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Permalink
https://escholarship.org/uc/item/5s60273q

Journal
Scientific reports, 7(1)

ISSN
2045-2322

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Publication Date
2017-07-21

DOI
10.1038/s41598-017-05128-z

Peer reviewed
Structural determinants at the M2 muscarinic receptor modulate the RGS4-GIRK response to pilocarpine by impairment of the receptor voltage sensitivity

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Membrane potential controls the response of the M2 muscarinic receptor to its ligands. Membrane hyperpolarization increases response to the full agonist acetylcholine (ACh) while decreasing response to the partial agonist pilocarpine. We previously have demonstrated that the regulator of G-protein signaling (RGS) 4 protein discriminates between the voltage-dependent responses of ACh and pilocarpine; however, the underlying mechanism remains unclear. Here we show that RGS4 is involved in the voltage-dependent behavior of the M2 muscarinic receptor-mediated signaling in response to pilocarpine. Additionally we revealed structural determinants on the M2 muscarinic receptor underlying the voltage-dependent response. By electrophysiological recording in Xenopus oocytes expressing M2 muscarinic receptor and G-protein-gated inwardly rectifying K+ channels, we quantified voltage-dependent desensitization of pilocarpine-induced current in the presence or absence of RGS4. Hyperpolarization-induced desensitization of the current required for RGS4, also depended on pilocarpine concentration. Mutations of charged residues in the aspartic acid-arginine-tyrosine motif of the M2 muscarinic receptor, but not intracellular loop 3, significantly impaired the voltage-dependence of RGS4 function. Thus, our results demonstrated that voltage-dependence of RGS4 modulation is derived from the M2 muscarinic receptor. These results provide novel insights into how membrane potential impacts G-protein signaling by modulating GPCR communication with downstream effectors.
term ‘desensitization’ does not imply an insensitivity of the GIRK channel itself. 

We previously revealed that the regulator of G-protein signaling (RGS) 4 protein modulates the cellular efficacy of pilocarpine. RGS proteins are known to negatively regulate G-protein signaling by accelerating GTP hydrolysis to GDP by G, subunit and thereby terminating the G-protein signal. Pilocarpine promotes the RGS4-mediated inhibition of M2 muscarinic receptor-activated G-protein signaling, leading to a smaller G-protein-gated inwardly rectifying K+ (GIRK) current than that of ACh. Importantly, membrane potential controls this phenomenon. However, how RGS4 is involved in the voltage-dependent behavior of M2 muscarinic receptor-activated signaling remains unclear.

Since RGS4 is not a membrane protein, RGS4 seems unlikely to function as a voltage sensor. This suggests the voltage sensitivity of RGS4 is derived from its coupled membrane protein, M2 muscarinic receptor. If so, the M2 muscarinic receptor would act as a voltage sensor not only toward its agonists but also toward the RGS4. The DRY motif and the intracellular loop 3 of the M2 muscarinic receptor are candidates for the structural determinants which contribute to the voltage-dependence of RGS4.

We next characterized the voltage-dependence of RGS4 modulation on M2 muscarinic receptor-activated signaling and revealed which receptor motif contributes to the underlying mechanism. With electrophysiologic recordings from Xenopus oocytes, we demonstrate that RGS4 triggers a voltage-dependent current decay of pilocarpine-induced GIRK current. By structure-function analysis of the DRY motif and the intracellular loop 3 of M2 muscarinic receptor using mutagenesis methods, we observed that mutations of charged residues in the DRY motif (D1203.49 and R1213.50), instead of intracellular loop 3, significantly impaired the voltage-dependence of RGS4 modulation. The results demonstrate a novel mechanism that membrane potential modulates RGS4 function through the voltage-sensitive M2 muscarinic receptor.

**Results**

**RGS4 modulation of M2 muscarinic receptor-mediated GIRK currents by pilocarpine is voltage-dependent.** To understand the effects of membrane potential on RGS4 function, we recorded the M2 muscarinic receptor-activated GIRK currents in rat atrial myocytes and Xenopus oocytes heterologously expressing the M2 muscarinic receptor, a cardiac-type GIRK channel (Kir3.1/Kir3.4 heterotetramer) and RGS4.

In atrial myocytes (Fig. 1a,b) and oocytes expressing RGS4 (Fig. 1c,d), pilocarpine-induced GIRK currents during membrane hyperpolarization reached a peak (Ip) and then decreased to steady state by pulse-end (Ip). This current decay suggests a desensitization of GIRK currents to pilocarpine at hyperpolarized potential in the presence of RGS4. The tau of current decay in the presence of 100 μM pilocarpine at −140 mV in RGS4-expressing oocytes was 0.47 ± 0.02 s (n = 6). This phenomenon was found to be due to the cessation of G-protein activation by modulation of GTP hydrolysis via RGS4 in our previous study using GTPS, a non-hydrolyzable analog of GTP. The current decay during hyperpolarization was not observed in the absence of RGS4 (Fig. 1e,f), suggesting that RGS4 is a crucial mediator in the hyperpolarization-induced desensitization of the GIRK channel. This suggests that the mechanism of desensitization is an RGS4-mediated reduction in active G-proteins. Note that the term ‘desensitization’ does not imply an insensitivity of the GIRK channel itself.

In oocytes expressing RGS4, with 100 μM pilocarpine, Ip during −140 mV pulses was smaller than Ip at hyperpolarized potentials (Fig. 1c). However, the current–voltage (I–V) curve of Ip was linear at hyperpolarized potentials, indicating GIRK currents are not yet desensitized immediately after hyperpolarization (Fig. 1d). The I–V curve of Ip showed a different shape from that of Ip. The differences in current amplitude between Ip and Ip indicate that desensitization increases as the membrane potential become more hyperpolarized (Fig. 1d). This phenomenon was not observed in the absence of RGS4 (Fig. 1e,f). The results demonstrate that hyperpolarization acts through RGS4 to desensitize GIRK channels to pilocarpine.

We next normalized the GIRK currents induced by different concentrations of pilocarpine with the same amplitude of Ip and then compared the level of Ip in the presence and absence of RGS4 (Fig. 2a,b). With RGS4, we observed that a higher concentration (100 μM) of pilocarpine had more impact on the normalized Ip level than a lower concentration (1 μM). The ratio Ip/I0 decreased at high concentrations of pilocarpine and hyperpolarized potentials in the presence of RGS4 (Fig. 2c), suggesting that RGS4 inhibits GIRK currents in a pilocarpine concentration-dependent manner. We then fitted the concentration-response curve of Ip and Ip at −100 mV in the presence of RGS4 with the Hill equation (Fig. 2d). There was no considerable difference in the −logEC50 of Ip and Ip (−logEC50 was 6.23 ± 0.12 M for Ip and 6.11 ± 0.02 M for Ip), indicating that the receptor affinity of pilocarpine was not substantially changed by the presence of RGS4 modulation. We observed the maximal inhibitory effect of RGS4 modulation in the presence of saturated concentration of pilocarpine (100 μM). These results suggest that RGS4–GIRK response is voltage-dependent, and this depends on pilocarpine binding to the M2 muscarinic receptor.

**Membrane potential rapidly modulates RGS4-mediated desensitization of M2 muscarinic receptor-GIRK signaling.** We next characterized the kinetics of the voltage-dependent response. The time course of GIRK current recovery from desensitization was examined with two hyperpolarizing pulses protocol (−100 mV for 2 s) which were separated by an interpulse (0 mV) of different durations (0.1, 0.3, 0.5, 0.7 and 0.9 s) (Fig. 3). Compared with the first hyperpolarizing pulse in the presence of RGS4, the pilocarpine-induced current showed a smaller Ip at the second hyperpolarizing pulse when the interpulse duration was 0.1 s (Fig. 3a). Prolonging the duration of interpulse to 0.5 s recovered the current amplitude of Ip. The tau of Ip recovery was
0.16 ± 0.01 s (n = 6) by exponential curve fitting. We did not observe hyperpolarization-induced desensitization in the absence of RGS4 (Fig. 3b). The results demonstrate that the M2 muscarinic receptor-GIRK signaling recovers from hyperpolarization-induced RGS4-mediated desensitization very rapidly.

Mutations in the DRY motif of the M2 muscarinic receptor, instead of intracellular loop3, impaired RGS4 modulation of GIRK current mediated by pilocarpine. To reveal the mechanism which underlies the voltage-dependence of RGS4 modulation, we focused on the voltage-sensitive M2 muscarinic receptor. We examined several charged amino acid residues of the M2 muscarinic receptor which are highly conserved in the rhodopsin-like GPCR family and may act as a voltage-sensor (Fig. 4a).

We first mutated charged residues D692.50 and D1033.32 (Fig. 4a,b), are in the transmembrane domains (TMs) of M2 muscarinic receptor and are relevant to voltage sensing and receptor activation4, 27, 28. Consistent with previous studies, we found that mutation of these residues to alanine (D69A and D103A) or asparagine (D69N and D103N) abolished ACh- and pilocarpine-induced GIRK currents in oocytes (Supplementary Fig. S1). This change may be due to the impairment of ligand binding in the mutation of D1033.32 or the reduced protein surface expression in the mutation of D692.50 4, 27.

We next mutated charged residues D120 3.49 and R121 3.50 located in the DRY motif of the M2 muscarinic receptor (Fig. 4a,b). Mutations that neutralized the charges of D120 3.49 (D120N) or R121 3.50 (R121N) singly, or both residues together (D120N/R121N), decreased the amplitude difference between the $I_p$ and $I_s$ of pilocarpine-evoked GIRK currents at −100 mV (Fig. 4d–f), as compared with that of wild-type (WT) M2 muscarinic receptor (Fig. 4c). This change may be due to the impairment of ligand binding in the mutation of D1033.32 or the reduced protein surface expression in the mutation of D692.50. 27.

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Such current decay during hyperpolarization was not seen in the absence of RGS4 (Fig. 4c–f, right panel). The ratios $I_s/I_p$ of the M2 muscarinic receptor mutants were significantly elevated compared with WT M2 muscarinic receptor (0.83 ± 0.03 for D120N; 0.91 ± 0.02 for R121N; 0.79 ± 0.01 for D120N/R121N; 0.53 ± 0.04 for WT, $P \leq 0.001$ for each mutant when compared with WT) (Fig. 4g). This suggests that neutralization of
D1203.49 or R1213.50 diminishes the hyperpolarization-induced desensitization of GIRK current by RGS4. To confirm whether mutations of D1203.49 and R1213.50 affect the pilocarpine-induced current, we calculated the concentration-response curves of these mutants. The concentration-response curves of D120N for pilocarpine- (Fig. 4h and Supplementary Fig. S2) and ACh-induced GIRK currents (Supplementary Fig. S3) were similar with WT, while the concentration-response curves of R121N for both pilocarpine and ACh were a rightward shift from that of WT. The 100 µM concentration of pilocarpine saturated current in all DRY mutants we tested. Although, as we shown in Fig. 2, the pilocarpine-bound M2 muscarinic receptor enhances RGS4-mediated desensitization of GIRK response at hyperpolarized potentials, the DRY motif mutants impaired this function. Therefore, the DRY motif of the M2 muscarinic receptor is an essential structural determinant of the voltage sensitivity of RGS4.

Since the intracellular loop 3 of the M2 muscarinic receptor is also involved in the voltage-dependence of ACh binding8, we examine the roles of the intracellular loop 3 in the voltage-dependence of RGS4 modulation of pilocarpine-induced GIRK current. Interestingly, we found that, in our oocyte expression system, the M2 muscarinic receptor ELAAL mutant retained voltage-dependent response of pilocarpine-induced GIRK currents in the presence of RGS4 (Fig. 5a). The I/Ip of ELAAL mutant (0.55 ± 0.05, n = 6) was similar to that of WT M2 muscarinic receptor (0.53 ± 0.04, n = 6) (Fig. 5b), suggesting that the N-terminal region of intracellular loop 3 is not essential for the voltage-dependence of RGS4 modulation. The current of ELAAL mutant fully recovered Ip.
from the desensitization when the interpulse duration was 0.9 s, longer than with the WT M2 muscarinic receptor (Fig. 5c). The tau for $I_p$ recovery was 0.16 ± 0.01 s for WT and 0.34 ± 0.01 s for ELAAL, respectively (n = 6 for each, $P < 0.001$ in Fig. 5d). The slow $I_p$ recovery of ELAAL mutant from the hyperpolarization-induced desensitization suggests that this mutant takes a longer time to recover the GIRK current from RGS4 modulation as compared with WT. The $I_s/I_p$ was, however, not influenced by mutation of KKDKK to ELAAL (Fig. 5b).

Concentration-response curves of ELAAL mutant for pilocarpine (Fig. 5e and Supplementary Fig. S2) and ACh (Supplementary Fig. S3) were slightly rightward shifted as compared with that of WT. Taken together, the results indicate that the N-terminal region of intracellular loop 3 is not essential for voltage-dependence of RGS4.

**Discussion**

In the present study, we find that membrane potential modulates RGS4 function through the voltage-sensitive M2 muscarinic receptor. Hyperpolarization enhanced the RGS4-mediated inhibition of pilocarpine-induced GIRK currents in both rat atrial myocytes and *Xenopus* oocytes (Fig. 1). This phenomenon is dependent on the pilocarpine concentration (Figs 1 and 2). A previous study suggested that hyperpolarization induces a decrease in M2 muscarinic receptor affinity toward pilocarpine in cat atrial myocytes5. However, in the absence of RGS4, pilocarpine-induced GIRK current only showed a negligible decrease in current amplitude during hyperpolarization. Therefore, the hyperpolarization-induced desensitization of GIRK channel appears to be due to the increase in the capability of RGS4 modulation, not a decrease in ligand affinity.

Analyses of gating charge-movement and reporter fluorescent signal associated with M2 muscarinic receptor have suggested that a voltage-induced conformational change in M2 muscarinic receptor occurs very rapidly, within 0.01 s4,12. This would precede the voltage-induced changes in RGS4 function. Our results showed that RGS4-mediated desensitization of GIRK currents recovers rapidly (tau of recovery time was about 0.16 s) at
holding membrane potential (Fig. 3), and this event would follow the receptor conformational change mediated by voltage change.

We showed that D1203.49 or R1213.50 in the DRY motif of M2 muscarinic receptor contribute to the voltage-dependence of RGS4 modulation of GIRK current. These charged residues are part of the underlying mechanism that enhances the capability of RGS4 modulation at hyperpolarized potentials (Fig. 4). This result indicates that the voltage-dependence of RGS4 is derived from the M2 muscarinic receptor. One possible mode of action for D1203.49 and R1213.50 is that these residues act as part of voltage sensors in the receptor for sensing membrane potential. Another possible mode is that these residues are required for its interaction with RGS4 because DRY motif is located on the cytosolic side of the plasma membrane. However, it is most likely that these charged residues support the pathway that mediates voltage-dependent conformational change of the M2 muscarinic receptor because these charged residues form a part of charge-charge interaction which stabilizes the receptor conformation14. These charged residues respectively interact with other residues located in the TM2 and TM6 as well14, 16, 27. Once the negative charge of D1203.49 is neutralized, the converse R1213.50 is probably free to move to find another ionic partner29 and vice versa. Therefore, neutralization of both D1203.49 and R1213.50 or only one of them disturbs the balance of charge-charge interaction within receptor and thus altering the voltage-sensitivity.

Other charged residues of the M2 muscarinic receptor are relevant to the voltage sensing, including D692.50 and D1033.32. However, mutations of D692.50 or D1033.32 appear to reduce both receptor expression and agonist

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**Figure 5.** Effects of mutations in the intracellular loop 3 of M2 muscarinic receptor on the voltage-dependent response of GIRK currents. (a) Pilocarpine (100 µM)-induced GIRK currents in oocytes expressing M2 muscarinic receptor mutant ELAAL with (left panels) and without (right panels) RGS4 were recorded at −100 mV as shown above. (b) The ratio of $I_\text{i}$ to $I_\text{p}$ ($I_\text{i}/I_\text{p}$) of M2 muscarinic receptor WT and ELAAL at −100 mV in the presence of pilocarpine (100 µM). (c) Recoveries of pilocarpine (100 µM)-induced GIRK currents from hyperpolarization-mediated desensitization of channels in oocytes expressing WT and ELAAL M2 muscarinic receptors were recorded with a protocol shown above the current traces. (d) The time constant (tau) of $I_\text{i}$ recovery times in c were calculated with exponential curve fitting. (e) Concentration-response curves of pilocarpine-induced GIRK currents were fitted with the Hill equation. The maximal value of pilocarpine-induced GIRK current was normalized to 1. The −logEC50 was 6.51 ± 0.02 for WT; 6.15 ± 0.02 for ELAAL. Data in b, d and e are means ± s.e.m., n = 6. ** Indicates a statistically significant difference (P < 0.001).
Concentration-response curve were fitted by Hill equation with SigmaPlot 12 (Hulinks).

Resistance of glass electrodes was 0.4–1.5 MΩ in Supplementary Figs S2 and S3. 100 μM pilocarpine-induced currents were obtained by digitally subtracting the baseline currents. The concentration–response curve was measured by sequential application of pilocarpine and ACh in bath solution for 30–60 s at each concentration when current amplitude reached a stable level as shown in Supplementary Figs S2 and S3. 100 μM Pilocarpine-induced current could be washed out within 1–2 min. Concentration-response curve were fitted by Hill equation with SigmaPlot 12 (Hulinks).
Data statistics. Data statistical analysis was performed with SigmaPlot 12 (Hulinks). Results are shown as mean ± s.e.m. from n cells. Statistical differences between each group were evaluated by Tukey’s test. Values of P < 0.05 were judged statistically significant. *, ** and *** indicate values of P < 0.05, 0.01 and 0.001, respectively.

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Acknowledgements
We are grateful to Dr. Jon Sack (University of California, Davis, USA) for critical reading of this manuscript. This study was supported by the Hiroshi and Aya Irisawa Memorial Promotion Award for Young Physiologists (to K.F.) from the Physiological Society of Japan, Grants-in-Aid for the Scientific Research on Innovative Areas 22136002 (to Y.K.), 15H01404 (to K.F.), and the Scientific Research (C) 15K08231 (to K.F.) and from the Ministry of Education, Science, Sports and Culture of Japan, and the Japan Society for the Promotion of Science.

Author Contributions
I.-S.C. performed the experiments, analyzed the data and wrote the manuscript. I.-S.C., K.F. and Y.K. designed the study, interpreted the results and revised the manuscript. All authors approved the manuscript.

Additional Information
Supplementary information accompanies this paper at doi:10.1038/s41598-017-05128-z

Competing Interests: The authors declare that they have no competing interests.

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