization (Figures 3A, 3B and 3F). Similarly, the co-expression of AGS3 also reduced TRPC4 current by ~45% without affecting the rate of current desensitization (Figures 3C–3F). Importantly, neither protein significantly altered the surface or total expression level of TRPC4 or M_2R (Figures 3G–3L), indicating again that the decreased current density was due to a change in functional coupling rather than maturation or trafficking of the expressed channel or receptor. Therefore, the GoLoco proteins also suppress TRPC4 currents, but they differ from the RGS proteins in that they do not affect the rate of current desensitization. This would be consistent with the role of GoLoco domain proteins in reducing the availability of $G\alpha_{i/o}$.

Noticeably, the degree of suppression on current density appeared to be less with the co-expression of GoLoco proteins than with RGS proteins. Since AGS3 cycles between membrane bound and cytosolic locations [33], the low efficiency of AGS3 in inhibiting TRPC4 could be due to a low level of AGS3 proteins associated with the plasma membrane. To overcome this potential problem, we used a mutant ASG3 construct that contains a consensus myristoylation sequence at the N-terminus to promote membrane targeting [33]. The shorter variant of AGS3, $AGS3^{sh}$ [34], which contains only three complete GoLoco motifs and lacks the tetratricopeptide repeat domains, was also used. Although co-expression of $AGS3^{sh}$ with TRPC4 and M_2R in HEK293 cells inhibited CCh-induced TRPC4 current by ~66% (Figures 3D and 3E), introduction of the myristoylation sequence to $AGS3^{sh}$ (Myr-$AGS3^{sh}$) led to complete suppression of the current (Figures 3D and 3E). Similarly, Myr-$AGS3^{sh}$ also strongly inhibited DAMGO-evoked TRPC4 current by more than 97% (Figure 4). We interpret these as the improved membrane targeting of AGS3 stabilized the interaction between $G\alpha_i$ and AGS3 [33], which in turn prevented receptor-induced formation of $G\alpha_i$-GTP and hence TRPC4 activation. As controls, neither $AGS3^{sh}$ nor Myr-$AGS3^{sh}$ affected the surface or total expression level of TRPC4 (Figures 3K and 3L).

Because the $AGS3^{sh}$ construct lacks the tetratricopeptide repeat domains, which are present in both LGN and AGS3-long [21,34], the above results also argue for the GoLoco motifs and their GDI activities to be responsible for the inhibitory effect on TRPC4 function. Notably, none of the GoLoco protein constructs, when co-expressed with TRPC4 and a $G_{i/o}$-coupled receptor, induced any constitutive TRPC4 current. Because GoLoco proteins cause $G\beta\gamma$ release from the heterotrimers without generation of $G\alpha_i$-GTP, these data also support the previous conclusion that the $G\alpha_{i/o}$ arm rather than the $G\beta\gamma$ arm of the $G_{i/o}$ signalling is responsible for TRPC4 activation [18].

Co-expression of RGS4 and LGN has an additive effect on suppressing TRPC4 currents

RGS and GoLoco proteins affect $G_{i/o}$ via different mechanisms. However, functional interactions might be possible between the GAP and GDI activities, resulting in the suppression of one activity in the presence of the other. For example, agonist induced decrease in AGS3-$G\alpha_{i1}$ interaction was abrogated by the co-expression of RGS4 [33]. To test whether RGS-mediated inhibition of TRPC4 currents was altered by the co-existence of

GoLoco proteins or vice versa, we compared DAMGO-evoked currents in the μOR stable cell line that transiently expressed TRPC4–GFP without or with either RGS4 or LGN individually or with both RGS4 and LGN together (Figure 5). When co-expressed individually with TRPC4 in μOR cells, RGS4 and LGN led to partial, but nonetheless significant, inhibition of DAMGO-induced TRPC4 currents via μOR (Figure 5E). As for CCh-evoked TRPC4 currents via M_2R, only RGS4, but not LGN, accelerated desensitization of the DAMGO-evoked currents (Figure 5F). Co-expression of RGS4 and LGN together with TRPC4, however, caused stronger inhibition of the DAMGO-induced currents, from ~72% and ~63% for RGS4 and LGN alone respectively, to ~90% when both were present (Figure 5E). These results suggest that the RGS protein and LGN probably acted separately on the G-proteins and the suppression of TRPC4 current was additive.

Supporting the idea that RGS and GoLoco domain proteins act at G-proteins rather than the channel, we found that the co-expression of RGS4, LGN or AGS3 with TRPC4 did not affect the current evoked by the direct TRPC4/C5 agonist, englerin A (Figure 6). Therefore, the RGS and GoLoco domain proteins specifically suppress $G_{i/o}$-mediated TRPC4 activation. These data also demonstrate that the co-expression of these proteins did not alter the surface expression of TRPC4, consistent with the assessment by surface biotinylation followed by Western blotting.

RGS12 and RGS14 completely suppress TRPC4 currents via GAP and GDI activities

The R12 subfamily of RGS proteins (RGS12 and RGS14) contains both RGS and GoLoco [also known as G-protein regulatory (GPR)] motifs in a single polypeptide (Figure 7J). Based on the results with co-expression of RGS4 and LGN, the R12 RGS proteins should be able to inhibit TRPC4 current more effectively. Indeed, co-expression of RGS12 or RGS14 with TRPC4 and M_2R completely abolished the CCh-induced TRPC4 currents (Figures 7A–7C and 7H), suggesting that having both the GAP and GDI activities can completely block TRPC4 channel function. Because RGS12 has a more complex domain organization than RGS14 (Figure 7J), we focused on RGS14 to examine whether both the RGS and GoLoco domains of these proteins are involved in the inhibition of TRPC4 activity.

We made three mutants of RGS14. E92A/N93A ($RGS14^{EN}$) disrupts RGS domain and therefore interferes with its GAP activity [35]; Q516A/R517A ($RGS14^{QR}$) interrupts the GoLoco-Gα interaction and hence eliminates the GDI activity [36]; E92A/N93A/Q516A/R517A ($RGS14^{ENQR}$) combined mutations at both the RGS and GoLoco motifs and therefore is believed to be defective at both the GAP and GDI activities [37]. Co-expression of $RGS14^{EN}$ or $RGS14^{QR}$ with TRPC4 and M_2R led to partial suppression of CCh-evoked TRPC4 currents (Figures 7D, 7E and 7G), averaging to ~66% and ~77% inhibition for the RGS-null and GoLoco-null mutants respectively (Figure 7H). Consistent with GAP activity being involved in current desensitization, the RGS-null mutant, $RGS14^{EN}$, markedly attenuated the desensitization of CCh-induced TRPC4 currents (Figures 7D and 7I). However, the GoLoco-null mutant, $RGS14^{QR}$, with the RGS motif kept intact, significantly accelerated the desensitization (Figure 7I). Importantly, mutations at both RGS and GoLoco domains, $RGS14^{ENQR}$, completely eliminated the inhibitory effect of RGS14 on CCh-evoked TRPC4 currents via M_2R (Figures 7F–7H).

Similar to the CCh-evoked TRPC4 activation via M_2R, the DAMGO-evoked activation of TRPC4 via μOR was also completely inhibited by the co-expression of wild-type RGS14 (Figure 8E). Mutation at the GoLoco domain, $RGS14^{QR}$, partially rescued the suppressed current by ~35% (Figures 8A, 8B, 8D and 8E), and the desensitization of the currents became faster than the control (Figure 8F). The co-expression of $RGS14^{ENQR}$ failed to decrease the DAMGO-evoked TRPC4 currents (Figures 8C–8E), neither did it affect the rate of current desensitization (Figure 8F). Therefore, the effects of the RGS and GoLoco domains of RGS14 on TRPC4 currents were mediated by $G\alpha_{i/o}$ proteins endogenously expressed in HEK293 cells rather than the specific receptor used for activating the G-proteins. Taken together, these results suggest that the RGS and GoLoco domains of RGS14 negatively regulate TRPC4 channel activities by acting at $G\alpha_{i/o}$ proteins.

DISCUSSION

TRPC channels are commonly thought of as receptor-operated channels activated downstream of either $G_{q/11}$/PLCβ or RTK/PLCγ signalling [13,14]. Our recent data, however, suggest that TRPC4 exhibits a unique dependence on PLCδ1, which co-ordinates with $G_{i/o}$ proteins to elicit TRPC4 currents [12]. The $G_{q/11}$/PLCβ and RTK/PLCγ pathways appear to be dispensable, although they do facilitate the activation kinetics in a PLCδ1-dependent manner. On the other hand, the pertussis toxin-sensitive $G_{i/o}$ proteins are absolutely required for TRPC4 activation [12]. Our data from the present study reveal several important features of $G_{i/o}$-mediated TRPC4 activation and how these may be involved in the physiological and pathological functions of TRPC4 channels.

$G\alpha_{i/o}$-dependence of TRPC4 function

Previously, TRPC4 was shown to physically interact with $G\alpha_{i2}$ via a C-terminal site, suggesting a direct effect by the $G\alpha_{i/o}$ subunits on TRPC4 activation [18]. Because the activity of $G\alpha_{i/o}$ proteins is suppressed by GAPs and GDIs, we reasoned that proteins containing these domains would probably have a negative impact on TRPC4 function. Indeed, we show that co-expression of either a RGS or a GoLoco domain protein with TRPC4 led to significant inhibition of TRPC4 currents evoked via stimulation of $G_{i/o}$-coupled receptors. The inhibition by RGS was due to its GAP domain as it also accelerated current desensitization, consistent with the increased GTPase activities (Figure 9), and was abolished by introducing mutations in the GAP domains of RGS proteins. On the other hand, the GoLoco domain proteins only caused current inhibition without affecting the desensitization kinetics, which is consistent with a GDI function that reduces $G\alpha_{i/o}$ availability. In particular, the GoLoco domain sequesters $G\alpha_{i/o}$-GDP with concomitant production of free Gβγ dimers [20,21]. For Gβγ -regulated GIRK1/2, the co-expression of LGN increased the basal current [25], but for TRPC4, neither LGN nor AGS3 caused an increase in basal current, suggesting that the free Gβγ dimers produced by the expression of GoLoco domain proteins do not cause TRPC4 activation. Together with the results that RGS proteins accelerated current desensitization, the overall inhibitory effect of GoLoco proteins lends further support to the notion that the Gα subunits of the $G_{i/o}$ proteins are responsible for stimulating TRPC4. In intestinal smooth muscle cells, the native muscarinic agonist-activated cation current, shown to be largely composed of TRPC4 [8] and co-dependent on

both M_2R and M_3R [38], was inhibited by intracellular dialysis of antibodies specific for $G\alpha_{i3}$/$G\alpha_o$ or $G\alpha_o$, but not for $G\beta$ [39], further arguing for the role of $G\alpha_{i/o}$ rather than $G\beta\gamma$ in supporting also the native TRPC4 currents.

Although $G\alpha_{i/o}$ proteins are abundantly expressed in the membrane of neurons [40], their functions are not completely understood. In addition to inhibiting adenylyl cyclases, $G_{i/o}$ signalling has been implicated in stimulating GIRK, inhibiting voltage-gated Ca^{2+} channels and so on. However, many of these functions appear to be mediated by $G\beta\gamma$ [41], leaving few confirmed functions to the $G\alpha_{i/o}$ subunits. The activation of TRPC4 represents a novel function of $G\alpha_{i/o}$, which, because of the cation permeability of TRPC4 channels, leads to membrane depolarization and hence excitation of the affected neuron. Therefore, through coupling to TRPC4, $G_{i/o}$ signalling, and the active forms of $G\alpha_{i/o}$ in particular, can be linked to neuronal excitation, as opposed to the conventional view that $G_{i/o}$ proteins were mainly associated with inhibitory neurological functions.

Inhibition by RGS proteins

The importance of RGS proteins in G-protein signalling has been increasingly recognized. The GAP function possessed by all RGS proteins serves to shorten the half-life of $G\alpha$-GTP and thereby terminate its actions on the effectors. This explains both the overall current reduction and the acceleration of desensitization by the RGS proteins. Importantly, RGS proteins only inhibited TRPC4 currents activated via receptor stimulation, but not those induced by the direct channel agonist englerin A (Figure 6), suggesting that these proteins exert their effect through G-proteins. Moreover, only the $G_{i/o}$-targeting RGS4 and RGS6 [31,42,43] were able to inhibit TRPC4 currents (Figures 1, 2 and 9). The G_q-targeting RGS2 [30] did not have any effect (Figure 1D), further supporting the importance of $G_{i/o}$ proteins in TRPC4 activation.

The effect of RGS proteins on TRPC4 current desensitization is similar to that on GIRK channels [23,34,44]. However, in addition to accelerating deactivation [23], RGS proteins also increased the activation kinetics of GIRK channels either without changing [44] or by strongly increasing [23,24] the maximal current amplitude. For TRPC4, we only observed a decreased current amplitude in response to receptor stimulation and no obvious effect on activation kinetics with the overexpression of RGS proteins. This could suggest a specific role of RGS on GIRK activation, perhaps through $G\alpha_o$ as previously shown [24]. Alternatively, because GIRK channels are activated by $G\beta\gamma$ [23], the modulation of $G\alpha_{i/o}$-GTP by RGS might only `indirectly' affect these channels. Particularly, the time lapse between GTP hydrolysis and $G\alpha_{i/o}$-GDP association with the free $G\beta\gamma$, as well as the possible conformational difference between freshly formed $G\alpha\beta\gamma$ trimers and older ones [23], could all contribute to the changes in GIRK currents in the presence of RGS proteins. By contrast, TRPC4 probably responds to $G\alpha_{i/o}$-GTP directly, hence factors that affect heterotrimer reassociation and their `readiness' for dissociation, as suggested previously [23], may have little impact on the channel activity.

Inhibition by GoLoco domain proteins

The GoLoco domains act as GDI to inhibit GDP release from Gα, but at the same time cause Gβγ dissociation from the heterotrimer, which results in a reduced availability of $G\alpha_{i/o}$-GTP but an increased level of free Gβγ in the cell [20,21]. Therefore, the reduction in $G_{i/o}$-mediated activation of TRPC4 currents in the presence of the GoLoco proteins LGN and AGS3 is consistent with the $G\alpha_{i/o}$-GTP-dependence of TRPC4 activation (Figure 9). The facilitation of this inhibition by membrane targeting of $AGS3^{sh}$ via myristoylation further suggests the dependence on $G\alpha_{i/o}$ in a membrane-delimited fashion. Importantly, the overexpression of GoLoco domain proteins did not alter cell-surface expression of TRPC4 as assessed biochemically by surface biotinylation and electrophysiologically by englerin A stimulation (Figure 6). The critical involvement of GoLoco motifs in this regulation was also demonstrated with the use of $AGS3^{sh}$, which contains only the GoLoco motifs, and the Q516A/R717A mutant of RGS14 ($RGS14^{QR}$), which has a disrupted GDI function (Figures 3, 7 and 8). Importantly, unlike RGS proteins, the GoLoco domain proteins did not alter desensitization kinetics of TRPC4, supporting the idea that they only reduce the availability, not the half-life, of $G\alpha_{i/o}$-GTP.

The effects of GoLoco domain proteins on TRPC4 differ markedly from that on GIRK channels. Co-expression of LGN with GIRK1/2 increased the basal GIRK current but reduced the receptor-induced current, whereas suppression of LGN expression in neurons increased excitability under basal conditions [25]. The change in the basal activity is consistent with the activation of GIRK currents by free Gβγ subunits. The decreased response to receptor stimulation was also anticipated from the sequestration of $G\alpha_{i/o}$ into the GDP-bound form, which will reduce the available heterotrimers to enter the G-protein cycle. Ironically, pipette dialysis of GoLoco domain peptides to cells during whole-cell recordings failed to alter the basal GIRK current and the initial response to receptor stimulation, but consistently dampened the receptor-induced GIRK current in response to repeated stimulation [26]. This suggested that GoLoco domain proteins sequester Gα-GDP mainly from active G-protein cycles. In the co-expression studies, basal turnover of the G-proteins during culture probably allowed the expressed GoLoco proteins to bind to Gα-GDP subunits and render them unavailable to re-enter the cycle. This explains the reduction in receptor-evoked channel activation. The lack of effect of GoLoco domain proteins on TRPC4 basal current contrasts with their effect on GIRK, supporting the notion that Gβγ is not involved in TRPC4 activation.

Fine-tuning TRPC4 function with RGS and GoLoco domain proteins

Our results indicate that, unless specifically membrane targeted, a GoLoco domain protein or a RGS protein with only RGS domain (e.g. RGS4 or RGS6) could only achieve partial inhibition on TRPC4 currents. The co-expression of both GoLoco and RGS proteins exhibited an additive effect (Figure 5). Therefore, it is interesting that RGS12 and RGS14, which contains both RGS and GoLoco domains, nearly completely suppressed receptor-induced TRPC4 activation (Figures 7 and 8). Selective disruption of the RGS and GoLoco domains of RGS14 partially abolished its inhibitory effect, resulting in similar levels of TRPC4 current suppression as when a RGS or GoLoco domain protein was expressed alone (Figures 7 and 8). More importantly, disruption of just the GoLoco domain ($RGS14^{QR}$)

allowed revealing of the RGS effect on current desensitization, reminiscent of the co-expression of RGS4 or RGS6 with the channel. By contrast, $RGS14^{EN}$, which contains the GoLoco but not the RGS domain, behaved just like LGN or AGS3. Therefore, although both acting through $G\alpha_{i/o}$ subunits, the RGS and GoLoco domains modulate TRPC4 activity via separate mechanisms and the effects are additive. These results reveal the complexity of receptor-operated TRPC4 activation that allows for fine-tuning of the channel function in native systems, such as the central neurons. By varying the expression and subcellular localization of various RGS and GoLoco domain proteins, the vital functions regulated by TRPC4 channels in the central nervous system can be precisely tuned to fulfil their physiological roles.

Indeed, TRPC4 channels have been shown to play roles in synaptic transmission, neuronal excitability and neurodegeneration [3–6,45,46]. TRPC4 is expressed throughout CA1, CA2, CA3 and dentate gyrus areas of hippocampus [47]. RGS and GoLoco proteins are also naturally present in hippocampal neurons where they may exert effects in fine-tuning TRPC4 function in regional and developmentally regulated fashions. For example, RGS14 expression occurs gradually during development, being largely undetectable until postnatal day 7 and reaching peak level only in adulthood [48]. The period of no or low RGS14 expression coincides with the time of active dendritic growth and branching during early postnatal development. In adult brain, RGS14 is enriched in the CA2 and fasciola cinerea areas of hippocampus with sporadic presence also in CA1 [48,49]. The CA2 area is functionally different from other CA regions in that it typically lacks synaptic long-term potentiation and has a negative impact on hippocampus-related spatial learning and object recognition memory. All of these were reverted by the deletion of *RGS14* gene [49]. It would be interesting in future studies to test whether TRPC4 is involved in these functions. The CA2 pyramidal neurons are also more resistant to cell loss associated with temporal lobe epilepsy than CA1 and CA3 neurons [50,51]. This could be, at least in part, due to suppression of TRPC4 function by the high RGS14 levels in the CA2 neurons since TRPC4 is implicated in epilepsy-induced neuronal death [3,4]. Thus, the ability of RGS14 to fine-tune $G_{i/o}$-mediated TRPC4 activation and thereby regulate neuronal functions warrants further investigation.

In summary, we show that TRPC4 channels are negatively regulated by RGS and GoLoco domain proteins in line with their established roles in promoting GTPase activity and sequestering $G\alpha_{i/o}$ respectively. Our data support the notion that the $G\alpha$, but not the $G\beta\gamma$, arm of $G_{i/o}$ signalling is involved in TRPC4 activation. These findings reveal additional layers of complexity of TRPC4 channel modulation, which have significant implications in the regulation of neurons and other cell types where TRPC4 exerts functions.

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Abbreviations

AGS3	activator of G-protein signalling 3
AGS3^{sh}	AGS3-short
CCh	carbachol or carbamoylcholine
DAMGO	[D-Ala^2, *N*-MePhe^4, -Gly-ol]-enkephalin
GAP	GTPase-activating protein
GDI	guanine-nucleotide-dissociation inhibitor
GEF	guanine-nucleotide-exchange factor
GIRK	G-protein-activated inwardly rectifying K^+
GoLoco	G$\alpha_{i/o}$-Loco
GPCR	G-protein-coupled receptor
GPR	G-protein regulatory
GPSM	G-protein signalling modulator
HA	haemagglutinin
HEK	human embryonic kidney
M_2R	M_2 muscarinic receptor
M_3R	M_3 muscarinic receptor
Myr-AGS3^{sh}	myristoylatable AGS3 short form
μOR	μ-opioid receptor
PEI	polyethyleneimine
PLC	phospholipase C
PSS	physiologically relevant external solution
RGS	regulator of G-protein signalling
RTK	receptor tyrosine kinase
TRPC	transient receptor potential canonical.

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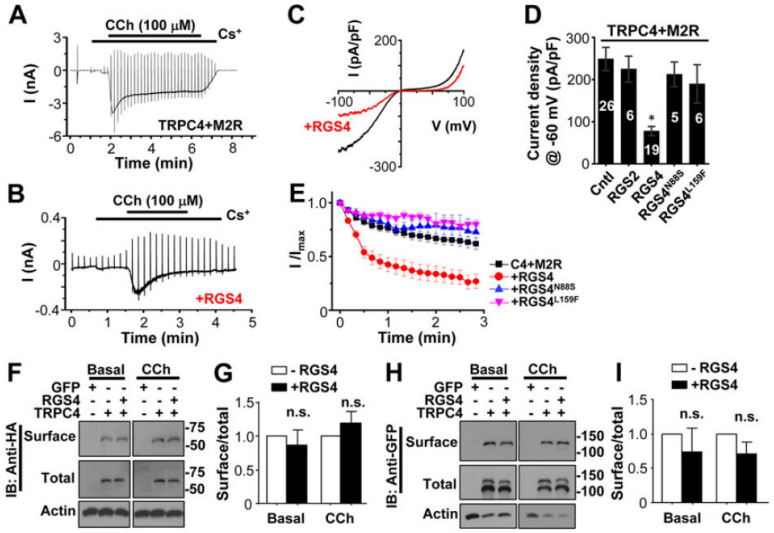

Figure 1. RGS4 suppresses TRPC4 currents activated via stimulation of M_2R and accelerates current desensitization

(**A** and **B**) Representative traces of currents at −60 mV in cells that co-expressed TRPC4 and M_2R without (**A**) and with (**B**) RGS4. A Cs^+ -rich solution and muscarinic agonist, CCh, were applied as indicated. Voltage ramps from +100 to −100 mV in a duration of 500 ms were applied every 10 s, which gave rise to the vertical lines. (**C**) Representative *I–V* relationships of CCh-evoked currents, as revealed by the voltage ramp (in all panels), from control (black line) and RGS4-expressing (red line) cells. (**D**) Summary of peak current density at −60 mV evoked by CCh (basal current in Cs^+ -rich solution subtracted for this and all similar plots) in cells that co-expressed TRPC4 and M_2R in the absence (Cntl) or presence of RGS2, RGS4 or RGS4 mutant, N88S or L159F. Data are means ± S.E.M. for the numbers of cells indicated; $*P<0.05$ compared with Cntl. (**E**) Normalized currents (I/I_{max}) at −60 mV after reaching the peak of CCh-evoked activation. Data are means ± S.E.M. for cells that co-expressed TRPC4 and M_2R in the absence or presence of RGS4 or its mutant, N88S or L159F. The number of cells is shown in (**D**). Note the faster decline of currents in cells that expressed RGS4. (**F**) Representative images of Western blots for surface-biotinylated and total HA–M_2R co-expressed in HEK293 cells with GFP or TRPC4–GFP ± RGS4 as indicated. Cells were untreated (basal) or treated with 100 μM CCh for 3 min (CCh) before biotinylation. HA–M_2R was detected using anti-HA. Actin was used for a loading control. (**G**) Quantification of surface/total ratios of HA–M_2R in cells that co-expressed TRPC4–GFP plus RGS4 normalized to that without RGS4 under basal and CCh-stimulated conditions. Data are means ± S.E.M. from three experiments. (**H**) Similar to (**F**), but TRPC4–GFP levels were measured using anti-GFP. (**I**) Quantification of surface/total ratio of TRPC4–GFP in cells that co-expressed TRPC4–GFP plus RGS4 normalized to that without RGS4 under basal and CCh-stimulated conditions. Data are means ± S.E.M. from three experiments; IB, immunoblotting; n.s., not significantly different from –RGS4.

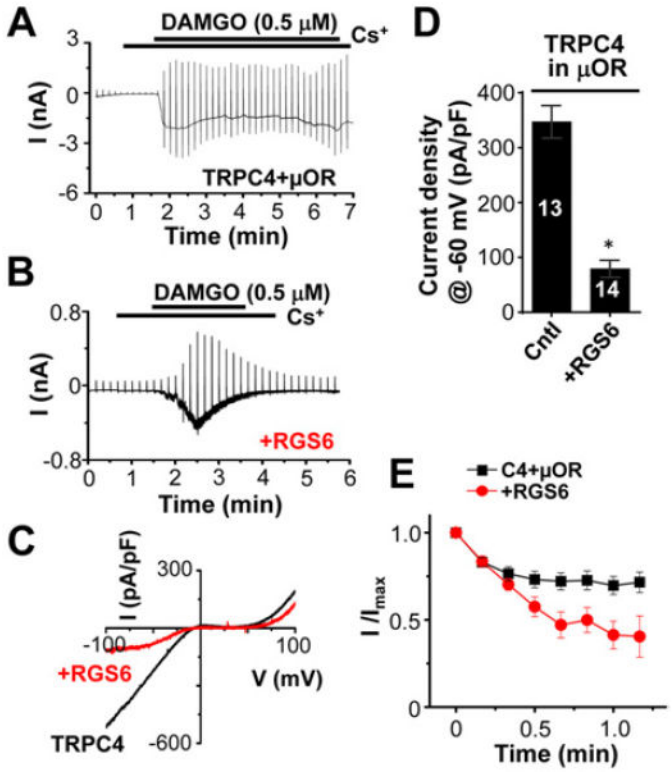

Figure 2. RGS6 suppresses TRPC4 currents activated via stimulation of μOR and accelerates current desensitization

(**A** and **B**) Representative traces of currents at −60 mV in cells that stably expressed μOR and transiently transfected with TRPC4 without (**A**) and with (**B**) RGS6. The Cs^+ -rich solution and μOR agonist DAMGO were applied as indicated. (**C**) Representative *I–V* relationships of DAMGO-evoked currents in control (black line) and RGS6-expressing (red line) cells. (**D**) Summary of peak current density at −60 mV evoked by DAMGO in μOR cells that expressed TRPC4 without (Cntl) or with RGS6. Data are means ± S.E.M. for the number of cells indicated; **P*<0.05 compared with Cntl. (**E**) Normalized currents (I/I_{max}) at −60 mV after reaching the peak of DAMGO-evoked activation for control and RGS6-expressing cells. Data are means ± S.E.M. for the number of cells shown in (**D**).

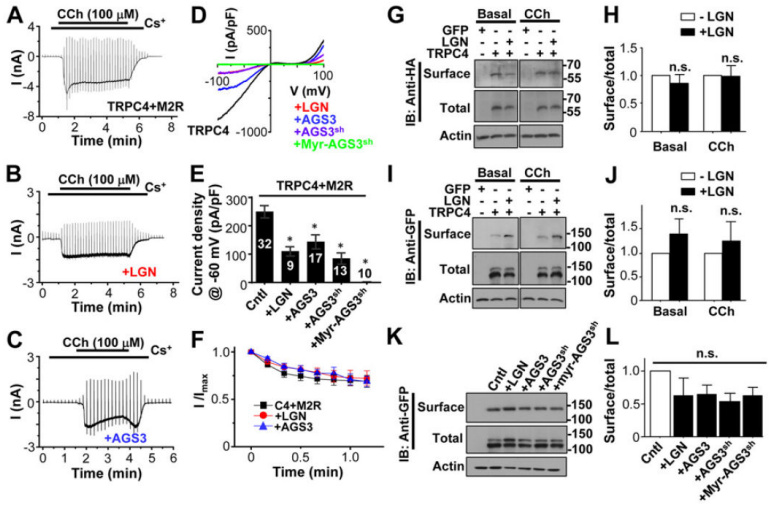

Figure 3. GoLoco domain proteins inhibit receptor-activated TRPC4 currents

(**A**–**C**) Representative traces of currents at −60 mV in cells that co-expressed TRPC4 and M_2R without (**A**) or with LGN (**B**) or AGS3 (**C**) and stimulated with CCh as indicated. (**D**) Representative *I*–*V* relationships of CCh-evoked currents in a control cell (black line) and cells that co-expressed LGN (red), AGS3 (blue), $AGS3^{sh}$ (purple) or Myr-$AGS3^{sh}$ (green). (**E**) Summary of peak current density at −60 mV evoked by CCh in cells that co-expressed TRPC4 and M_2R in the absence (Cntl) or presence of LGN, AGS3, $AGS3^{sh}$ or Myr-$AGS3^{sh}$. Data are means ± S.E.M. for the number of cells indicated; **P*<0.05 compared with Cntl. (**F**) Normalized currents (I/I_{max}, means ± S.E.M.) at −60 mV after reaching the peak of CCh-evoked activation for cells that co-expressed TRPC4 and M_2R in the absence or presence of LGN or AGS3. (**G**) Representative images of Western blots for surface-biotinylated and total HA–M_2R co-expressed in HEK293 cells with GFP or TRPC4–GFP ± LGN as indicated. Cells were untreated (basal) or treated with 100 μM CCh for 3 min (CCh) before biotinylation. HA–M_2R was detected using anti-HA. Actin was used for a loading control. (**H**) Quantification of surface/total ratios of HA–M_2R in cells that co-expressed TRPC4–GFP plus LGN normalized to that without LGN under basal and CCh-stimulated conditions. Data are means ± S.E.M. from three experiments. (**I**) Similar to (**G**) but TRPC4–GFP levels were measured using anti-GFP. (**J**) Quantification of surface/total ratio of TRPC4–GFP in cells that co-expressed TRPC4–GFP plus LGN normalized to that without LGN under basal and CCh-stimulated conditions. Data are means ± S.E.M. from three experiments. (**K**) Representative images of Western blots for surface biotinylated and total TRPC4–GFP co-expressed in HEK293 cells with M_2R (Cntl) and the indicated GoLoco domain proteins. Cells were untreated before biotinylation and TRPC4–GFP detected using anti-GFP. (**L**) Quantification of surface/total ratio of TRPC4–GFP in cells that co-expressed TRPC4–GFP plus the indicated GoLoco domain proteins normalized to Cntl (M_2R plus TRPC4–GFP only). Data are means ± S.E.M. from three experiments; IB, immunoblotting; n.s., not significantly different from Cntl.

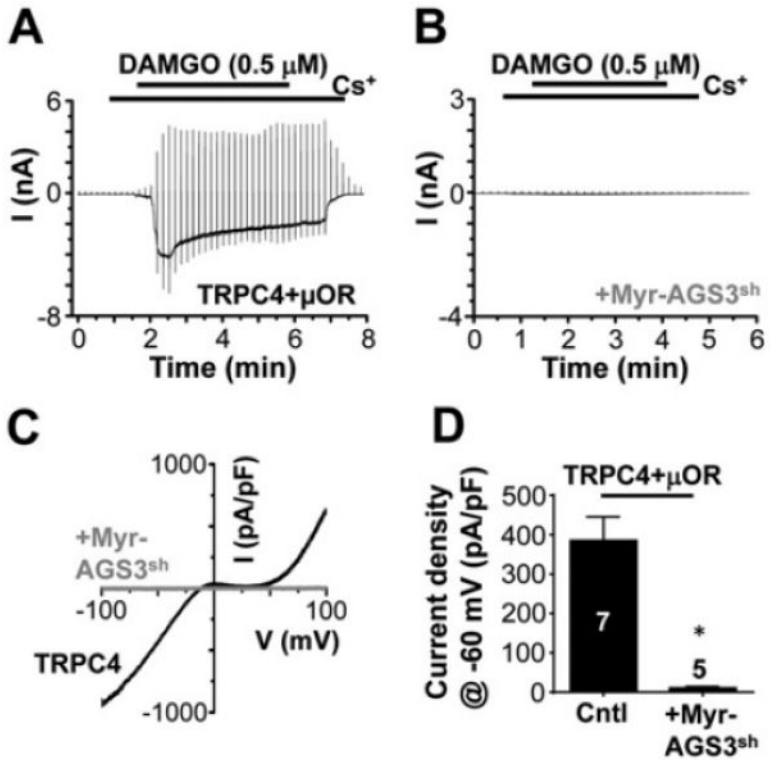

Figure 4. Myr-AGS3^{sh} completely inhibits TRPC4 currents evoked by μOR activation

(**A** and **B**) Representative traces of currents at −60 mV in μOR stable cells that expressed TRPC4 without (**A**) or with (**B**) Myr-AGS3^{sh} and stimulated with DAMGO as indicated. (**C**) Representative *I–V* relationships of DAMGO-evoked currents in control (black line) and Myr-AGS3^{sh}-expressing (grey line) cells. (**D**) Summary of peak current density at −60 mV evoked by DAMGO in μOR cells that expressed TRPC4 without (Cntl) or with Myr-AGS3^{sh}. Data are means ± S.E.M. for the number of cells indicated; **P*<0.05 compared with Cntl.

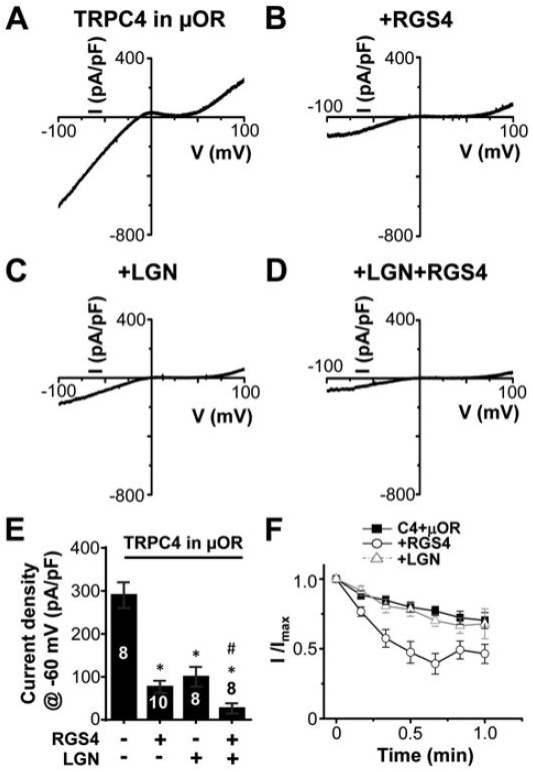

Figure 5. RGS4 and LGN additively inhibit TRPC4 currents evoked by μOR activation

(**A**–**D**) Representative *I*–*V* relationships of DAMGO (0.5 μM)-evoked currents in μOR cells that expressed TRPC4 in the absence (**A**) and presence of RGS4 (**B**), LGN (**C**) or RGS4 plus LGN (**D**). (**E**) Summary of current density at −60 mV evoked by DAMGO in μOR cells that expressed TRPC4 without (Cntl) or with RGS4 and LGN either individually or in combination. Data are means ± S.E.M. for the number of cells indicated; $^{*}P<0.05$ compared with Cntl, $^{\#}P<0.05$ compared with RGS4 or LGN alone by ANOVA. (**F**) Normalized currents (I/I_{max}, means ± S.E.M.) at −60 mV after reaching the peak of DAMGO-evoked activation for μOR cells that expressed TRPC4 without or with RGS4 or LGN. Note that RGS4 facilitated current desensitization.

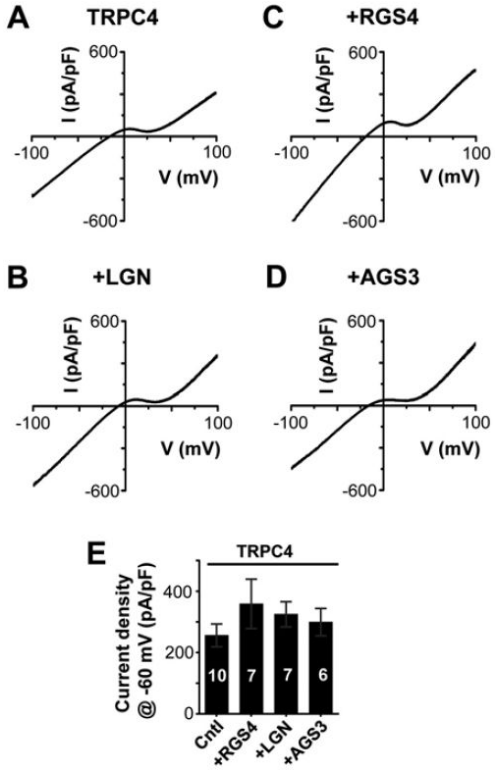

Figure 6. RGS and GoLoco domain proteins do not affect TRPC4 currents activated by englerin A

(**A**–**D**) Representative *I*–*V* relationships of englerin A (1 μM)-evoked currents in HEK293 cells that expressed TRPC4 in the absence (**A**) and presence of RGS4 (**B**), LGN (**C**) or AGS3 (**D**). (**E**) Summary of current density at −60 mV evoked by englerin A in cells that expressed TRPC4 without (Cntl) or with RGS4, LGN or AGS3. Data are means ± S.E.M. for the number of cells indicated.

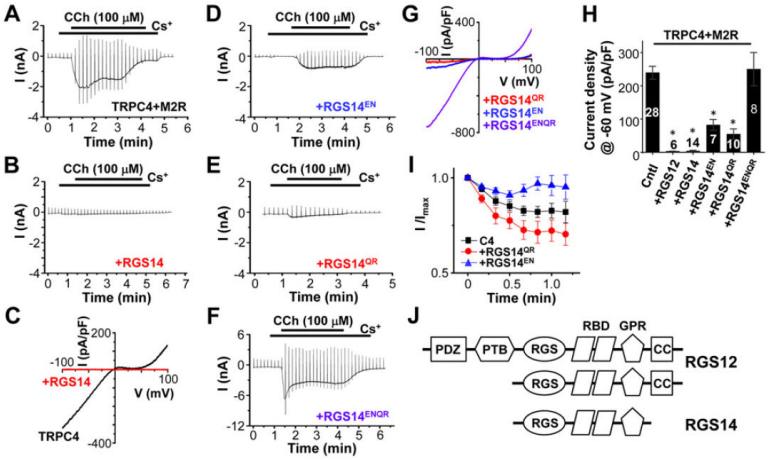

Figure 7. R12 family RGS proteins completely inhibit M_2R-mediated TRPC4 activation via RGS and GoLoco domains

(**A** and **B**) Representative traces of currents at −60 mV in cells that co-expressed TRPC4 and M_2R without (**A**) or with (**B**) RGS14 and stimulated with CCh as indicated. (**C**) Representative *I–V* relationships of CCh-evoked currents in control (black line) and RGS14-expressing (red line) cells. (**D**–**F**) Similar to (**A**) but the cell co-expressed TRPC4 and M_2R in the presence of a RGS14 mutant, E92A/N93A (RGS14^{EN}) (**D**), Q516A/R517A (RGS14^{QR}) (**E**) or E92A/N93A/Q516A/R517A (RGS14^{ENRQ}) (**F**). (**G**) *I–V* relationships of CCh-evoked currents for cells shown in (**D**–**F**) (RGS14^{QR}, red line; RGS14^{EN}, blue line; RGS14^{ENRQ}, purple line). (**H**) Summary of current density at −60 mV evoked by CCh in cells that co-expressed TRPC4 and M_2R in the absence (Cntl) and presence of RGS12, RGS14 or a RGS14 mutant, RGS14^{QR}, RGS14^{EN} or RGS14^{ENQR}. Data are means ± S.E.M. for the number of cells indicated; **P* <0.05 compared with Cntl. (**I**) Normalized currents (*I*/I_{max}, means ± S.E.M.) at −60 mV after reaching the peak of CCh-evoked activation for cells that co-expressed TRPC4 and M_2R in the absence or presence of RGS14^{QR} or RGS14^{EN}. Note the obvious difference in desensitization kinetics. (**J**) Diagram showing the relative positions of structural domains of RGS12 (upper two) and RGS14 (bottom). PDZ, PSD95/Dlg1/ZO-1; PTB, phosphotyrosine-binding; RBD, Ras-binding domain, GPR (same as GoLoco); CC, coiled coil.

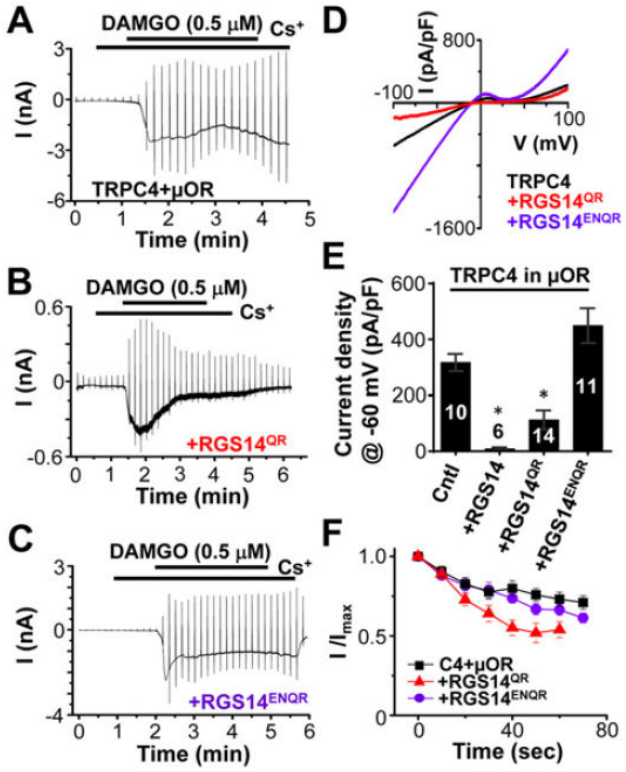

Figure 8. RGS14 completely inhibits μOR-mediated TRPC4 activation

(**A**–**C**) Representative traces of currents at −60 mV in μOR cells that expressed TRPC4 without (**A**) or with a RGS14 mutant, RGS14^{QR} (**B**) or RGS14^{ENRQ} (**C**), and stimulated with DAMGO as indicated. (**D**) *I–V* relationships of DAMGO-evoked currents for cells shown in (**A**–**C**) (control, black line; RGS14^{QR}, red line; RGS14^{ENRQ}, purple line). (**E**) Summary of current density at −60 mV evoked by DAMGO in μOR cells that expressed TRPC4 in the absence (Cntl) and presence of RGS14 or its mutant, RGS14^{QR} or RGS14^{ENQR}. Data are means ± S.E.M. for the number of cells indicated; **P* <0.05 compared with Cntl. (**F**) Normalized currents (I/I_{max}, means ± S.E.M.) at −60 mV after reaching the peak of DAMGO-evoked activation for μOR cells that expressed TRPC4 without or with RGS14^{QR} or RGS14^{ENQR}.

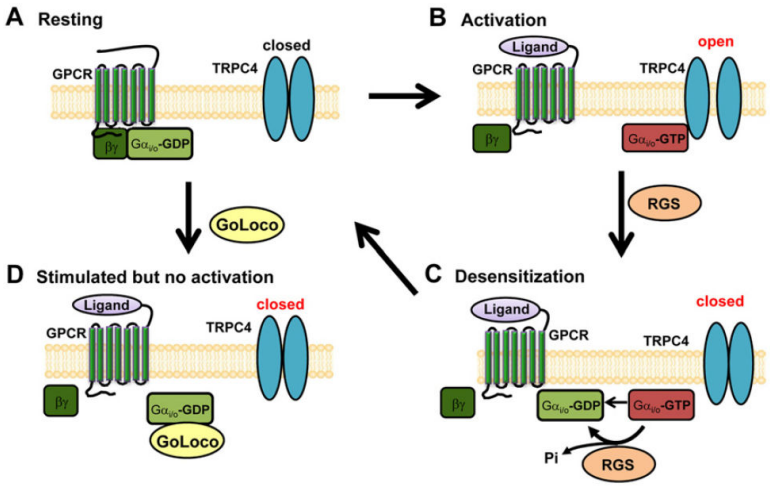

Figure 9. Schematic diagram of RGS- and GoLoco-mediated inhibition of TRPC4 channels

(**A** and **B**) Receptor-operated TRPC4 activation involves ligand binding to $G_{i/o}$-coupled receptors, GDP to GTP exchange in $G\alpha_{i/o}$, $G\alpha_{i/o}$-GTP dissociation from $G\beta\gamma$, and subsequent interaction of $G\alpha_{i/o}$-GTP to TRPC4 to open the channel. Note that only two of the four subunits of the TRPC4 channel complex are shown. (**C**) Introduction of a RGS protein increases the GTPase activity of $G\alpha_{i/o}$ and thereby shortens the life-time of $G\alpha_{i/o}$, resulting in TRPC4 channel closing in the continued presence of the receptor ligand. Therefore, RGS proteins cause an overall decrease in TRPC4 current density and acceleration in current desensitization. (**D**) Introduction of a GoLoco domain protein decreases the availability of $G\alpha_{i/o}$ by locking them to the GDP-bound form and at the same time produces more free $G\beta\gamma$ dimers. The free $G\beta\gamma$ dimers are inconsequential to TRPC4 activity, but the reduction in available $G\alpha_{i/o}$ dampens receptor-operated TRPC4 activation.