Gi/o-coupled muscarinic receptors co-localize with GIRK channel for efficient channel activation

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Abstract

G protein-gated inwardly rectifying K⁺ (GIRK) channel regulates cellular excitability upon activation of Gi/o-coupled receptors. In Gi/o-coupled muscarinic M₂R, the intracellular third loop (i3) is known as a key domain for Gi/o coupling, because replacement of i3 of Gq-coupled muscarinic M₁R with that of M₂R enables the chimeric receptor (MC9) to activate the GIRK channel. In the present study, we showed that MC9, but not M₁R, co-localizes with the GIRK channel and Gαi1 by Förster resonance energy transfer (FRET) analysis. When M₁R was forced to stay adjacent to the channel through ligation with short linkers, M₁R activated the GIRK channel. FRET analysis further suggested that the efficacy of channel activation is correlated with the linker length between M₁R and the GIRK channel. The results show that co-localization is an important factor for activating the GIRK channel. In contrast, for MC9 and M₂R, the GIRK channel was activated even when they were connected by long linkers, suggesting the formation of a molecular complex even in the absence of a linker. We also observed that replacement of 13 amino acid residues at the N-terminal end of i3 of MC9 with those of M₁R impaired the co-localization with the GIRK channel as well as channel activation. These results show that localization of the receptor near the GIRK channel is a key factor in efficiently activating the channel and that the N-terminal end of i3 of M₂R plays an important role in co-localization.

Introduction

G protein-gated inwardly rectifying potassium (GIRK) channel is a key protein that regulates cellular excitability and is activated by interacting with free Gβγ released from the heterotrimeric Gαβγ complex upon the activation of G protein-coupled receptors (GPCRs) [1]. The conformational rearrangement between the GIRK channel and Gβγ has been observed upon the receptor activation on the living cell membrane by Förster or bioluminescence resonance energy transfer analyses (FRET or BRET, respectively) [2, 3] and the GIRK-Gβγ complex formation was demonstrated by structural analyses [4]. The GIRK channel is also known to bind
to G\(\alpha_i\) and G\(\alpha_o\) in their resting and active forms [5–8], suggesting that G\(\beta\gamma\) released from G\(\alpha_i\) or G\(\alpha_o\) immediately interacts with and activates the GIRK channel. In addition, several Gi/o-coupled receptors (Gi/o-Rs) have been reported to localize near Gi/o by FRET and BRET analyses [9–11]. Therefore, Gi/o-Rs, Gi/o and the GIRK channel have been suggested to form a ternary pre-signaling complex to effectively activate the GIRK channel.

Gi/o-Rs activate the GIRK channel through releasing G\(\beta\gamma\) from pertussis toxin (PTX)-sensitive G\(\alpha_i/o\) [1, 12]. When the receptors are highly expressed, the GIRK channel is activated by receptors that stimulate PTX-resistant G protein, such as the Gq-coupled muscarinic receptors (M\(_1\)R and M\(_3\)R) or Gs-coupled adrenergic receptors (\(\beta_1\)-AR and \(\beta_2\)-AR) [13, 14]. When surface expression of M\(_i\)R is very high, the chance of M\(_i\)R-Gi/o coupling may increase [13] and/or the distance between M\(_i\)R and GIRK channel may be shortened, which could allow G\(\beta\gamma\) released from Gq to activate the channel. Interestingly, Gs-coupled \(\beta_2\)-AR has been reported to functionally and physically interact with GIRK channels [15, 16]. In addition, the rate of GIRK channel activation was thought to correlate with the co-localization of Gi/o-Rs with the channel [17]. We thought that the distance between GPCR and the GIRK channel is a critical factor for effective channel activation.

In this study, we first showed by FRET analyses that a chimeric receptor of M\(_i\)R and M\(_2\)R (MC9) [18], which activates the GIRK channel [19], locates adjacent to the channel and G\(\alpha_i1\) in transfected HEK293T cells. Next, we examined whether Gq-coupled M\(_i\)R activates the GIRK channel when these molecules are forced to stay close to each other by ligating them with short linkers. The key amino acid residues in complex formation for activating the GIRK channel were further investigated by analyzing the changes in co-localization of MC9 mutants with the GIRK channel.

**Materials and methods**

**Constructs and expression system**

The chimeric MC9 receptor was constructed by replacing the last eight residues of the 5th transmembrane (TM) and most of i3 of M\(_i\)R with those of M\(_2\)R (M\(_2\)R-i3) as previously reported [18]. YFP was fused at the C-tail of M\(_i\)R, M\(_2\)R, and MC9 (M\(_i\)R-YFP, M\(_2\)R-YFP, and MC9-YFP, respectively). To control the subunit composition of the GIRK channel, GIRK1 and GIRK2 were ligated (GIRK1/2) [20], by a glycine-rich linker of 34 amino acids (a.a.) (see S1 Method). For FRET analysis, CFP was attached to the C-tail of GIRK1/2 (GIRK1/2-CFP). Fluorescent protein (FP) was inserted into mouse G\(\alpha_i1\) between Ala121 and Glu122 (G\(\alpha_i1\)-FP) with a short linker (A121-SGGGS-V2). . .K212-SGGGS-E122) [21] or into mouse G\(\alpha_q\) between Phe123 and Glu124 with the SGGGS linker. M\(_i\)R-YFP and MC9-YFP were ligated to GIRK1/2 or GIRK1/2-CFP by junctional glycine-rich linkers, with varying lengths of 34, 100, 265, and 535 a.a. residues (S1 Method). The chimeric G\(\alpha_{q5}\) was constructed by replacing the last five residues of G\(\alpha_q\) with those of G\(\alpha_i1\) [22]. Each construct was subcloned into the pcDNA3.1(-) expression vector and the DNA sequence was confirmed. The cDNA construct of CFP fused with the PH domain (CFP-PH) was kindly gifted from Dr. Jalink [23]. HEK293T cells were transfected with the plasmid DNAs (\(\mu\)g for 4 x 10\(^4\) cells) of M\(_i\)R-YFP (0.8), MC9-YFP constructs (0.8), M\(_2\)R-YFP (1.2), GIRK1/2 (0.7), GIRK1/2-CFP (1.2), G\(\alpha\)-FPs (1.1), G\(\alpha_{q5}\) (1.0), G\(\beta_1\) (0.8), G\(\gamma_2\) (0.5), M\(_i\)R, MC9 or M\(_2\)R tandem constructs (1.2), CFP-PH (1.1) or prestein-YFP (1.0) [24] using LipofectAMINE 2000 (1 \(\mu\)L, Invitrogen, Carlsbad, CA, USA). The transfected cells were seeded onto cover glasses or glass bottom dishes 8–24 h after the transfection, as previously reported [25]. G protein subunits were not additionally transfected for the electrophysiological experiments and FRET analysis between receptor-YFPs and GIRK1/2-CFP, in order to analyze the interaction between receptor and endogenous Gi/o. Experiments were carried
out 24–72 h after transfection. For PTX treatment, the cells were cultured with PTX (300 ng/mL) for more than 16 h and then the experiments were performed.

**Electrophysiology**

Macroscopic membrane currents were recorded from cells expressing fluorescent constructs by the whole cell patch clamp technique using Axopatch 200B amplifiers, Digidata1332A, and pClamp 9 software (Molecular Devices, San Jose, CA, USA). After establishing the whole cell configuration, the cell was held at -80 mV and ramp pulses (from -120 to +40 mV for 400 s) were applied every 5 s. The bath solution was composed of the following: 140 mM NaCl, 4 mM KCl, 1 mM CaCl₂, 0.3 mM MgCl₂, 10 mM HEPES (pH 7.4 adjusted with NaOH). The internal solution was composed of: 130 mM KCl, 5 mM Na₂-ATP, 3 mM EGTA, 0.1 mM CaCl₂, 10 mM HEPES, 4 mM MgCl₂, 0.3 mM GTP-Li₃ (pH 7.3 adjusted with KOH). An agonist of muscarinic receptors, oxotremorine M (oxo-M), was applied by using fast perfusion system (VC-77SP, Warner Instruments, Hamden, CT, USA) as previously described [26]. To record the GIRK channel current, Na⁺ ions in the bath solution were substituted with K⁺ to enhance the driving force of K⁺ and oxo-M was applied in 140 mM K⁺ bath solution. The average of the holding current amplitude for 100 ms before applying the ramp pulse was measured and then normalized to the cell capacitance to calculate the current density (I). The basal current density (I₀) before application of oxo-M (10 μM) was subtracted from the maximum value (I_max) after agonist application to evaluate the agonist-induced current density (∆I_max). To analyze the decay of the GIRK current, the ratio of the agonist-induced current density before washout of the agonist (∆I_last) to (∆I_max) was calculated. We also analyzed the onset of GIRK channel activation, with the application of oxo-M for 5 s controlled by the combination of Clampex9 and VC-77SP. The time to half-maximum current amplitude (t₁/₂) was then measured for each trace.

**Imaging and FRET analysis**

Fluorescent images were obtained from cells expressing FP-fused constructs using a total internal reflection fluorescence (TIRF) microscope (Olympus, Tokyo, Japan) as previously described [25]. MetaFluor imaging software (Molecular Devices, Sunnyvale, CA, USA) was used to control the excitation of CFP and YFP and to acquire images. For FRET analysis, five images were acquired before and after acceptor bleaching, which was performed by excitation of YFP with a 515-nm laser line for 1 min (emission intensity of YFP was decreased by less than 5% in the bleaching procedure). Averaged emission intensities of CFP (I_{CFP}) and YFP (I_{YFP}) before and after acceptor bleaching were measured in each cell by subtracting their background intensities and were then normalized to the cell size. To calculate FRET efficiency, the increase in I_{CFP} after acceptor bleaching was normalized to the total I_{CFP} after bleaching in each cell. To make the CFP emission images, five CFP images before and after acceptor bleaching were averaged and their subtraction was performed by using MetaMorph software (ver 6, Molecular Devices).

To evaluate the activation of Gqi5 by MC9 constructs, the intensity of CFP-PH (I_{CFP-PH}) was measured under TIRF illumination. Cells expressing the fluorescent constructs were continuously perfused with bath solution by gravity at a rate of approximately 3 mL/min, and various concentrations of oxo-M were applied by changing the perfusion solution. The baseline I_{CFP-PH} was the averages of I_{CFP-PH} measured from 13 images before oxo-M application. The maximal decrease in I_{CFP-PH} upon application of oxo-M (∆I_{CFP-PH}) was normalized to the baseline I_{CFP-PH} in each cell.
Statistical analysis

All data are expressed as the means ± S.E., with n indicating the number of data. Statistical significance between two groups was examined by unpaired Student’s t-test and that between more than two groups was tested by one-way analysis of variance (ANOVA) followed by Tukey’s test. Values of p ≤ 0.05 were considered statistically significant (*: p < 0.01, **: p < 0.001, ***: p ≤ 0.001, n.s.: p > 0.05). Pearson correlation coefficients were calculated using Microsoft Excel2013.

Results

i3 of M2R is a key structure in activating and staying adjacent to the GIRK channel

Application of an agonist oxo-M increased the amplitude of the inward current density in cells transfected with the GIRK channel and Gi/o-coupled M2R, but not with the channel and Gq-coupled M1R (Fig 1A and 1B, left and center panels). The amplitude of the agonist-induced GIRK channel current density (ΔI_{max}) was decreased to almost null by treating the cells with PTX (300 ng/mL) (Fig 1C left panels), showing that the effect of M2R is mediated by the activation of Gi/o. Replacement of the i3 of M1R with that of M2R (M2R-i3) enabled the chimeric receptor (MC9) to activate the GIRK channel, as previously reported [19]. The effect of MC9 was inhibited by PTX treatment (Fig 1C right panels) and the speed of current increase induced by the MC9 activation did not differ from that by M2R (S1 Fig), indicating that M2R-i3 is a critical structure for Gi/o coupling and GIRK channel activation. We then examined whether or not MC9 locates adjacent to the GIRK channel by analyzing FRET from GIRK1/2-CFP to receptor-YFP. In HEK293T cells expressing these constructs, acceptor bleaching resulted in the increases in intensities of CFP (I_{CFP}) (Fig 2A). Interestingly, the FRET efficiency from GIRK1/2-CFP to MC9-YFP was larger than that to M1R-YFP (Fig 2B left panels). As the surface expression level of the FP-tagged constructs were not different (S1 Table), MC9 but not M1R was suggested to co-localize with the GIRK channel. The FRET efficiency did not change by application of oxo-M (Fig 2B), suggesting that activation of receptor does not change the co-localization. We also analyzed the FRET from G_{αi1}-CFP to receptor-YFP in HEK293T cells transfected together with G_{β1} and G_{γ2}. The FRET efficiency from G_{αi1}-CFP to MC9-YFP was higher than that to M1R-YFP both in the absence and presence of the agonist (Fig 2B bottom bars). The results support pre-coupling of MC9 with G_{αi1} and show that pre-coupling is not changed by MC9 activation. The interaction between GIRK1/2 and G_{αi1} showed higher FRET efficiency than that between GIRK1/2 and G_{αq} (Fig 2B, right panel), which is consistent with previous reports [5–8]. As a negative control for the FRET analysis, we chose a member of an anion-transporter family, prestin [24], since prestin is not expected to selectively interact with the GIRK channel and G protein. FRET values from CFP tagged constructs to prestin-YFP were similar to those to M1R-YFP (Fig 2), suggesting that M1R does not specifically interact with GIRK channel and G_{αq}. Taken together, MC9, GIRK, and Gi/o are adjacent to each other and may form a molecular complex.

Distance between receptor and GIRK1/2 channel is a key determinant for channel activation

The FRET analysis raised a question about the functional significance of co-localization. To address this question, Gq-coupled M1R was forced to stay adjacent to the GIRK channel by connecting them with a short linker composed of 34 glycine-rich amino acid (a.a.) residues (Fig 3A). Application of oxo-M (10 μM) elicited a rapid increase in the amplitude of the inward
current in cells transfected with M₁R-YFP. 34-GIRK1/2 (Fig 3B red lines). This effect remained after PTX treatment (Fig 3C, Table 1), suggesting that Gβγ released from PTX-resistant Gαq activated the channel. The amplitude of the GIRK channel current decreased by approximately 30% at 25 s after application of oxo-M, which may have resulted from negative regulation induced by Gq signaling [27]. When the linker length was elongated, the GIRK channel was not activated; ΔI_{max} was decreased in accordance with the increase in the linker length of the
M₁R tandem constructs (Fig 3D open circles). In contrast, the application of oxo-M evoked a rapid increase in the inward current, even when MC9-YFP was connected to GIRK1/2 by a long linker of 535 a.a. residues (Fig 3E and 3F). The response was totally mediated by PTX-sensitive Gi/o (Fig 3G, Table 1). Similar results were observed when the linker length was 100 or 265 a.a. (Fig 3H, Table 1), indicating that the effect of MC9 does not change by the linker length. ΔI_{max} in the MC9 tandem constructs were 30% smaller than that in non-linker combination, but the difference was not statistically significant (Fig 3H). The ligation of the receptor and the GIRK channel may have a slight inhibitory effect on the expression and/or activation of the GIRK channel. These results suggest that MC9, but not M₁R, is in proximity of the GIRK channel, even when the linker is long, presumably by forming a molecular complex.

Next, the FRET efficiency between GIRK1/2-CFP and M₁R- or MC9-YFP was measured for each tandem construct. FRET efficiency in the M₁R tandem construct was decreased in
accordance with the increase in the linker length (Fig 4A open diamonds), whereas that in the MC9 construct was not changed by the change of the linker length in the presence or absence of the agonist (Fig 4B triangles). For M₁R, FRET efficiency was well-correlated with ΔI_{max} (Fig 4C, Pearson correlation coefficient is 0.98). The results suggest that receptor-GIRK signaling tightly depends on their distance. For MC9, the FRET efficiency and ΔI_{max} did not change when the linker length was changed (Fig 4C, Pearson correlation coefficient is 0.53). These results support that MC9, but not M₁R, forms a complex with the GIRK channel for efficient channel activation.

**M₂R forms a complex with GIRK channel**

M₂R-i3 is the key structure in complex formation with the GIRK channel and Gi/o (Figs 1 and 2). Thus, the results of the M₂R tandem constructs connected to the GIRK channel by various
linker residues were expected to be similar to those observed in MC9 constructs (Figs 3H and 4B). As expected, $\Delta I_{\text{max}}$ as well as FRET efficiency were not changed with the increases in the linker length (Fig 5A and 5B) and $\Delta I_{\text{max}}$ was not positively correlated with FRET efficiency (Fig 5C, Pearson correlation coefficient is -0.79). GIRK current increase induced by $M_2R$ was

Activation of GIRK channel was evaluated as the amplitude of the agonist-induced increase in the inward current density ($\Delta I_{\text{max}}$) at the holding potential of -80 mV. Numbers of cells are indicated in parentheses.

**p < 0.001, *p < 0.01, n.s. p > 0.05

Table 1. Effects of PTX treatment on the GIRK channel activation of $M_1R$-YFP and MC9-YFP tandem constructs.

| Constructs               | $\Delta I_{\text{max}}$ (pA/pF) PTX (-) | $\Delta I_{\text{max}}$ (pA/pF) PTX (+) |
|--------------------------|------------------------------------------|------------------------------------------|
| $M_1R$-YFP-34-GIRK1/2    | 11.3 ± 2.5 (11)                          | 8.5 ± 2.2 (12) **                       |
| $M_1R$-YFP-100-GIRK1/2   | 9.9 ± 1.5 (16)                           | 8.0 ± 1.5 (15) **                       |
| $M_1R$-YFP-265-GIRK1/2   | 6.5 ± 1.2 (12)                           | 4.4 ± 1.7 (7) **                        |
| MC9-YFP-100-GIRK1/2      | 43.7 ± 12.3 (12)                         | 0.4 ± 0.2 (3) **                        |
| MC9-YFP-265-GIRK1/2      | 34.6 ± 10.7 (11)                         | 0.4 ± 0.3 (3) **                        |
| MC9-YFP-535-GIRK1/2      | 36.0 ± 10.4 (9)                          | 0.1 ± 0.1 (3) **                        |

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Fig 4. Distance from $M_1R$ to GIRK channel correlates with efficacy of channel activation. (A) and (B) FRET analyses. Schematic diagrams in the top panel illustrate the tandem construct for the FRET analysis. Symbols represent FRET efficiency in each tandem construct in the absence of oxo-M (A, open diamonds for $M_1R$ tandem constructs, B, open triangles for MC9 constructs) or presence of oxo-M (B, filled triangles for MC9 constructs). Dashed lines indicate FRET efficiency when the receptor-YFP and GIRK1/2-CFP were co-expressed (no linker, data form Fig 2B). Numbers of data are shown in the parentheses. **p < 0.001, n.s.: p > 0.05 (C) Correlation between $\Delta I_{\text{max}}$ and FRET efficiency. $\Delta I_{\text{max}}$ is plotted as a function of the FRET efficiency for each tandem construct (open symbols) or each combination (no linker, filled symbols). The number of junctional linker residues is indicated on each symbol. The results of $M_1R$ tandem constructs are expanded in the inset to highlight the correlation between FRET efficiency and the GIRK channel activation.

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completely inhibited by the PTX treatment (Table 2), indicating that the effects were mediated by the activation of Gi/o in the M₂R tandem constructs. The FRET efficiency from GIRK to M₂R was smaller than that to MC9, which may be due to the difference in the C-terminal tail of the receptor at which YFP is tagged. Gi/o was suggested to be located in proximity to M₂R.

Table 2. Inhibitory effects of PTX treatment on the GIRK channel activation by M₂R-YFP tandem constructs.

| construct              | ΔI_{max} (pA/pF) PTX (-) | ΔI_{max} (pA/pF) PTX (+) |
|------------------------|---------------------------|---------------------------|
| M₂R-YFP-100-GIRK1/2    | 62.0 ± 7.7 (12)           | 0.9 ± 0.4 (3) ***         |
| M₂R-YFP-265-GIRK1/2    | 49.0 ± 11.3 (7)           | 1.2 ± 0.5 (3) **          |
| M₂R-YFP-535-GIRK1/2    | 49.3 ± 10.0 (12)          | 0.4 ± 0.3 (3) ***         |

Activation of GIRK channel was evaluated as the amplitude of the agonist-induced increase in the inward current density (ΔI_{max}) at the holding potential of -80 mV. Numbers of cells are indicated in parentheses.

***p<0.001,
**p<0.01<p<0.001

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as well as the GIRK channel; the FRET efficiency from Goq1-R-CFP to M2R-YFP was 2.8 ± 0.5 (n = 12, p = 0.001 compared to FRET from Goq1-CFP to prestin-YFP in Fig 2B). Taken together, these results indicate that M2R, Gi/o, and the GIRK channel form a molecular complex.

**Dual roles of M2R-i3 in the activation of and co-localization with GIRK**

We then evaluated the residues in M2R-i3 responsible for activation of and co-localization with the GIRK channel and activation of Gi/o. It was previously reported that most of the long M2R-i3 is not necessary for Gi/o coupling [28]. Indeed, the replacement of the long chain in the middle of M2R-i3 (K221-P379) with SGGGS did not decrease ΔI_{max} and the FRET efficiency (ΔI_{max} = 75.1 ± 12.5 pA/pF, n = 10; FRET = 5.5 ± 0.9%, n = 15, cf Figs 1 and 2). Thus, the proximal N- and distal C-terminal residues of M2R-i3 may be critical regions; the charged residues at the proximal N-terminal end of M2R-i3 as well as Val and Thr residues at the distal C-terminal end have been suggested to play important roles in Gi/o coupling [19, 28, 29]. Residues at the N-terminal end were replaced with those of M1R (MC9A-YFP and MC9B-YFP, Fig 6A) and also double mutations (V387A/T388A) were introduced at the C-terminal end. The emission intensities of YFP (I_{YFP}) fused to these constructs under TIRF illumination did not differ (I_{YFP} in S2 and S3 Tables), indicating that their surface expression levels were similar.

We first measured ΔI_{max} and FRET from GIRK1/2-CFP to chimeric and mutant receptor-YFP. Although it was not statistically significant, ΔI_{max} in VT/AA mutants was 30% smaller than that in MC9-YFP (Fig 6B), possibly due to the impairment of the activation of Gi/o. Interestingly, ΔI_{max} in MC9B-YFP was significantly smaller than that in MC9-YFP (Fig 6B). Similarly, the FRET efficiency from GIRK1/2-CFP to MC9B-YFP, but not to MC9-VT/AA-YFP, was smaller than that to MC9-YFP (Fig 6C). ΔI_{max} and FRET values appeared to be correlated in MC9-YFP chimeric constructs (Fig 6D): Pearson correlation coefficient is 0.79 (+VT/AA) and 0.97 (-VT/AA). Next, we examined the effect of the chimeric and double mutation on the Gi/o activation by using Goq5, which is activated by Gi/o-Rs and stimulates Gq-phospholipase C (PLC) signaling [22]. As the last five a.a. residues of Goq5 are derived from Goq1 and include the ADP-ribosylation site, Goq5 is inhibited by PTX. The activity of Goq5 signaling was monitored as the decrease in fluorescent intensity of the CFP-tagged PH domain (CFP-PH) under TIRF illumination (Fig 6E upper panel). Upon PIP2 hydrolysis, CFP-PH translocates from the membrane into the cytosol, where TIRF illumination cannot reach [23, 26], resulting in a decrease in the fluorescent intensity of CFP-PH (I_{CFP-PH}). Indeed, activation of MC9-YFP constructs decreased I_{CFP-PH} in the presence of Goq5 (Fig 6E, lower left open circles). The amplitude of the decrease in I_{CFP-PH} (ΔI_{CFP-PH}) was almost null when cells were treated with PTX (Fig 6E filled circles and Table 3), suggesting that ΔI_{CFP-PH} may reflect the efficacy of the receptor to activate Gi/o. As previously reported in studies of M2R [28, 29], VT/AA attenuated Goq5 activation: ΔI_{CFP-PH} in the VT/AA mutant was smaller than that in MC9-YFP (Fig 6F). In contrast, ΔI_{CFP-PH} was not changed by chimeric mutations of MC9A and MC9B (Fig 6F). These results suggest a possibility that all MC9 constructs are equally not co-localized with Goq5 and/or PLC. Another possibility is that there is a difference in the extent of co-localization with Goq5 between the MC9, MC9A and MC9B, and that the difference of the co-localization does not change the extent of PLC activation. If this is the case, co-localization might not be critical for the PLC activation, highlighting the significance of co-localization for the GIRK channel activation. We also analyzed FRET from Goq1-CFP to MC9B-YFP and found that co-localization with Goq1 appeared to be attenuated, but the difference was not significant (S3 Table). Taken together, at least the 13 a.a. residues at the N-terminal end of M2R-i3 play...
Fig 6. N-Terminal end of M1R-i3 plays important roles in the activation of and co-localization with GIRK channel. (A) Amino acid sequences of i3 of mutant and chimeric constructs of MC9-YFP (black: M1R sequences, red: M2R sequences). (B) Summary of ΔI\textsubscript{max}. Circles represent agonist-induced GIRK channel current density (ΔI\textsubscript{max}) upon application of oxo-M (10 μM). (C) Summary of FRET efficiency between MC9-YFP constructs and GIRK1/2-CFP in the absence of the agonist. FRET efficiency was calculated by acceptor bleaching (see experimental procedures). Fluorescent intensities of YFP and CFP in each combination under TIRF illumination were measured and are summarized in S2 Table. (D) Correlation between ΔI\textsubscript{max} and FRET efficiency. ΔI\textsubscript{max} is plotted as a function of FRET efficiency for each MC9-YFP construct (open circles). (E) Activation of Gqi5 by
MC9-YFP constructs. Schematic diagram of monitoring of the activation of Gqi5 is shown in the upper panel. Under TIRF illumination, application of oxo-M decreased the intensity of CFP tethered at the PH domain ($I_{\text{CFP-PH}}$), as shown in lower traces in the absence of PTX treatment (open circles). The inhibitory effects of PTX (filled circles) are summarized in Table 3. Summary of agonist-induced decrease in $I_{\text{CFP-PH}}$ ($\Delta I_{\text{CFP-PH}}$). Circles represent $\Delta I_{\text{CFP-PH}}$ upon application of oxo-M (10 μM). Numbers of experiments are indicated in parentheses. *0.01 < p ≤ 0.05, **0.001 < p ≤ 0.01, n.s.: p > 0.05.

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important roles in co-localization with the GIRK channel as well as channel activation, while Val and Thr residues at the C-end are required for Gq signaling by $\alpha_{qi5}$ activation.

Coupling properties of MC9B and MC9-VT/AA

The oxo-M concentration dependence of MC9B-YFP and MC9-VT/AA-YFP was then investigated. Examination of the concentration-$\Delta I_{\text{CFP-PH}}$ relationship (Fig 7A) revealed that EC$_{50}$ did not differ between MC9 and MC9B. In the case of VT/AA mutant, 100 μM oxo-M was not the saturating concentration which made the estimation of EC$_{50}$ impossible and support that VT/AA mutations impaired the $\alpha_{qi5}$ activation. The concentration-$\Delta I_{\text{max}}$ of MC9-VT/AA-YFP showed that EC$_{50}$ of the mutant was 5-fold larger than that of MC9-YFP (Fig 7B). The lower affinity may represent impaired Gi/o activation. In the case of MC9B, the EC$_{50}$ was 2-fold larger than that of MC9 (Fig 7B filled circles). These results indicate that the coupling efficacy between MC9B and the GIRK channel was reduced. As the speed of the GIRK channel activation is another index of signaling efficacy, we analyzed the activation speed by using a motor-driven fast-perfusion system to control agonist application. The time to reach the half-maximal current (t$_{1/2}$) upon activation of MC9B was longer than that of MC9 (Fig 7C), consistent with the results showing that GIRK and MC9B are distant from each other [17]. Taken together, the N- and C-terminal ends of M$_2$R-i3 were shown to have different roles in receptor-GIRK signaling.

Discussion

We showed that M$_2$R-i3 enables the chimeric MC9 to locate adjacent to $\alpha_{qi1}$ and the GIRK channel, that Gq-coupled M$_1$R activates the GIRK channel when it is in proximity to the

Table 3. Inhibitory effects of PTX treatment on the activation of Gqi5 or GIRK channel currents by MC9-YFP constructs.

| GIRK channel activation | $\Delta I_{\text{max}}$ (pA/pF) PTX (-) | $\Delta I_{\text{max}}$ (pA/pF) PTX (+) |
|-------------------------|-------------------------------------|-------------------------------------|
| MC9-YFP                 | 101.9 ± 18.9 (7)                    | 1.3 ± 0.9 (3) ***                   |
| MC9A-YFP                | 77.0 ± 11.5 (8)                     | 0.1 ± 0.6 (3) ***                   |
| MC9B-YFP                | 47.4 ± 9.1 (8)                      | 2.5 ± 1.7 (3) ***                   |
| MC9-YFP-VT/AA           | 71.5 ± 14.0 (7)                     | 1.7 ± 0.6 (3) ***                   |
| Gqi5 activation         | $\Delta I_{\text{CFP-PH}}$ (%) PTX (-) | $\Delta I_{\text{CFP-PH}}$ (%) PTX (+) |
| MC9-YFP                 | 20.8 ± 3.8 (10)                     | 3.6 ± 1.0 (6) ***                   |
| MC9A-YFP                | 24.1 ± 3.3 (10)                     | 5.9 ± 1.0 (4) ***                   |
| MC9B-YFP                | 22.9 ± 4.0 (11)                     | 5.5 ± 1.2 (5) **                    |
| MC9-YFP-VT/AA           | 8.2 ± 1.2 (10)                      | 3.7 ± 0.3 (7) **                    |

Activation of GIRK channel was evaluated as the amplitude of the agonist-induced increase in the inward current density ($\Delta I_{\text{max}}$) at the holding potential of -80 mV. Activation of Gqi5 was evaluated as the decrease in the intensity of CFP-PH ($\Delta I_{\text{CFP-PH}}$) under TIRF illumination. Numbers of cells are indicated in parentheses.

***p ≤ 0.001,

**0.001 < p ≤ 0.01

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Fig 7. Properties of MC9B and MC9-VT/AA receptors. (A) Oxo-M concentration-ΔICFP-PH relationships. I_{CFP-PH} was decreased upon application of various concentrations of oxo-M in cells transfected with MC9-YFP constructs. ΔICFP-PH is plotted as a function of oxo-M concentration (MC9-YFP, open circles; MC9B-YFP, filled circles; MC9-VT/AA-YFP, diamonds). EC_{50} value is 0.82 ± 0.20 μM (n = 6) for MC9-YFP and 0.99 ± 0.32 μM (n = 5) for MC9B-YFP (p > 0.05). EC_{50} was not calculated for MC9-VT/AA-YFP because of the small changes of I_{CFP-PH}. (B) Oxo-M concentration-ΔI_{max} relationships. The amplitude of the GIRK channel current density (ΔI_{max}) induced by various concentrations of oxo-M is plotted as a function of oxo-M concentration (symbols are indicated in A). EC_{50} value is 0.22 ± 0.06 μM (n = 4) for MC9-YFP, 0.59 ± 0.10 μM (n = 5) for MC9B-YFP (p > 0.05, vs MC9-YFP) and 1.16 ± 0.13 μM (n = 5) for MC9-VT/AA-YFP.
channel, and that the distance between the receptor and GIRK channel is a key determinant in effective channel activation.

Interaction between Gi/o-coupled muscarinic receptors, GIRK and Gi/o

Localization of MC9 or M₂R in proximity to the GIRK channel was shown by FRET analysis and experiments using tandem constructs (Figs 2–5). These results were consistent with those of the previous FRET study of metabotropic receptor GABAβR, Gi/o, and GIRK [10]. As suggested for other Gi/o-Rs [9–11], MC9 and M₂R are likely to be pre-coupled with Gi/o (Fig 2B). Because Gαi/o binds to the GIRK channel either in GTP-bound form or GDP-bound form [5–8, 30], the Gi/o-GIRK coupling is thought to play important roles in the ternary complex formation. In fact, replacement of the helical domain of Gαq with that of Gαi was reported to enable the chimeric G protein to interact with the GIRK channel and the Gq-coupled M₁R to activate the channel in cells treated with PTX [30]. Therefore, M₂R and MC9 were suggested to localize near the GIRK channel by pre-coupling with Gi/o. In the case of MC9 chimeric mutants, MC9B showed significant impairment of the activation of and co-localization with the GIRK channel (Fig 6). Although it was not statistically significant in MC9B, the co-localization with Gαi1 was attenuated (S3 Table). The lack of statistical significance (MC9B-YFP vs MC9-YFP, S3 Table) was possibly due to the small FRET efficiency between Gαi1-CFP and YFP tagged constructs relative to the background level. We think that the proximal N-terminal residues of M₂R-i3 is a key region for the pre-coupling of M₂R and MC9 with Gi/o. However, another possibility that the proximal N-terminal residues may play some roles in direct interaction with the GIRK channel cannot be excluded.

FRET efficiency between MC9, Gi/o, and the GIRK channel was not changed by receptor activation (Figs 2B and 4B). These results were different from those of previous FRET studies, in which agonist-induced increases in the FRET efficiency was shown between Gq-Rs and Gαq [25, 26] or between adenosine receptor A₁R and Gial [31]. Similar FRET increases have been reported between adrenergic receptors (α₂A-AR and β₁-AR) and G protein [32, 33]. As revealed in a recent single-molecule imaging study of G proteins and α₂A-AR or β₁-AR, receptors and G proteins dynamically associate with each other [34]. These reports supported the notion that the activated receptors associate with and activate G protein, which is detected as agonist-induced increases in FRET between receptor and G protein. In contrast, agonist-induced FRET increases were not detected in several Gi/o-Rs, which were suggested to pre-couple with Gi/o [9–11]. These reports are consistent with the results showing that pre-coupling is not changed by receptor activation. Interestingly, an agonist-induced decrease in FRET was observed between the opioid receptor and Gαi1 [9]. Therefore, the receptor-G protein interaction may differ depending on the receptor type.

Difference in efficacy of GIRK channel activation between M₁R and MC9

M₁R activates the GIRK channel only when it is in proximity of the channel by linking them with a short linker (Fig 3), but ΔI_{max} upon activation of M₁R was lower than that of MC9 (Fig 3D and 3F). As FRET efficiency did not differ between the M₁R and MC9 constructs ligated with the GIRK channel by a short linker (Fig 4A and 4B), the relative distance from M₁R to the channel is likely to be similar to that from MC9. The weak effect of M₁R on the GIRK channel activation may be because of the low abundance of the endogenous Gαq subunit, as
co-expression of G\(\alpha_q\) markedly increased the GIRK channel current amplitude for \(\beta_2\)-AR [16]. We thus co-expressed G\(\alpha_q\) with M\(_2\)-R-YFP-100-GIRK1/2, but co-expression failed to increase the amplitude; \(\Delta_{I_{\text{max}}}\) was 6.4 \(\pm\) 4.5 pA/pF (n = 3). This may be because of the inhibitory effects of G\(\alpha_q\) on the GIRK channel [27, 35–37]. In fact, co-expression of G\(\alpha_q\) with M\(_2\)-R-YFP-100-GIRK1/2 inhibited the effect of M\(_2\)-R on the GIRK channel; \(\Delta_{I_{\text{max}}}\) was 3.6 \(\pm\) 2.5 pA/pF (+G\(\alpha_q\), n = 4) and 78.2 \(\pm\) 15.9 pA/pF (-G\(\alpha_q\), n = 5, \(P = 0.002\)). Therefore, the weak effect of M\(_2\)-R on the GIRK channel activation can be explained by the inhibitory effects of G\(\alpha_q\) on the GIRK channel.

### Localization in proximity is a key factor for activating GIRK channel

In MC9-YFP, the EC\(_{50}\) of GIRK channel activation was 4-fold lower than that of G\(\alpha_{q15}\) activation (Fig 7). This can be interpreted as the functional role of receptor-GIRK channel co-localization; localization of MC9 in the proximity to the GIRK channel enhances the efficacy of receptor-GIRK channel signaling through increasing a probability for MC9 to associate with complex of Gi/o and GIRK channel (Fig 2C G\(\alpha_{i1}\) vs. G\(\alpha_{q}\)). Interestingly, in the case of MC9B which impaired co-localization with the GIRK channel (Fig 6C), the difference of EC\(_{50}\) between \(\Delta_{I_{\text{CFP-PH}}}\) and \(\Delta_{I_{\text{max}}}\) was attenuated. The EC\(_{50}\) of GIRK channel activation was 2-fold lower than that of G\(\alpha_{q15}\) activation (Fig 7A and 7B), which may be results of the decreases in a probability for MC9B to associate with Gi/o-GIRK channel complex. In contrast, mutations at the C-terminal end of M\(_2\)-R-i3 (VT/AA mutation) impaired the activation of G\(\alpha_{q15}\) (Figs 6F and 7A), consistently with previous reports in which VT/AA mutation of M\(_2\)-R disrupted the Gi/o activation [27, 28]. As for the GIRK channel activation, the double mutation did not abolish \(\Delta_{I_{\text{max}}}\) but shifted the concentration-\(\Delta_{I_{\text{max}}}\) relationship rightwards (Fig 7B). As co-localization with the GIRK channel was not affected (Fig 6C), impairment of Gi/o activation in the double mutant may be compensated by co-localization, which enhances receptor-GIRK signaling.

In conclusion, we showed that the Gi/o-coupled muscarinic receptors co-localize with the GIRK channel and G\(\alpha_{i1}\), which is mediated by at least 13 a.a. residues at the N-terminal end of M\(_2\)-R-i3, and that co-localization is another determinant of the efficacy of channel activation.

### Supporting information

S1 Methods. Sequences of the glycine rich junctional amino acid residues.
(DOCX)

S1 Fig. Activation speed of GIRK channel induced by M\(_2\)-R and MC9.
(DOCX)

S1 Table. Fluorescent intensities of FPs fused at receptors, G\(\alpha_{i1}\) or GIRK1/2 and the FRET efficiency under TIRF illumination.
(DOCX)

S2 Table. FRET efficiency between GIRK1/2 -CFP and MC9-YFP constructs under the TIRF illumination.
(DOCX)

S3 Table. Fluorescent intensities of G\(\alpha_{i1}\)-CFP and MC9-YFP constructs under the TIRF illumination.
(DOCX)
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