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TRPC5-TRPC1와 TRPC5-TRPC5 콘카티머의 전기생리학적 특성에 대한 비교

Electrophysiological comparison of trpc5-trpc1 and trpc5-trpc5 concatemer

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강하나

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Abstract

Electrophysiological comparison of TRPC5-TRPC1 and TRPC5-TRPC5 concatemer

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Transient receptor potential canonical (TRPC) 5 is a nonselective cation channel that plays a significant role in membrane depolarization and calcium influx. TRPC5 assembles as not only a homotetramer but also heterotetramer with TRPC1. However, it has been hard to test and confirm the heterotetrameric channels with fixed ratios. Thus, the heteromeric concatemers of TRPC5 and TRPC1 should be made to get a fixed stoichiometry 1:1. The purpose of this study is to identify and recornfirm the characteristics of TRPC5 homomers and heteromers with 1:1 fixed stoichiometry. Overall characteristics were consistent with the previous studies but several specific features were different. TRPC5-TRPC1 concatemer was activated by EA bu not

carbachol or constitutively active Gi protein. TRPC5-TRPC1 concatemer was activated by carbachol only with internal GTPgS. TRPC5-TRPC5 concatemer responds to carbachol as well as EA. In concuson, we provide evidence that TRPC5-TRPC1 heteromeric conatemer with fixed stoichiometry need specific condition to rspond to carbachol whereas TRPC5-TRPC5 homomeric concatemer responds physiologically to carbachol.

keywords : TRP, TRPC5, TRPC1, Heteromer, Homomer, Concatemer, G protein, GPCR, Englerin A, Cell Permeability, GTPgS

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INTRODUCTION

Transient receptor potential (TRP) channels are calcium permeable, non selective cation channels that consist of seven members. Among the seven group members, TRPC1, TRPC4, TRPC5 channels are classified as a subgroup that have similar stimulation processes [1, 2]. The transient receptor potential canonical (TRPC) 1 channel is widely distributed in mammalian cells. In mammalian cells, TRPC5 has only single isoform but it can form heteromeric channels with TRPC1 in the mammalian brain [3]. Subsequently, TRPC1 forms heterotetrameric channels with either TRPC4 (we use the term TRPC4/TRPC1 for heteromer) or TRPC5 (TRPC5/TRPC1) subunits and is involved in regulating calcium permeability and membrane potential of the plasma membrane [4]. TRPC4/TRPC1 and TRPC5/TRPC1 have similar activation processes but slightly different desensitization [5]. TRPC4/TRPC1 heteromr was desensitized via PIP2 depletion and Ca, whereas TRPC5/TRPC1 via PIP2 depletion and PKC [6].

Molecular mechanisms of tetramerization among TRPC4 and TRPC5 channels were addressed using FRET method and size-exclusion chromatography [7, 8]. A part of 1st ankyrin repeat domain (ARD) at that time (69 - 98) was responsible for homotetramerization process of TRPC5 [7], and that parts of 3rd and 4th ARD (87 - 172) and

N-terminus coiled-coil domain at that time (254 - 304) are important in the homotetramerization process of TRPC4 [8]. According to recent up-to-date domains [9 - 14], they correspond to the 2nd ARD (69 - 98) of TRPC5, the 2nd and 3rd ARD (87 - 172), and helix-loop-helix (HLH) domain (254 - 304) of TRPC4. A recent study suggested similar results using FRET method and thorough electrophysiology. With a number of N-terminal truncation mutants of TRPC4 channels, we suggested that 98 - 124 residues in N-terminus (3rd ARD) and 700 - 728 residues in the C-terminus (connecting-helix) play a great role in the homotetramerization process of TRPC4 [15].

The molecular mechanistic study for heteromerization process is unfortunately limited despite of its significance. One study so far represented specific domains responsible for heteromerization process of TRPC1/4 and TRPC1/5 channels. Using FRET method and electrophysiological recording with various truncation mutants, we suggested that 700 - 728 residue (connecting-helix) of TRPC4 and 707 - 735 residue (connecting-helix) of TRPC5 are important in heteromerization with TRPC1 [16]. It is surprising, however, that TRPC1 utilizes different domains for TRPC4 and TRPC5. For TRPC1/4, 725 - 745 region of TRPC1 is used as an inter-subunit interface, while the 673 - 725 region is used for TRPC1/5. Topologic analysis based on sequence alignment and Cryo-EM structure of

TRPC4 and TRPC5 suggests that both regions of TRPC1 correspond to putative connecting-helix of TRPC1 channel. The N terminal regons of TRPC1 (CCD or HLH regon: 188-278 residue) and the N terminal regons of TRPC4 (CCD or HLH regon: 228-257 residue) or TRPC5 (CCD or HLH regon: 229-250 residue) were also involved in heteromerization [16]. Thus, ARD-HLH domains-Rib helix-CCD are involved in the heteromer formations. Since the cytosolic ankyrin repeat domain (ARD) embraces the coiled coil domain (CCD) in the center and the connecting (or rib) helix contacts with ARD from next subunit, our previous results match well with recent results of TRPC4 [9, 10, 13] or TRPC5 structure [11, 12, 14].

TRPC1 plays a tricky role in channel field of TRP1/4/5 subfamily. First, TRPC1 acts as a negative regulator for TRPC4/5. In neurodegenerative diseases like Huntinton's disease (HD) and Parkinson' disease (PD), TRPC1 protects neuronal cell death by reducing Ca2+ influx [17]. Following this hypothesis, a larger effect of TRPC1 knockout on cellular activity occurs in HD and PD than TRPC heteromer knock-out. Interestingly, TRPC1 depletion induced double-rectifying I-V curve in synovial sarcoma cells [18]. Second, TRPC1 acts as a positive regulator for Na+ influx through TRPC channels to induce cell death in A498 and HS578T cells [19]. Lastly, in many cases, TRPC1/C4 and TRPC1/C5 heteromers contribute to

cell excitability by depolarizing the membrane potentials in neurons [20 -22]. It is rather surprising though, that the effect of homomeric knock out (TRPC4 or TRPC5) and heteromeric double knock out (TRPC1/4 or TRPC1/5) are similar in terms of neuronal activity.

However, it has been hard to test and confirm the heterotetrameric channels with fixed ratios [23]. Previous experiments were done by coexpressing of TRPC5 and TRPC1 channels and determining the ratio by examining the shape of current-voltage curve (I-V curve) or fluorescent density of the fluorescent protein tagged to TRPC channels. Certain fluorescent ratios seemed to form heteromers based on the previous studies [5] but this also was not enough to authenticate the 1:1 fixed ratio of TRPC5/TRPC1 heteromers as the structure was not stable. Thus, the heteromeric concatemers of TRPC5 and TRPC1 should be made to get a fixed stoichiometry 1:1. We used the term TRPC5-TRPC1 conatemer for this construct.

Here, we started to test TRPC5-TRPC1 concatemer provided by David Beech and then continued to test the effects of GTPgS on the one provided by Byoung-Cheol Lee. To provide a reasonable comparison, TRPC5-TRPC5 homotetrameric channels were tested along TRPC5-TRPC1. At the same day, we tested two types of concatemers for both TRPC5-TRPC1 heteromer and TRPC5-TRPC5 homomer. Characteristics of TRPC5-TRPC1 and TRPC5-TRPC5

concatemeric channels were tested to confirm the regulation of the channels by GPCR pathway and direct stimulation of the channel (see also 24). Englerin A was tested at the end of each experiment for positive control to reconfirm rather that the concatemeric channels function properly. We also tested whether Gαq(Q209L) activation inhibits activity of both TRPC15-TRPC1 and TRPC5-TRPC5 and Gαi2(Q205L) activates concatemeric channels with fixed stoichiometry. Furthermore, we investigated whether GTPgS could significantly increase the current in both TRPC5-TRPC5 and TRPC5-TRPC1.

MATERIALS AND METHODS

Cell culture

HEK293 cells that were stably expressing tetracycline-regulated human TRPC5-TRPC1 have been described [25 - 27]. TRPC5-TRPC1 heteromeric concatemers were stably expressed in T-REx293 cells. All cells were grown at 37C in a 5% CO2 incubator and cultured with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with heat activated fetal bovine serum (FBS; 10%), penicillin (100 units/ml), and streptomycin (100ug/ml). The modified HEK 293 cells (T-REx293 cells) were supplemented with selection antibiotics blasticidin (5 ug/ml) and Zeocin (250 ug/ml) (Invitrogen). [26] To induce expression of channels in T-REx293 cells, 1 ug/ml tetracycline was added to the media before it was seeded in a 12-well plate for whole-cell patch clamp recordings.

Transfection of T-REx293 TRPC5-TRPC1 stable cell and HEK 293 cells

Modified HEK293 cells, T-REx293 cells with stably expressing human TRPC5-TRPC1 were maintained in the given medium above. 150 ul of 70-80% confluent 100 pi plate was seeded to 1 well/12 well each. After tetracycline inducement, TRPC5-TRPC1 were performed using TurboFect transfection agent. TRPC5-TRPC1 stable cells were

transfected at 60-70% confluence with 0.5 μg/well of pcDNA3 vector containing the cDNA for human TRPC5 was mixed with 50 - 100 ng/well of pEYFP-N1 (Clontech) and transfected using the transfection reagent TurboFect, 1:2 ratio of DNA to the reagent, as detailed in the manufacturer's protocol.

TRPC5-TRPC5 homomeric concatemers and TRPC5-TRPC1 heteromeric concatemers were transfected to HEK293 cells using FuGENE6 and TurboFect, respectively. TRPC5-TRPC5 homomeric concatemers were human TRPC5-TRPC5-EGFP cDNA so extra fluorescent protein was not added. When using FuGENE6 transfection agent, 1:3 ratio of DNA to the reagent was necessary for ideal procedure. On the other hand, during TRPC5-TRPC1 transfection with TurboFect, pEYFP-N1 was also added. Coexpression of TRPC channels with G-proteins or receptors was achieved through a channel to G-protein transfection ratio of 1:1. After 24h, the cells were trypsinized and transferred to a small recording chamber (RC-11, Warner Instruments) for whole-cell recording.

Generation of TRPC5-TRPC1 stable cell

Human TRPC5 - TRPC1 concatemer was cloned with a ten amino acid linker (ASASASASAS) flanked by AgeI and SacII restriction sites was introduced into pcDNATM4/TO between EcoRI and XhoI restriction sites using Gibson Assembly® (New England Biolabs)

(forward oligonucleotide: 5′ CCACTAGTCCAGTGTGGTGGAATTCA CCGGTGCCAGCGCATCCGCTTCTGCCTCCG 3′, and reverse oligonucleotide: 5′ GTTTAAACGGGCCCTCTAGACTCGAGCCGCGG GATGCGGAGGCAGAAGCGGATGCG 3′). TRPC5, including an N-terminal Kozak sequence, was inserted upstream of the linker between KpnI and AgeI restriction sites, using hTRPC5/pcDNA™ 4/TO [25, 28] as a PCR template (forward primer: 5' GCTTGGTACCGCCACCATG 3' and reverse primer: 5' TGACACCGGTGAGGCGAGTTGTAACTTGTT CTTC 3'). TRPC1 was inserted downstream of the linker between SacII and XbaI restriction sites. HEK 293 cells stably expressing the TRPC5-TRPC1 construct was then generated for tetracycline-regulated expression as for TRPC5 HEK 293 Tet Cells. [25] First, the plasmid DNA was transfected and then was transducted.

Electrophysiology

The whole cell patch clamp was performed to measure the TRPC channel current in HEK293 cells. The transfected cells were trypsinized from the 12 well and attached to coverslips in the small chamber on an inverted microscope(IX70, Olympus, Japan) for 7-10 minutes prior to patch recording. The currents were recorded using an Axopatch 200B amplifier (Axon Instruments). Patch pipettes were made from borosilicate glass and had resistance of 3-5MΩ when

filled with normal intracellular solutions. The normal tyrode(NT) contained 135 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM glucose, and 10 mM HEPES with a pH that was adjusted to 7.4 using NaOH. The internal solution contained 140 mM CsCl, 10 mM HEPES, 0.2 mM Tris-guanosine 5′-triphosphate, 0.5 mM EGTA, and 3 mM Mg-adenosine 5′-triphosphate with a pH that was adjusted to 7.3 with CsOH. A voltage ramp pulse from +100 mV to −120 mV was applied for 500 ms at a −60 mV holding potential. Experiments were performed at room temperature (19 - 25 °C). The recording chamber was continuously perfused at a flow rate of 1 - 2 ml/min.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 was analyzed using pCLAMP (version 10.7) and Origin software (Microcal origin, version 8).

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

Ion permeability and IV curve of TRPC5-TRPC1 heteromeric concatemers

To investigate the electrophysiological properties of TRPC5-TRPC1 heteromeric concatemers, we initially transiently transfected, but failed to get typical current from concatemer. Thus, we made stable cell lines and screened colonies. Stable human embryonic kidney (HEK) 293 cell lines inducibly expressing a TRPC5-TRPC1 concatemer were established. For screening, we used Englerin A as an agonist [29]. We found 14 stable cell lines showing typical IV cuve of TRPC1/TRPC5 heteromers. 3 stable cell lines, no. 3 no. 9 no. 18, were selected and used for the following experiments (Fig. 2A, B, C). To provide a reasonable comparison, TRPC5-TRPC5 homotetrameric channels were tested along TRPC5-TRPC1. At the same day, we tested two types of concatemers for both TRPC5-TRPC1 heteromer and TRPC5-TRPC5 homomer. TRPC5-TRPC1 concatemer had outwardly rectifying shape of IV curve whereas TRPC5-TRPC5 concatemer had double rectifying shape; which is very typical for heteromers and homomers. TRPC5-TRPC5 concatemers had significantly bigger current sizes (Fig. 2). Measurement of changes in extracellular cesium concentration revealed that cesium increased conductance even in TRPC5-TRPC5 concatemer (Fig. 2D). On the

contrary, cesium did not increase the current in TRPC5-TRPC1 heterotetramers. We showed in the previous study that TRPC5-TRPC1 heterotetramers formed a strong pore field, which results in decrease in relative monovalent ion conductance to cesium [23]. We suggest that TRPC5-TRPC1 heteromeric concatemers have limitations in pore opening as in coexpressing TRPC5 and TRPC1, meaning that cesium is not enough to extend the pore permeability of heteromers.

TRPC5-TRPC1 concatemer was not activated by M receptor stimulation

Since we recorded typical currents from TRPC5-TRPC1 concatemers, we investigated whether muscarinic stimulation induces currents in TRPC5-TRPC1 concatemers. Both cells expressing TRPC5-TRPC1 concatemer, transiently and stably expressing cells did not show significant response to M3 stimulation. On the other hand, inward current simultaneously increases while outward current remains constant to M5 stimulation (Fig. 3A, B, right). However, the I/V curve of carbachol stimulation did not seem consistent to the previous studies. We observed linear I/V curve carbachol stimulation with expression of M5 receptor, which suggest that expression of M5 receptor induce some changes on others unknown rather than TRPC5-TRPC1 structure itself (Fig. 3B). To confirm the carbachol

activity, the effect of M3 stimulation on TRPC5-TRPC5 concatemer has been tested at the same time. Both the ALP and RDPP homomeric concatemers had an ideal I/V curve, that is, double rectifying shape (Fig. 3C).

Inactivation of TRPC5-TRPC1 concatemer after Gαq(Q209L) activation

Next, we investigated whether Gq, the downstream target of muscarinic stimulation, activates or inhibits TRPC5-TRPC1 concatemer. In our previous study, we showed that Gq predominantly activated TRPC5/TRPC1 heteromer when TRPC5 and TRPC1 were co-expressed [5]. Gαq(Q209L) is a constitutively active Q209L mutant of Gαq, which lacks intrinsic GTPase activity. This means it exists in the GTP-bound active conformation. The expression of the active mutant Gαq(Q209L) is known to activated PLCβ that depletes PIP2 [5, 24, 30, 31]. When Gαq(Q209L) is expressed to stable cell lines expressing TRPC5-TRPC1 concatemer, none of the cell lines had current increase even via EA 200 nM activation (Fig. 4A, B right). No current change was observed with external cesium gradients in TRPC5-TRPC1 concatemer (Fig. 4 right). Similar result was observed in TRPC5-TRPC5 concatemer. Interestingly, fast activation-deactivation was observed when activated via EA at TRPC5-TRPC5 concatemer with expression of Gαq(Q209L) form (Fig. 4D). The

currents were significantly smaller than the control, but the increase was worth considering (Fig. 4).

Gαi2(Q205L) increased the current in TRPC5-TRPC1 concatemer

We investigated the effect of Gαi2 which is another downstream target of muscarinic stimulation. Gαi2 isoform is known to be the most effective activator among the Gαi subunits of TRPC4 [24]. TRPC5 has a comparable structure to TRPC4 and similar response to Gαi2(Q205L) [24, 32]. No. 9 stable cell line expressing TRPC5-TRPC1 concatemer had enhanced current increase to EA stimulation but did not have cesium current increase. On the other hand, no. 3 stable cell line expressing TRPC5-TRPC1 concatemer showed enhanced current to cesium (Fig. 4B). None of the previous studies regarding heteromer current showed such a significant increase in cesium. Most of the studies showed an increase in cesium current when transiently transfected 'heteromer', TRPC4/TRPC1 had homomeric IV curve when TRPC4 and TRPC1 were co-expressed. This means heteromers with fixed stoichiometry never have been observed to have significant current increase in cesium.

In TRPC5-TRPC5 concatemer with expression of Gαi2(Q205L), the response to cesium was maximized compared to any other stimulation done. The current increase reached close to EA stimulation, which is

known as the strongest agonist of TRPC5. TRPC5-TRPC5 homomeric concatemer channel has been studied to have current increase in cesium, but this much of an enhancement was unusual. All three types, control, GiQL, and GqQL, had similar response to Englerin A stimulation, confirming that the result of the experiment with different co-expression is reliable.

Internal GTPγS facilitated the response to carbachol in TRPC5-TRPC1 concatemer

Finally, we investigated whether GTPγS, a universal actvator for TRPC channels, facilitates the response to cabachol in TRPC5-TRPC1 concatemer. It is known that activation of G protein can stimulate TRPC5/TRPC5 homomer while TRPC5/TRPC1 has not been studied specifically [1]. In TRPC5-TRPC5 homomeric concatemer, GTPγS induced an increase of the basal current recorded instantly after cell rupture. The TRPC5-TRPC5 concatemer current increased after external solution change (NT to Cs) (Fig. 5A, B). Additionally, EA effect was also enhanced as GTPγS is already acting as an activator intracellularly. TRPC5-TRPC5 homomeric currents activated by EA stimulation was maximal compared to any other stimulation for activation as well as the control (Figs. 2-4). External cesium-rich solution in TRPC5-TRPC5 homomer with GTPγS could reach to the amplitude similar to EA current (Fig. 5B, right).

HEK293 cells are reported to endogenously express functional M3 muscarinic receptors [33 - 36]. There was a slight current increase right after 100 uM carbachol stimulation and then it constantly decreased to basal after it reached the peak. Current increase by carbachol disappears without NT wash. To recheck if the peak of carbachol stimulation was successful, the I/V curve at the peak was obtained, which seemed to be an ideal outward rectifying shape. When GTPγS and carbachol react internally and externally at the same time, the synergistic effects of all G protein pathways for activation might be observed. EA of 200 nM was always used as a positive control at the end of the patch clamp. Interestingly, TRPC5-TRPC1 heteromeric concatemer had a similar IV curve to of 200 nM EA after being stimulated with carbachol, but the response was delayed to reach the peak (Fig. 5C-1). In TRPC5-TRPC1 heteromer, enhanced EA activation could be seen as well (Fig. 5C, C-2).

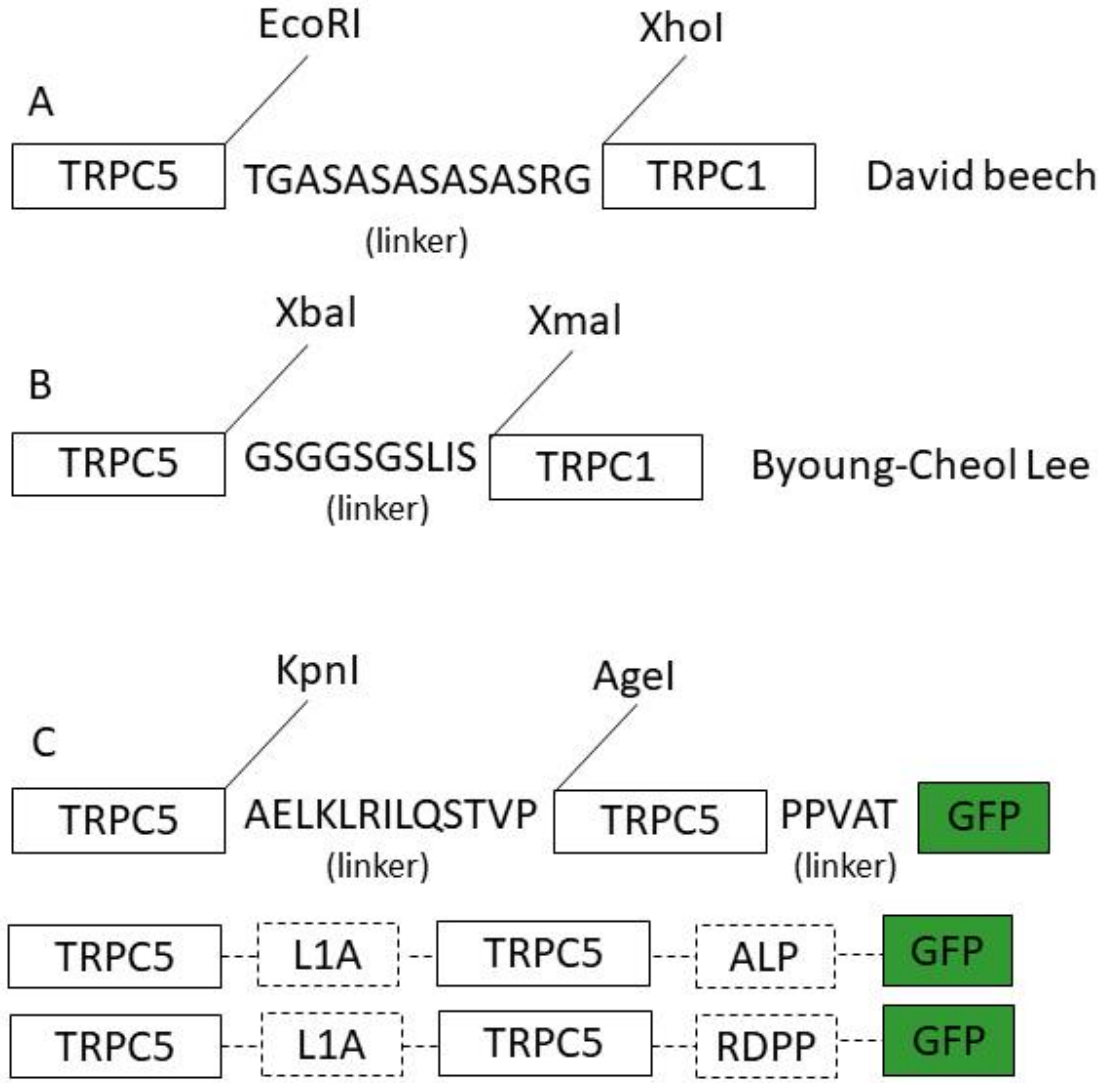

Figure 1. Schematic diagram of TRPC5-TRPC1 concatemer and TRPC5-TRPC5 concatemer

(A) TRPC5-TRPC1 heteromer provided by David Beech. Cloning of the TRPC5-TRPC1 concatemer was facilitated by flanking in ten amino acid linker (ASASASASAS) by AgeI and SacII restriction sites using pcDNATM4/TO between EcoRI and XhoI restriction sites using Gibson Assembly. Further explanation [19]. (B) Generation of TRPC5-TRPC1 concatemer provided by Byoung-Cheol Lee was done via using non-cutter human TRPC5, TRPC1 using Xbal and Xmal. Ten amino acid linker (GSGGSGSLIS) were flanked in between. SacI and SaII were used to insert the TRPC5-TRPC1 construct into pUC57 vector. Based on pUC57 vector, the TRPC5 gene was inserted to the head of the TRPC1 construct. This concatemer construct was placed on the mammalian expression vector. (C) TRPC5-TRPC5 concatemer construct was produced by the former Ph.D student flanking in AELKLRILQSTVP (L1A) linker and PPVAT linker using NheI, XhoI and AgeI with EGFP tagged to the second linker. Based on pEGFP-N1 vector, L1A was inserted in between human TRPC5. Linker ALP indicates ALPVAT, and RDPP does RDPPVAT instead of PPVAT linker between TRPC5 and GFP.

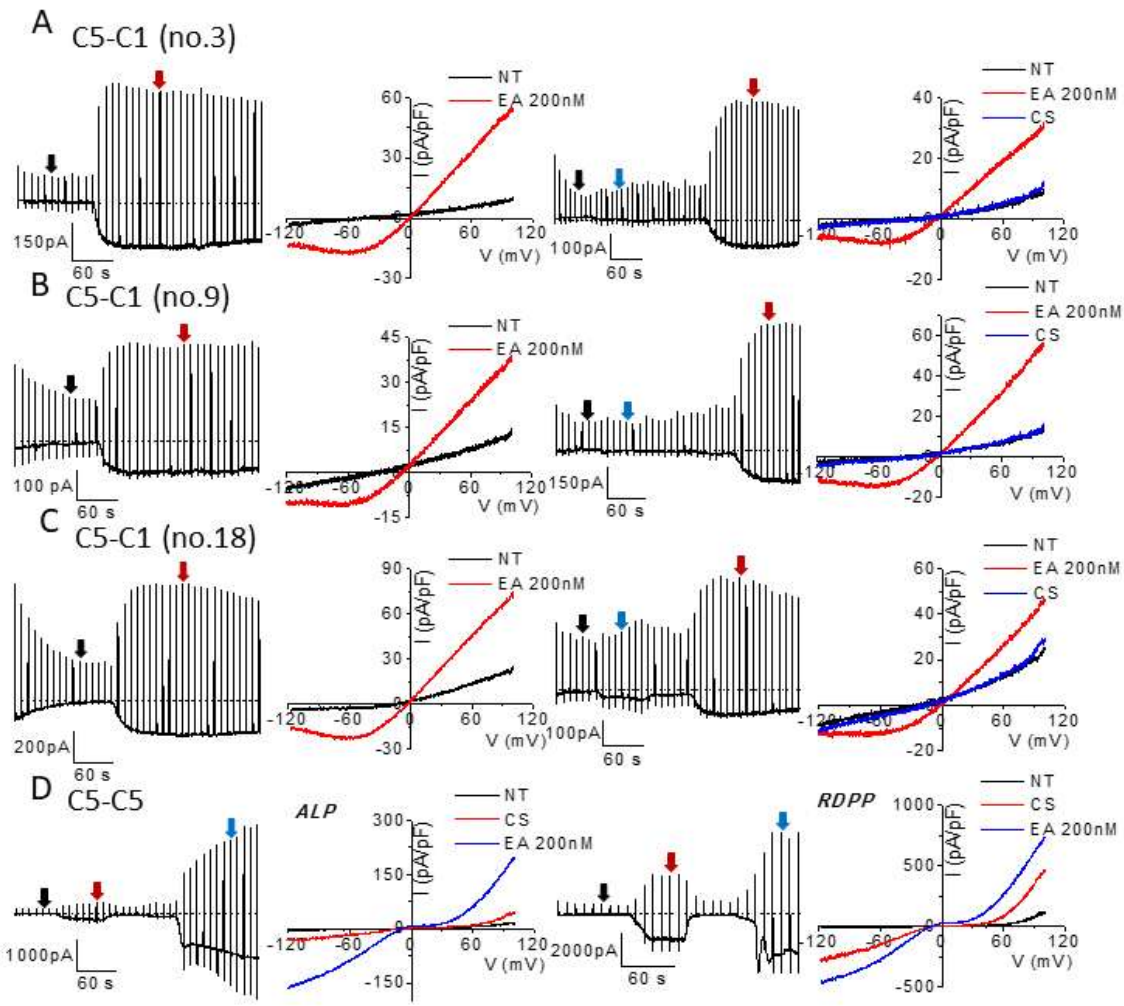

Figure 2. Cesium and Englerin A induced currents of TRPC5-TRPC1 and TRPC5-TRPC5 concatemers.

14 cell lines were tested prior to TRPC5-TRPC1 heteromer stable cell usage (A, B, C). Three cell lines with the biggest current inducements were no.3, no.9 and no.18. All three of the cell lines were tested with EA 200nM at first to confirm the heteromeric current. After, the external solution was changed from NT to Cesium rich solution for recording the current. The currents were recorded in TRPC5-TRPC1 stably expressing tetracycline induced HEK293 cells using the whole-cell patch clamp technique. Tetracycline inducement was done 24h before the patch clamp recording.(D)Transient transfected TRPC5-TRPC5 concatemers with EGFP tagged HEK293 cells were used for whole cell patch clamp recording. Cells with similar brightness of EGFP were patched prior to recording. At the holding potential of -60mV, the ramp pulse was applied from 100mV to -120mV at every 20 second. I/V curve of the current was taken at the peak of each stimulation and excluded currents from the NT washing state.

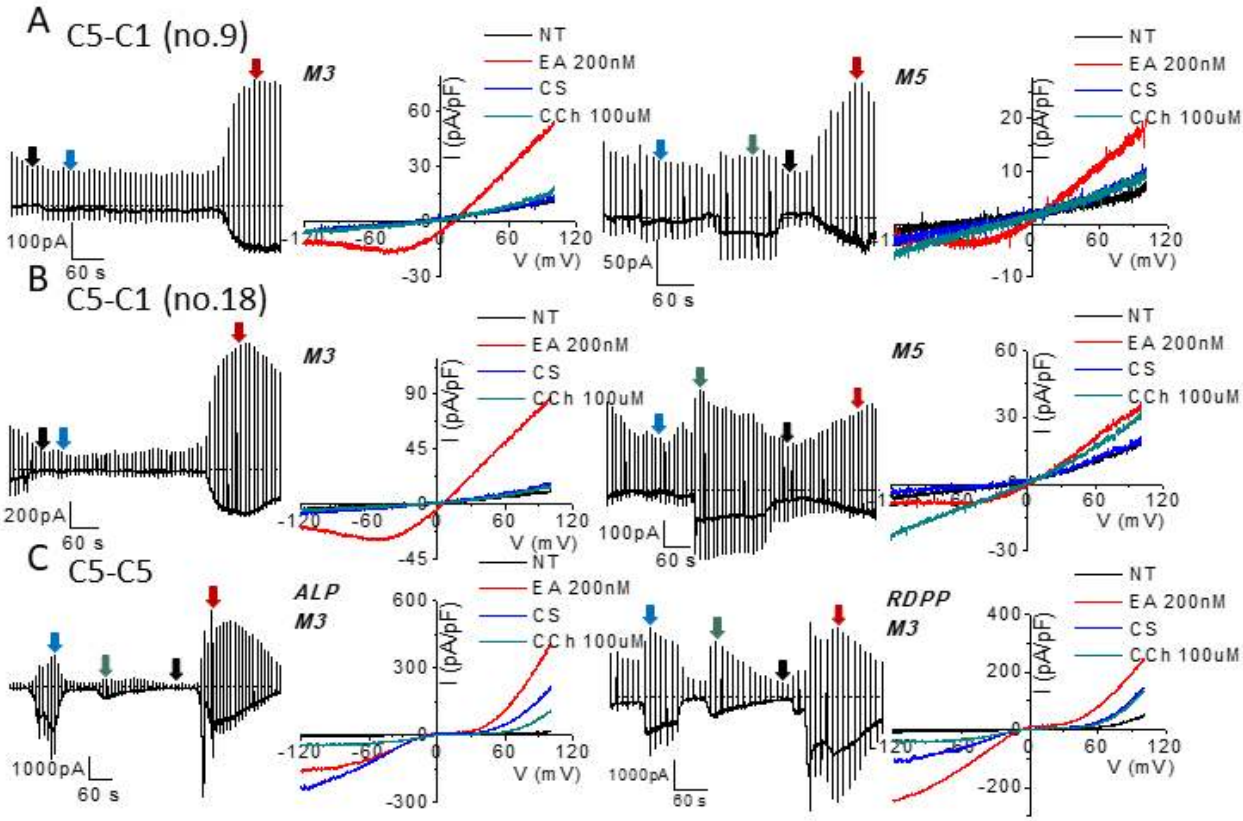

Figure 3. Effect of 100uM Carbachol on TRPC5-TRPC1 and TRPC5-TRPC5 Muscarinic receptor expressed cells.

Full traces and I/V curves of heterotetrameric (A)TRPC5-TRPC1 no.9 (B)TRPC5-TRPC1 no.18 following external solution change to Cesium rich solution, 100uM Carbachol and then 200nM Englerin A stimulation. M3 receptor expressing (A,B left) cells show good positive control with Englerin A activation but no Carbachol stimulation at all. M5 receptor expressing (A.B right) cells had inward specific activation but did not have heteromeric current. Smaller Englerin A stimulation was observed as the cells were affected via Carbachol stimulation. (C)TRPC5-TRPC5 M3 receptor expressing cells with Cesium external solution change and Carbachol stimulation followed by Englerin A activation. Englerin A activation was the largest, Cesium current increase was the second and then the Carbachol stimulation led to smallest current inducement.

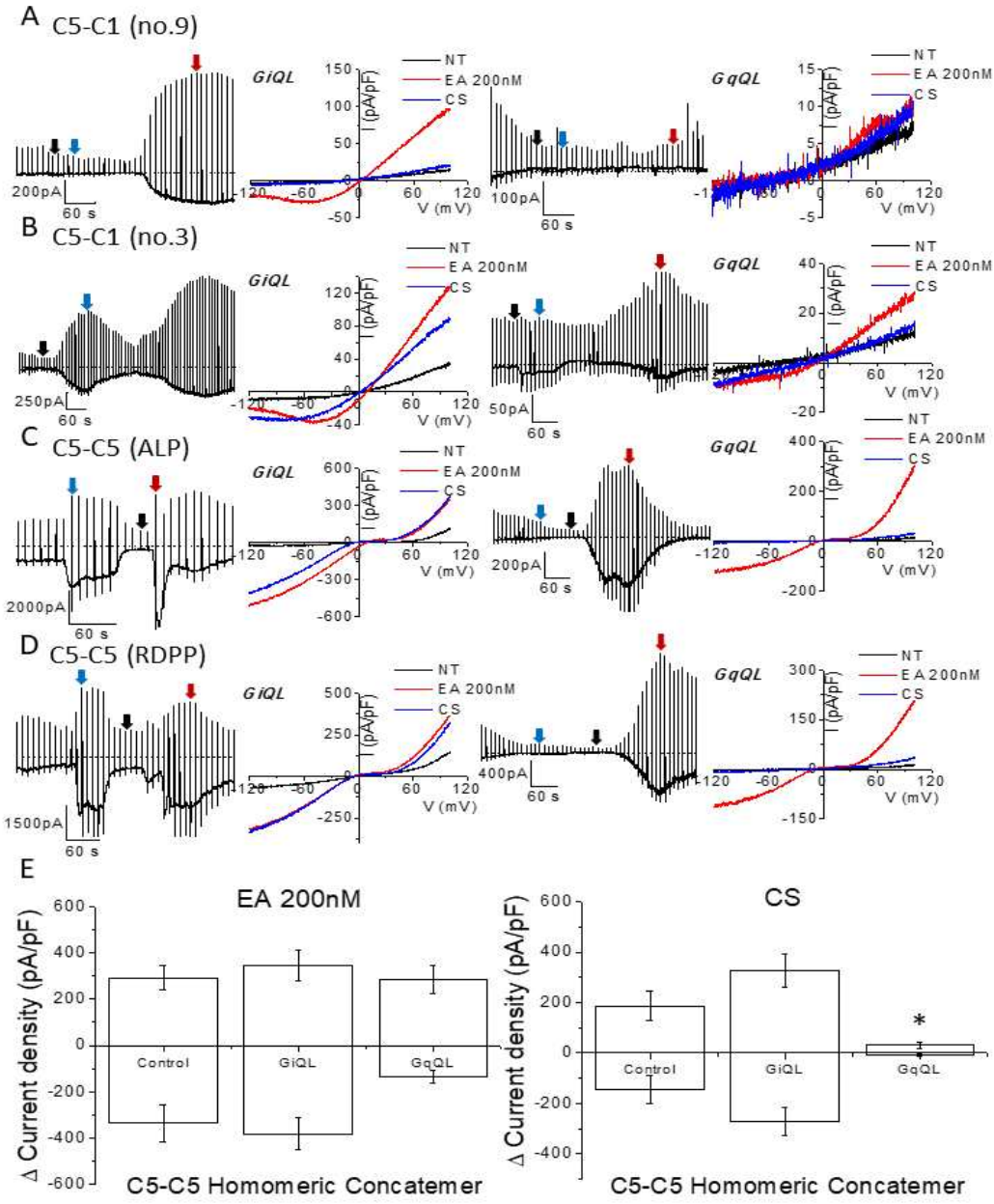

Figure 4. Inhibition of TRPC5-TRPC1 and TRPC5-TRPC5 by Gαq(Q209L) and activation of TRPC5-TRPC1 and TRPC5-TRPC5 by Gαi2(Q205L)

(A,B right) TRPC5-TRPC1 channels coexpressed with Gαq(Q209L) were stimulated by 200nM EA at +100mV. When the external solution was changed to Cesium rich solution, no or small change in current was observed. There was no EA stimulation observed. (A,B left) TRPC5-TRPC1 channels coexpressed with Gαi2(Q205L) were stimulated with the same protocol and showed significant increase. The color of the arrow, the peak or basal of each stimulation, corresponds to the I/V curve color. (E) A summarized current density at -100mV and +100mV of TRPC5-TRPC5 stimulated by 200nM EA and external high cesium concentration. Englerin A was used as a positive control at the end of the experiment. Under Cs condition, 100 mV, control 186.6 ± 60.2 (n=7); GiQL 327.1 ± 64.7 (n=9); GqQL 30.0 ± 10.6 (n=5). Under EA stimulation at 100 mV, control 292.5 ± 51.1 (n=7); GiQL 348.1 ± 65.6 (n=9); GqQL 285.3 ± 60.3 (n=5). * P value <0.05 (0.02).

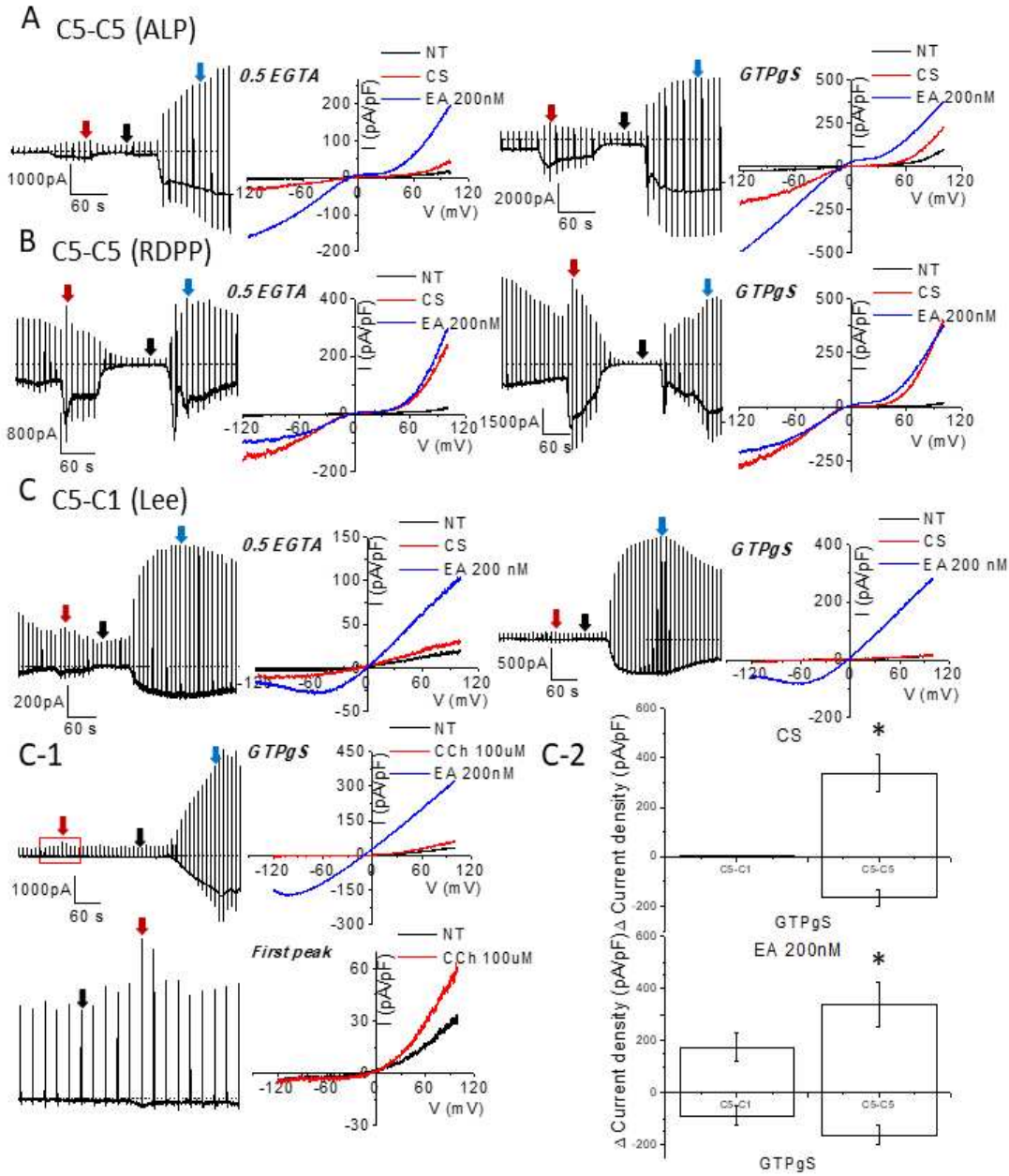

Figure 5. Effects of GTPgS

(A,B)TRPC5-TRPC5 homomer comparison of intracellular GTPgS and control internal solution. Both TRPC5-TRPC5 homomeric currents increase about twice with internal GTPgS in both external Cesium solution and 200nM Englerin A stimulation. (C)TRPC5-TRPC1 heteromer comparison showed significant increase in 200nM Englerin A stimulation whereas there was no change in Cesium rich external solution. (C-1) upon stimulation with 100uM Carbachol, current activity was slightly enhanced. I/V curve showed heteromeric current. (C-2) GTPɣS effect leads to the max current regardless of the kind of current enhancement. TRPC5-TRPC1 pore cannot be losen in a Cesium rich solution by affecting G protein permeability. Under Cs condition, C5-C1 conatemer 3.0 ± 0.1 (n=3); C5-C5 conatemer 338.1 ± 75.3 (n=5). Under EA stimulation, C5-C1 conatemer 172.7 ± 108.7 (n=3); C5-C5 conatemer 338.1 ± 168.3 (n=5). **P value <0.01 (0.004).

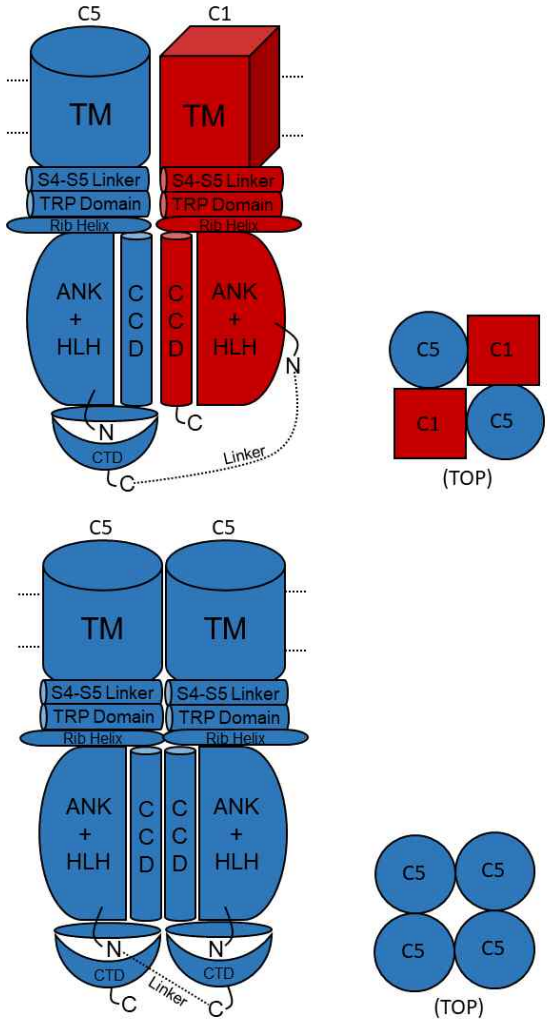

Figure 6. Schematic presentation of TRPC5-TRPC1 and TRPC5-TRPC5 concatemer structure.

The TRPC channels are shown schematically. TM: transmembrane domain, ANK: ankyrin repeated domain, CCD: coiled coil domain, CTD: cytosolic domain, HLH: helix loop helix domain

DISCUSSION

In the present study, we showed that 1) muscarinic stimulation and GiQL stimulation did not induce any current in TRPC5-TRPC1 heteromeric concatemer, 2) EA induced a large current in TRPC5-TRPC1 heteromeric concatemer, and 3) muscarinic stimulation can induce a small current with outward rectifying IV curve in TRPC5-TRPC1 concatemer when GTPgS was included in the pipette.

One big question from this study was why the TRPC5-TRPC1 concatemers did not react to the carbachol even with M3 or M5 receptor expression (Fig. 3). The TRPC5-TRPC1 concatemer was activated by EA and showed the typical outward rectifying IV curve (Fig. 2). Interestingly TRPC5-TRPC1 concatemer expressed with M5 showed inward current increase to carbachol but the IV curve does not seem ideal to be confirmed as a heteromer current (Fig. 3). After getting this result, we reevaluated the previous results from other research groups [Beech data]. There was no carbachol experiment done with the specific TRPC5-TRPC1 concatemer. This question is still unknown and has not been figured out. Considering the recent TRPC4 structure [9, 10, 13], the rib helix may be the reason for the failure to carbachol reaction. The rib helix is involved in the G protein pathway. Calmodulin also binds to the rib helix of TRPC4

that leads to the limitation of channel conformation. This fixation may lead to rib helix that affects the channel activation [13]. TRPC1 structure does not have the region after CCD compared to TRPC5 struture. As in Figure 6, homotetramer is able to fully function with a short linker. On the other hand, in heterotetramer, TRPC1 might require a longer linker with TRPC5 (Fig. 1). The short linker in TRPC5-TRPC1 concatemer constructis may result in the malfunction of the rib helix.

Another possibility is that the activation mechanism of EA is different from that by carbachol. Even in C553/C558 mutant of TRPC5, the same result was obtained. The C553/C558 mutant was activated by Englerin A [9, 11, 29], but not by carbachol or GiQL [37]. Previous studies showed that Gαq(Q209L) completely inhibited TRPC5 when activated with agonist, carbachol or GiQL [24]. However, when the homomeric concatemers were tested with cesium or EA, cesium did not increase current but EA was able to activate the channel. The EA-induced current decreased with fast deactivation, resulting in V shaped activation-deactivation (Fig. 4). We showed that EA binds to the intersubunit interface and increased the current [12 - 14, 29]. Independent of muscarinic receptor stimulation via rib helix, EA might increase the current by acting on selectivity filter itself.

Lastly, in TRPC5-TRPC1 concatemer, carbachol activated the typical current when carbachol and GTPgS were extracellularly and intracellularly applied, respectively. Other G proteins besides Gi seem involved in regulation of TRPC5-TRPC1 concatemer response to carbachol [30, 38, 39].

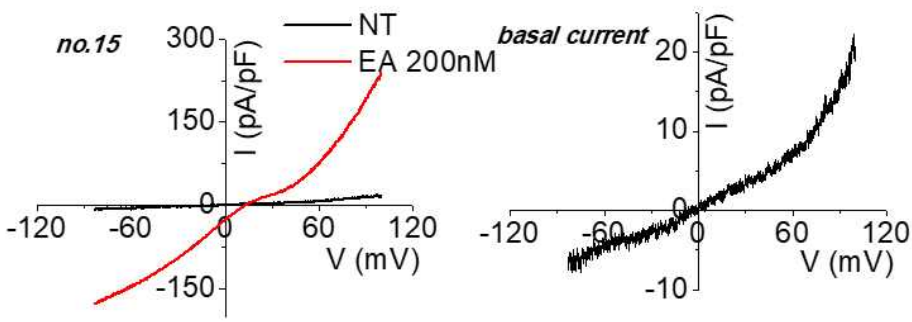

Figure 7. Homomeric current observation in the process of TRPC5-TRPC1 heteromer stable cell generation

During stable cell generation and testing, few of the cell lines showed homomeric current when activated (Fig. 7). Even though the cell lines were all from TRPC5-TRPC1 concatemer, half of them did not activate and one third of the cell lines did not activate enough to test other characteristics than EA stimulation. and the other 1/6 had homomeric current such as the figure above (Fig. 7). Maybe they may form octamer with the part of TRPC5 from TRPC5-TRPC1 concatemer facing the pore part. In this case, the channel would show the homomeric IV curve. The hexamer which have 3 TRPC5

and 1 TRPC1 at the pore region seems possible, although the IV curve was not shown from such a possibility.

Co-expression of TRPC5-TRPC1 with Gαi2(Q205L) significantly enhanced outwardly rectifying current, especially at stable cell line no. 3 (Fig. 4). Both cesium current and EA current increased, stating heteromeric concatemers may have less pore fixation than the transfected unstable heteromers from the past [23]. However, high passage cell lines of all three of the cell lines no. 3, no. 9, no. 18 did not function as heteromers and did not even react to EA. TRPC5-TRPC1 Gαq(Q209L) showed current inhibition in both cesium and EA (Fig. 4). Current even decreased when compared to the basal.

As in the past, FuGENE6 was considered to be the novel transfection agent to TRPC channels [5, 17, 24, 31], but when transfecting concatemer from david beech, no heteromeric characteristics were observed even when fluorescence protein was clearly seen. We tried cells with and without fluorescence protein for a while but could not figure out why the transfection did not process. We tried TurboFect transfection agent that is known to be used for harder transfecting DNA [see also 40 - 42]. Compulsively, DNA was fully functioning when transfected with TurboFect. David Beech also used FuGENE HD, which is slightly different from FuGENE6 [18, 19, 25, 26, 27, 28]. This confirmed that slight differences in the usage of Transfection

agents may lead to huge differences when handling structurally different DNA than the previously used.

In conclusion, using concatemers with fixed stoichiometry could reconfirm the characteristics of TRPC5-TRPC1 heteromers and TRPC5-TRPC5 homomers even with more precise current transitions. With this fixed structure, there is no doubt that TRPC5 heteromer and homomer would be fully understood.

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국문 초록

TRPC5-TRPC1와 TRPC5-TRPC5 콘카티머의 전기생리학적 특성에 대한 비교

TRPC5 이온채널은 비선택적 양이온 채널로 막전압의 탈분극과 칼슘 유입에 중요한 역할을 한다. TRPC5는 TRPC5 호모테트라머를 이루어 생리학적 기능을 하지만 TRPC1과 헤테로머를 이루어서도 중요한 생리학적 기능을 수행한다. TRPC5-TRPC1 헤테로머의 기능을 알아보기위해 두 유전자를 함께 발현하여 실험을 진행하여 왔으나 이 경우 여러 조합의 헤테로머가 생기거나 호모머와 함께 세포막에 이온통로들이 발현하여 전기생리학적으로 혼합된 전류-전압 곡선이 나왔다. 이에 TRPC5-TRPC1 헤테로머 콘카티머를 만들어 고정된 구조를 가지게 하였고, 대조군으로 TRPC5-TRPC5 호모머 콘카티머도 만들었다. 이 두 컨카디머를 갖고 이제까지 알려진 TRPC5 호모머와 헤테로머의 특징을 비교 분석하였다. 전체적인 특성은 여태 알려진 것과 일관되었지만 조금 더 들여다 보았을 때 세세한 차이점을 발견할 수 있었다. 예를 들어, TRPC5-TRPC1 헤테로머 콘카티머는 EA에는 잘 반응하였지만 무스카린성 수용체 자극에 대해서는 반응하지않았다. 활동성이 있는 Gi 단백에 의해서도 활성화되지 않았다. 단 GTPgS를 세포 내에 투입하고 무스카린 수용체를 자극한 경우에만 반응을 하였다. 이러한 결과를 바탕으로 다음과 같은 결론을 얻을 수 있었다. TRPC5-TRPC5 호모머 콘카티머는 링커가 있더라도 EA, 무스카린 수용체 자극, G 단백, 세슘 및 GTPγS에

잘 반응하였지만 고정된 구조를 가진 TRPC5-TRPC1 헤테로머 콘카티머의 경유 생리학적 자극에 반응하지않았고 GTPγS와 무스카린 수용체 자극이 동시에 있는 경우에만 반응하였다. TRPC5-TRPC1 헤테로머 콘카티머가 생리학적 자극에 반응하기 위해서는 링커 길이를 바꾸면서 저절한 링커를 찾거나 GTPγS에 의해 어떤 기전이 활성화되어 무스카린 수용체 자극에 반응하는지 보다 구체적인 활성화 기전에 대한 연구가 필요하다.

주요어 : TRP, TRPC5, TRPC1, 헤테로머, 호모머, 콘카티머, G protein, GPCR, Englerin A, 기공(세포) 투과성, GTPgS

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