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RGS4 regulates partial agonism of the M2 muscarinic receptor-activated K^+ currents

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Key points

- Partial agonists produce a smaller response than full agonists even at 100% receptor occupancy.
- In G-protein-coupled receptor signalling, the submaximal efficacy of partial agonists is determined by the conformational change of the agonist–receptor complex, which reduces effector activation. However, it remains unclear whether the regulator of G-protein signalling (RGS) proteins contribute to the partial agonism.
- By analysing the M2 muscarinic receptor (M2R)-activated G-protein-gated K^+ inwardly rectifying (K_G) currents in a *Xenopus* oocyte expression system, pilocarpine acted as a partial agonist in the presence of RGS4, as it did in rat atrial myocytes, while it acted like the full agonist ACh in the absence of RGS4.
- Functional couplings within the agonist–M2R complex/G-protein/RGS4 system controlled the relative efficacy of the agonists.
- Our findings help us to understand the molecular components and mechanism underlying the partial agonism of M2R-mediated physiological responses.

Abstract Partial agonists are used clinically to avoid overstimulation of receptor-mediated signalling, as they produce a submaximal response even at 100% receptor occupancy. The submaximal efficacy of partial agonists is due to conformational change of the agonist–receptor complex, which reduces effector activation. In addition to signalling activators, several regulators help control intracellular signal transductions. However, it remains unclear whether these signalling regulators contribute to partial agonism. Here we show that regulator of G-protein signalling (RGS) 4 is a determinant for partial agonism of the M2 muscarinic receptor (M2R). In rat atrial myocytes, pilocarpine evoked smaller G-protein-gated K^+ inwardly rectifying (K_G) currents than those evoked by ACh. In a *Xenopus* oocyte expression system, pilocarpine acted as a partial agonist in the presence of RGS4 as it did in atrial myocytes, while it acted like a full agonist in the absence of RGS4. Functional couplings within the agonist–receptor complex/G-protein/RGS4 system controlled the efficacy of pilocarpine relative to ACh. The pilocarpine–M2R complex suppressed G-protein-mediated activation of K_G currents via RGS4. Our results demonstrate that partial agonism of M2R is regulated by the RGS4-mediated inhibition of G-protein signalling. This finding helps us to understand the molecular components and mechanism underlying the partial agonism of M2R-mediated physiological responses.

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Abbreviations 2-BP, 2-bromopalmitate; cRNA, complementary RNA; D2R, D2 dopamine receptor; GAPs, GTPase-activating proteins; GEF, guanine nucleotide exchange factor; GPCR, G-protein-coupled receptor; K_G, G-protein-gated inwardly rectifying K^+; M2R, M2 muscarinic receptor; PTX, pertussis toxin; RGS, regulator of G-protein signalling.

Introduction

Partial agonists are agonists that produce smaller responses than full agonists even at 100% receptor occupancy. Therefore, they can also act as a competitive antagonist in the presence of a full agonist by competing for receptor occupancy with higher affinity (Zahn *et al.* 2002; Zhu, 2005). Clinically, partial agonists are used to avoid overstimulation of receptor-mediated signalling, thereby decreasing side effects, and can be used in addiction treatments, such as μ-opioid partial agonist buprenorphine (Jasinski *et al.* 1978; Zhu, 2005).

In G-protein-coupled receptor (GPCRs) signalling, effector activation is dynamically and oppositely tuned by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs promote the formation of the active GTP-bound state of G_α subunits, while GAPs accelerate GTP hydrolysis in the G_α subunits. Previous studies suggested the partial agonism is due to the lower efficiencies of GEFs when partial agonists bind to specific receptors (Tota & Schimerlik, 1990; Seifert *et al.* 2001). Partial agonists induce a different conformational change of the receptor from that induced by full agonists (Seifert *et al.* 2001; Lape *et al.* 2008). The binding of partial agonists stabilizes the receptor conformation and decreases the affinity of the ligand–receptor complex for G_α subunits, thereby reducing the ability to activate the GEFs relative to full agonists (Tota & Schimerlik, 1990; Seifert *et al.* 2001).

Although the role of GEFs in partial agonism has been presented in many studies as described above, the role of GAPs in partial agonism has not been discussed. The regulator of G-protein signalling (RGS) proteins are a family of well-known GAPs that negatively regulate various G-protein-mediated signal pathways (Hepler, 1999; Ross & Wilkie, 2000). The physiological roles of RGS proteins, especially RGS4, have been established in studies of mammalian cardiomyocytes (Cifelli *et al.* 2008; Hibino *et al.* 2010). RGS4 is involved in parasympathetic signalling and heart rate control by modulating physiological responses to the natural full agonist ACh via M2R and G-protein-gated inwardly rectifying K^+ (K_G) channels (Fujita *et al.* 2000; Inanobe *et al.* 2001; Ishii *et al.* 2001; Cifelli *et al.* 2008). It is conceivable that RGS-mediated negative regulation of G-protein signals may also contribute to the partial agonism of muscarinic ligands.

In the present study, we investigated the effect of RGS4 on the M2R agonist-evoked K_G currents in rat atrial myocytes and *Xenopus* oocytes that expressed M2Rs and cardiac-type K_G channels (Kir3.1/Kir3.4) with or without RGS4. Relative to the M2R full agonist ACh, the M2R partial agonist pilocarpine produces submaximal responses in several muscarinic signalling pathways (Bymaster *et al.* 1999; Zahn *et al.* 2002). We found that deficient RGS4 function elevated the relative efficacy of pilocarpine to ACh on K_G currents. Disturbing the interaction within the agonist–receptor complex/G-protein/RGS4 system impaired the RGS4-mediated regulation and eliminated the divergent efficacy between pilocarpine and ACh. By co-expression of M2Rs and D2 dopamine receptors (D2Rs), we found that pilocarpine conspicuously suppressed the dopamine-evoked K_G currents via M2Rs and RGS4. This indicates that the binding of pilocarpine to M2Rs promotes the RGS4-mediated negative regulation of G-protein signalling. Our finding suggests that the RGS-mediated inhibition of G-protein signals is one of the components that underlies the partial agonism of M2R-activated K_G currents.

Methods

Ethical approval

All animal experiments were performed in accordance with the guidelines for the use of laboratory animals of Osaka University Graduate School of Medicine. The experimental protocol, including the use of a combination anaesthetic, was approved by the Institutional Animal Care and Use Committee and the Animal Experiments Committee of Osaka University. Adult male Wistar rats (200–300 g) were adequately anaesthetized by a combination anaesthetic (I.P., 0.3 mg kg^{-1} of medetomidine, 4.0 mg kg^{-1} of midazolam, and 5.0 mg kg^{-1} of butorphanol; Kawai *et al.* 2011) after injecting heparin (I.P., 1000 U kg^{-1}).

Preparation of isolated atrial myocytes

Single atrial myocytes were enzymatically isolated from hearts that were removed from rats as described previously (Ishii *et al.* 2001). Briefly, a cannula was inserted into the aorta, and the heart was perfused in a retrograde manner through the coronary arteries. The heart was digested by collagenase (Sigma, St. Louis, MO, USA) in nominally Ca^{2+}-free solution at 37°C for 10 min. Dissociated myocytes were maintained in KB solution, which contained (mM): 10 taurine, 10 oxalic acid, 70 glutamic acid, 25 KCl, 10 KH_2PO_4, 0.5 EGTA, 11 glucose, 10 Hepes; pH 7.3 with KOH. Freshly isolated cells were used on the day.

Preparation of *Xenopus* oocytes expressing receptors, channels and RGS proteins

Isolation and maintenance of the oocytes of frogs (*Xenopus laevis*) and injection with complementary RNA (cRNA) were performed as described previously (Inanobe *et al.* 2001). The following constructs were used in this study: porcine M2R, mouse Kir3.1, rat Kir3.4, rat RGS4 and human D2R. Truncated and site-directed mutants of RGS4 were produced as described in Inanobe *et al.* (2001). Oocytes were injected with cRNAs for M2R (80 ng oocyte^{-1}), Kir3.1 (8 ng oocyte^{-1}), Kir3.4 (8 ng oocyte^{-1}), wild-type RGS4 (160 ng oocyte^{-1}) and various RGS4 mutants (160 ng oocyte^{-1}). For co-expression of D2R and M2R, the injected concentrations (ng oocyte^{-1}) were 80 for D2R and 0.08–0.8 for M2R. After injection, oocytes were incubated at 18°C in ND96 solution, which contained (mM): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$ and 5 Hepes; pH 7.6 with NaOH, and supplemented with gentamicin (50 μg ml^{-1}) and sodium pyruvate (2.5 mM). Currents were recorded 3–5 days after the cRNA injection. In some experiments, oocytes were incubated in 2–bromopalmitate (2–BP, 100 μM) for $\geq$2 h before electrophysiological recordings.

Patch-clamp analysis

Membrane currents of rat atrial myocytes were recorded in the whole-cell configuration of the patch-clamp technique by a patch-clamp amplifier (EPC10, HEKA Electronics, Lambrecht, Germany) at room temperature. Data were reproduced, low-pass-filtered at 1 kHz (−3 dB) by an eight-pole Bessel filter, sampled at 5 kHz, and analysed offline with PatchMaster (HEKA Electronics) and FitMaster (HEKA Electronics). The bath solution contained (mM): 115 NaCl, 20 KCl, 1.8 $CaCl_2$, 0.53 $MgCl_2$, 5.5 glucose and 5.5 Hepes; pH 7.4 with NaOH. The pipette solution contained (mM): 150 KCl, 5 EGTA, 1 $MgCl_2$, 3 K_2ATP, 0.1 Na_2GTP and 5 Hepes; pH 7.3 with KOH. The tip resistance of the glass electrodes was 2–5 MΩ when filled with the pipette solution.

Two-electrode voltage clamp

Membrane currents of oocytes were recorded using the two-electrode voltage clamp by a GeneClamp 500 amplifier (Molecular Devices, Sunnyvale, CA, USA) at room temperature. Data were reproduced and analysed with pCLAMP 10 (Molecular Devices) and Clampfit 10.2 (Molecular Devices). The bath solution contained (mM): 90 KCl, 3 $MgCl_2$, 0.15 niflumic acid and 5 Hepes; pH 7.4 with KOH. The tip resistance of the glass electrodes was 0.4–1.5 MΩ when filled with the 3 M KCl pipette solution.

Data analysis

Agonist-induced currents were obtained by digitally subtracting currents recorded under control conditions from those recorded in the presence of agonists. Results are shown as mean values obtained from n cells, and error bars represent SEM. Statistical differences were evaluated by Student's t tests. Significance was indicated by $P < 0.05$.

Chemicals

Chemicals were purchased from Sigma-Aldrich. CCG-50014 was purchased from Calbiochem. CCG-4986 was kindly provided by Dr Yoshiyuki Hari (Osaka University). CCG-50014 and CCG-4986 were dissolved in DMSO and diluted to a final solvent concentration $\leq$1% in pipette solution and bath solution. 2–BP was dissolved in ethanol and diluted to a final solvent concentration $\leq$0.1% in bath solution.

Results

Effects of RGS4 on ACh- and pilocarpine-evoked K_G currents

We recorded the M2R full agonist ACh-evoked K_G currents in rat atrial myocytes and found that 1 μM ACh evoked a maximal K_G current and showed a time-dependent increase in current amplitude upon membrane hyperpolarization (Fig. 1*A*, black trace), which is a characteristic of ACh-evoked K_G currents called 'relaxation' (Noma & Trautwein, 1978; Yamada *et al.* 1998; Fujita *et al.* 2000). We next examined several M2R partial agonists (including pilocarpine, bethanechol and oxotremorine) and found that a clinically used cholinomimetic agent, pilocarpine, gave the smallest K_G currents relative to the full agonist ACh-evoked K_G currents (Fig. 1*A* and Fig. S1*A* and *B* in the online Supporting information). Therefore,

we investigated the mechanism underlying the partial agonism of pilocarpine.

Pilocarpine (100 μM) evoked a smaller saturated K_G current than did ACh in the same atrial myocyte and showed a time-dependent decrease in current amplitude from peak to steady state upon membrane hyperpolarization (Fig. 1*A*, red trace). Applying M2R antagonist atropine or K_G channel blocker tertiapin-Q completely inhibited the pilocarpine-evoked currents (Fig. 1*B* and *C*).

To investigate whether RGS4 influences partial agonism of M2R, we tried to reconstitute the cardiac K_G currents in *Xenopus* oocytes that expressed cardiac-type K_G channels, the Kir3.1/Kir3.4 heteromultimer (Krapivinsky *et al.* 1995), and M2Rs with or without RGS4. The basal receptor-independent K_G currents were not influenced by RGS4 expression. In the presence of RGS4, pilocarpine evoked the submaximal response of steady-state current that resembles the K_G currents in atrial myocytes at −100 mV (Fig. 1*D*). In the absence of RGS4, pilocarpine evoked a larger steady-state current that almost equalled the maximal response of ACh-evoked currents (Fig. 1*E*). We observed a similar time-dependent decrease of pilocarpine-evoked currents and the time-dependent current increase of ACh-evoked currents (Fig. 1*D*) to that of the atrial myocytes shown in Fig. 1*A*. However, such a characteristic difference between pilocarpine- and ACh-evoked currents was not shown in the oocytes lacking RGS4 (Fig. 1*E*). This indicates that RGS4 is essential for reconstituting the cardiac M2R-activated K_G currents. The elevation of steady-state current in the absence of RGS4 suggests RGS4 inhibits M2R-activated K_G currents in response to pilocarpine. We also made similar observations in other M2R partial agonist-evoked K_G currents (Supporting information Fig. S1*C*–*F*).

We tried to examine the involvement of RGS4 in partial agonism in rat atrial myocytes by several small-molecule inhibitors of RGS proteins, such as CCG-4986 and CCG-50014 (Roman *et al.* 2007; Blazer *et al.* 2011). However, these compounds showed no or little effect on ACh- and pilocarpine-evoked K_G currents in both atrial myocytes and oocytes (data not shown), suggesting these compounds could not influence the RGS4 function in modulating K_G currents in our experimental conditions.

Concentration–response relationship of agonist-evoked K_G currents

By applying ACh (0.001–10 μM) or pilocarpine (0.1–100 μM), we observed faster activations and deactivations of K_G currents in the RGS4-expressing oocytes than control oocytes (without RGS4; Fig. 2*A* and *C*). This is consistent with previous findings that RGS4 accelerates the time course of agonist-induced activation and deactivation of K_G currents (Doupnik *et al.* 1997; Fujita *et al.* 2000). According to the concentration–response curve, we found that RGS4 suppressed the efficacy of pilocarpine relative to ACh, with values of 53% (with RGS4, Fig. 2*B*) compared to 94% (without RGS4, Fig. 2*D*) at −60 mV. The $-\log EC_{50}$ of ACh was 7.95 ± 0.13 M with RGS4 and 8.23 ± 0.07 M without RGS4; the $-\log EC_{50}$ of pilocarpine was 6.08 ± 0.08 M with RGS4 and 6.36 ± 0.12 without RGS4. There was

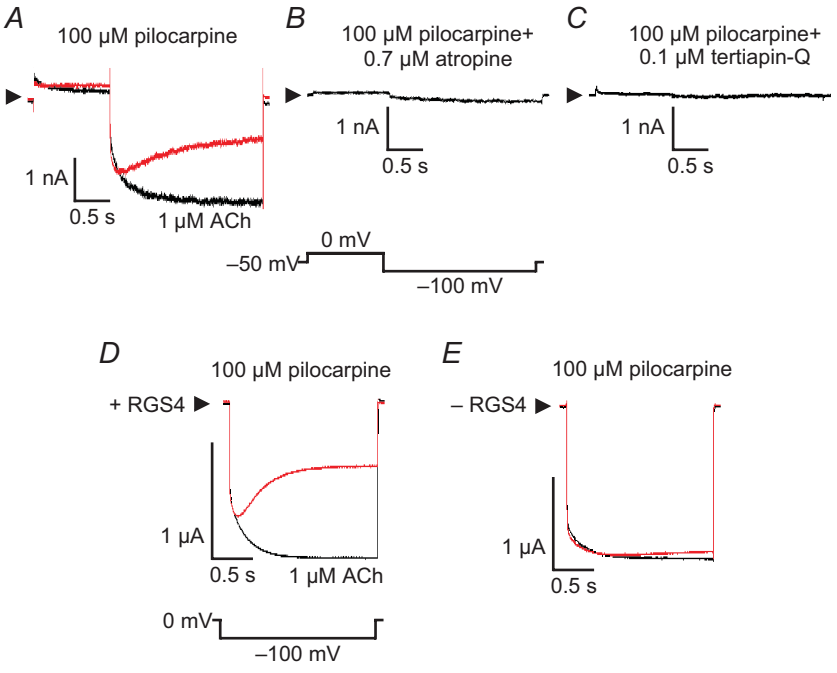

Figure 1. Effects of RSG4 on ACh- and pilocarpine-evoked K_G currents
A, 100 μM pilocarpine-evoked K_G currents (red) relative to 1 μM ACh-evoked K_G currents (black) in the same rat atrial myocyte. Whole-cell currents were recorded with a voltage pulse protocol as showed below the current traces and basal currents were subtracted. Arrowheads indicate the zero current level. Pilocarpine (100 μM)-evoked currents were blocked in the presence of 0.7 μM atropine (*B*) or 0.1 μM tertiapin-Q (*C*) in atrial myocytes. Agonist-evoked currents were recorded in the same oocyte expressing M2Rs and the Kir3.1/Kir3.4 heteromultimer with (*D*) and without (*E*) RGS4 at −100 mV.

no significant difference in the $-\log EC_{50}$ of ACh and pilocarpine regardless of the presence or absence of RGS4. This indicates that the agonist affinity at M2R was not influenced by RGS4 but the relative efficacy of pilocarpine to ACh was elevated in the absence of RGS4. The different efficacy between pilocarpine and ACh was almost eliminated in the deficiency of RGS function, suggesting RGS4-mediated inhibition is essential for partial agonism of M2R-activated K_G currents.

To test the idea in atrial myocytes, we examined the effect of intracellular GTPγS, a non-hydrolysable analogue of GTP. We found that the time-dependent decrease of pilocarpine-evoked K_G currents could be eliminated by applying GTPγS (Supporting information Fig. S2). The difference between the current amplitudes of ACh- and pilocarpine-evoked K_G currents was much smaller in the presence of GTPγS when compared with control conditions (Fig. 1). This suggests RGS4-mediated inhibition of K_G currents may be overcome by the GTPγS-induced irreversible activation of K_G channels.

Voltage dependence of response of pilocarpine compared to ACh

Pilocarpine-evoked K_G currents showed a distinct current change upon the hyperpolarizing pulse compared to the ACh-evoked K_G currents in the presence of RGS4 (Fig. 1*D*). To examine the effect of membrane voltage on partial agonism, we recorded the responses induced by 1 μM ACh and 100 μM pilocarpine in the same oocyte with a step protocol (−20 mV to −100 mV, −20 mV increments, 2 s) and evaluated the relative response of pilocarpine to ACh at several membrane potentials. We found that RSG4-mediated inhibition of K_G currents in response to pilocarpine was enhanced during membrane hyperpolarization (Fig. 3*A*) and therefore the relative K_G currents evoked by pilocarpine to ACh were smaller at the more negative potentials in the presence of RGS4 (Fig. 3*C*). Such voltage dependence in K_G currents evoked by pilocarpine relative to ACh was not observed in the absence of RGS4, because the responses induced by pilocarpine and ACh were about the same at each potential (Fig. 3*B* and *C*). This suggests that the partial agonism of M2R-activated K_G currents is voltage dependent and RGS4 plays a crucial role in the underlying mechanism.

Requirements of RGS4 domain for partial agonism

To clarify how RGS4 influences partial agonism, we investigated the functional structure of RGS4 for partial agonism. RGS proteins contain a conversed region, the RGS domain, which is composed of 120–130 amino acid

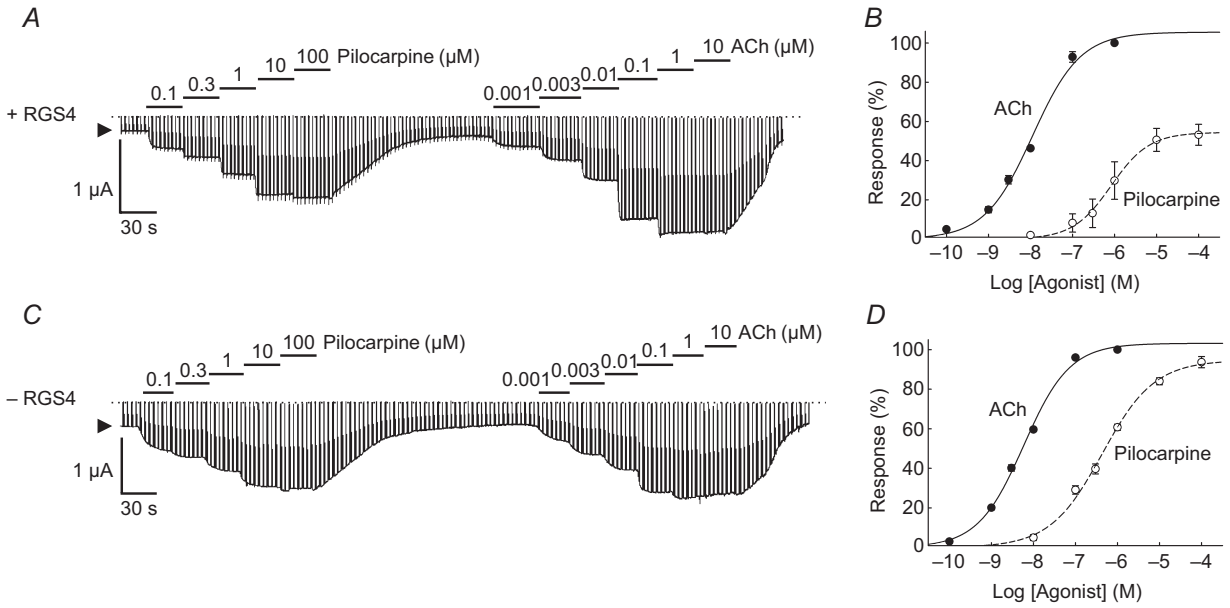

Figure 2. Concentration–response curve of pilocarpine and ACh
Currents were recorded in the presence of pilocarpine (0.1–100 μM) and ACh (0.001–10 μM) with a holding potential of −60 mV in oocytes with (*A*) and without (*C*) expression of RGS4. The bars above each trace indicate the periods and concentrations of application of ACh or pilocarpine. Dashed lines indicate the zero current level and arrowheads indicate the basal current level. Concentration–response curves of pilocarpine and ACh in oocytes with (*B*) or without (*D*) expression of RGS4. The average maximal response of ACh-evoked K_G currents was set as 100%. The vertical axis indicates the relative percentages of response that were induced by each concentration of agonists. Data show the means ± SEM, $n = 6$ at each concentration in both with and without RGS4 groups. All symbols have attached error bars, but some of them are smaller than the symbol.

residues that mediate interaction with pertussis toxin (PTX)-sensitive G_α subunits and the effect of RGS4 on the agonist concentration-dependent relaxation of K_G channels (Inanobe *et al.* 2001). On the other hand, regions outside the RGS domain are divergent and may regulate subcellular localization (Zheng *et al.* 1999). To determine the structural requirements for partial agonism, we examined several truncated RGS4 mutants.

Oocytes expressing wild-type RGS4 showed a partial response to pilocarpine, but absence of RGS4 yielded a response similar to the effect of the full agonist ACh at −60 mV (Fig. 4*Aa*, *b* and *C*). The truncated RGS4 mutants and a point mutation in RGS domain were constructed as illustrated in Fig. 4*B*. The mutant RGS4/51-177 is composed of only the RGS domain. Oocytes expressing RGS4/51-177 showed a similar time-dependent decrease of pilocarpine-evoked currents and the relative response of pilocarpine to ACh (47%) as shown in oocytes expressing wild-type RGS4 (Fig. 4*Af* and *C*). Mutants containing the RGS domain and N-terminus (RGS4/1-177) or RGS domain and C-terminus (RGS4/51-205) also produced a similar relative response (51% and 48%; Fig. 4*Ad*, *e* and *C*). However, the mutants that did not contain the full RGS domain (RGS4/1-50 and RGS4/85-177) showed elevated relative responses (94% and 96%), which are closer to the value observed in the absence of RGS4 (Fig. 4*Ac*, *g* and *C*). Lack of the full RGS domain eliminated the efficacy difference between pilocarpine and ACh, indicating that the RGS domain is essential for RGS4-mediated inhibition to control the partial agonism of M2R-activated K_G currents.

We next examined the effect of a point mutation in the RGS domain (asparagine 128 to histidine, N128H; Fig. 4*B*). The residue N128 is necessary for RGS4 function and controls GAP activity. The RGS4/N128H mutation impairs the effect of RGS4 by interfering in the interaction with G_α subunits (Natochin *et al.* 1998; Srinivasa *et al.* 1998; Inanobe *et al.* 2001). In oocytes expressing RGS4/N128H, pilocarpine evoked an inward current that mildly decreased during hyperpolarization; the relative response (91%) was much higher than the wild-type RGS4 group (Fig. 4*Ah* and *C*). This suggests that interaction between the RGS domain and G_α subunits modulates the partial agonism of M2R-activated K_G currents.

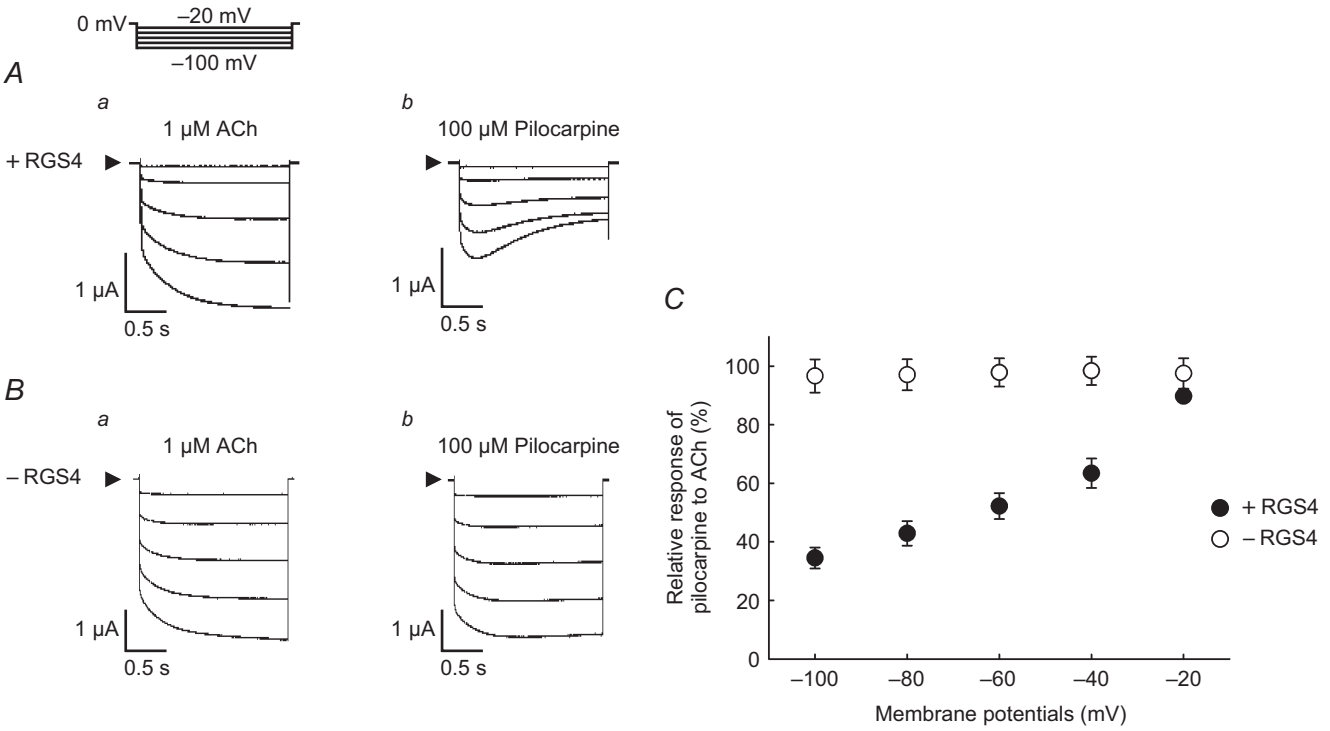

Figure 3. Voltage-dependence of relative response of pilocarpine to ACh
ACh (1 μM, *a*) and pilocarpine (100 μM, *b*)-evoked K_G currents in oocytes with (*A*) and without (*B*) expression of RGS4. Whole-cell currents were recorded with a step protocol as showed above the current traces and basal currents were subtracted. Arrowheads indicate the zero current level. *C*, the pilocarpine (100 μM)-evoked K_G currents as a percentage of ACh (1 μM)-evoked K_G currents at each membrane potential were calculated from steady state. Filled circles indicate the oocytes expressing RGS4; open circles indicate absence of RGS. The graph shows the means ± SEM, $n = 6$ for each condition.

Functional couplings of RGS4 to plasma membrane

In addition to the RGS–G_α subunit interaction, the functional coupling of the pilocarpine–M2R complex and RGS proteins may also play an important role in partial agonism because RGS proteins have been indicated to interact with GPCRs, including M2R–RGS4 coupling (Abramow-Newerly *et al.* 2006; Jaén & Doupnik, 2006). To investigate the influence of M2R–RGS4 coupling in partial agonism, we disturbed the membrane localization of RGS4 by application of 2–bromopalmitate (2-BP), an inhibitor of palmitoyl-CoA transferase. 2–BP inhibits the palmitoylation of RGS4 and results in the redistribution of RGS4 from plasma membrane to cytosol (Wang *et al.* 2010; Bastin *et al.* 2012).

To confirm the effect of 2–BP, rat atrial myocytes and *Xenopus* oocytes were pretreated with 2–BP (100 μM) for longer than 2 h before electrophysiological recording. The 2–BP treatment severely weakened the plasma membrane of myocytes and we failed to record the current precisely, while we found in oocytes that application of 2–BP decelerated the activation and deactivation of ACh-evoked K_G currents when compared with the vehicle ($\leq$0.1% ethanol) group in the RGS4-expressing oocytes (Fig. 5*A* and *B*). This suggests that RGS4 function in this system is indeed impaired by applying 2–BP. We next examined the effect of 2–BP in partial agonism: pilocarpine evoked smaller K_G currents than ACh-evoked K_G currents in the vehicle group (Fig. 5*C*), while pilocarpine evoked larger K_G currents that almost equalled ACh-evoked K_G currents in the 2–BP-treated group (Fig. 5*D*). The efficacy difference between pilocarpine and ACh was eliminated by 2–BP-mediated redistribution of RGS4. These results suggest that deficient coupling of RGS4 to the membrane target impaired the regulatory function of RGS4 and thus failed to control the partial agonism of M2R-activated K_G currents.

Dual effects of pilocarpine on G-protein signalling

Our results demonstrated that RGS4-mediated inhibition of G-protein signalling plays an important role in the mechanism of partial agonism of M2R. A previous study indicated that RGS proteins are able to influence the potency and maximal effect of μ–opioid receptor agonists and suggested RGS proteins may be more effective when the receptor/G-protein/effector system is signalling at submaximal levels (Clark *et al.* 2003). Indeed, we found that RGS4 suppressed

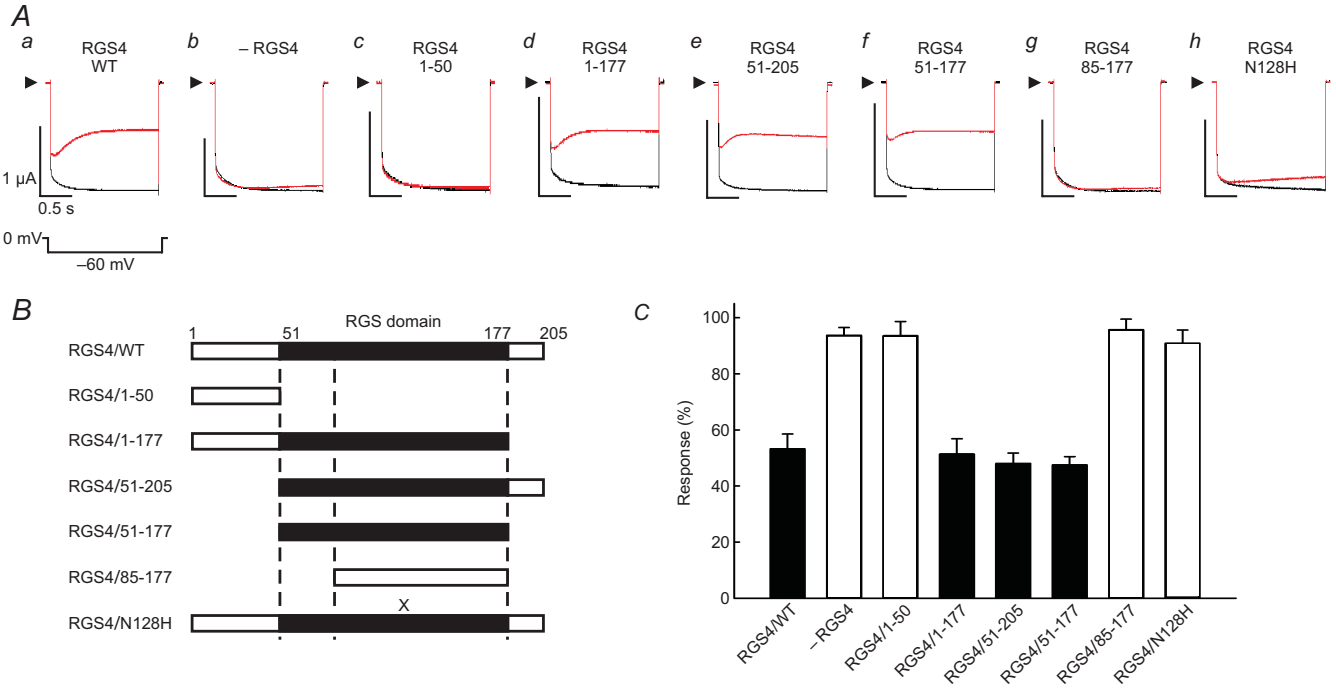

Figure 4. Requirements of RGS4 domain for partial agonism of the M2R-activated K_G currents
A, ACh (1 μM)-evoked K_G currents (black) and pilocarpine (100 μM)-evoked K_G currents (red) in oocytes expressing wild-type RGS4 (*a*), without RGS4 expression (*b*) and expressing various RGS4 mutants (*c–h*) at –60 mV. *B*, formations of truncated mutants of RGS4 and a point mutation in RGS domain. Black portions represent the region of RGS domain. *C*, the response of pilocarpine-evoked K_G currents relative to ACh-evoked K_G currents was calculated from steady state at –60 mV. The bar graph shows the means ± SEM, n = 6–9. The filled bars indicate that the intact RGS domain is included in the mutants; the open bars indicate absence of the intact RGS domain and a significant difference ($P < 0.05$) from the wild-type RGS4 group.

K_G currents in a pilocarpine concentration-dependent manner, which reflects the controlling role of pilocarpine in RGS4-mediated inhibition (Supporting information Fig. S3). We assumed that the submaximal response of the partial agonist is caused by promoting RGS-mediated inhibition of G-protein signals.

To test this idea, we investigated the influence of pilocarpine on RGS4-mediated inhibition. We analysed the effect of pilocarpine on K_G currents in the oocytes that co-expressed with M2Rs and D2Rs because the agonist concentration-dependent relaxation of M2R- and D2R-activated K_G currents were both modulated by RGS4 (Supporting information Fig. S4). To examine the effect of pilocarpine on the common G-protein-mediated signalling, we recorded the D2R- and M2R-activated K_G currents and ensured that the total current was below saturated state (Fig. 6). Co-expressions of M2R and D2R in oocytes were separated into two groups by injecting different concentrations of M2R cRNA: the injected cRNA ratios of D2R to M2R were 100 for the high M2R-expressing group (D/M = 100 group) and 1000 for the low M2R-expressing group (D/M = 1000 group).

With co-treatment of dopamine and pilocarpine, dopamine-evoked K_G currents were defined by subtracting the pilocarpine-evoked currents from total currents. In the presence of RGS4, 0.1 μM dopamine-evoked K_G currents in the presence of pilocarpine were smaller than in the absence of pilocarpine (D/M = 100 group, Fig. 6*A* and *E*). However, the presence or absence of pilocarpine yielded no difference in the dopamine-evoked K_G currents in the absence of RGS4 (D/M = 100 group, Fig. 6*B* and *E*). This shows the dual effects of pilocarpine: (1) like GPCR agonists, it activates G-protein signal transduction; (2) it inhibits G-protein-mediated activation of K_G currents via RGS4. Reduced or no expression of M2R produced mild or no suppression of dopamine-evoked currents in the presence of RGS4, respectively (D/M = 1000 group, Fig. 6*C* and *E*). This suppression was not observed at low concentrations of pilocarpine (data not shown). Certainly, dopamine cannot evoke K_G currents in oocytes deficient in D2R (Fig. 6*D* and *E*). These results suggest that the binding of pilocarpine to M2R promotes RGS4-mediated inhibition of K_G currents.

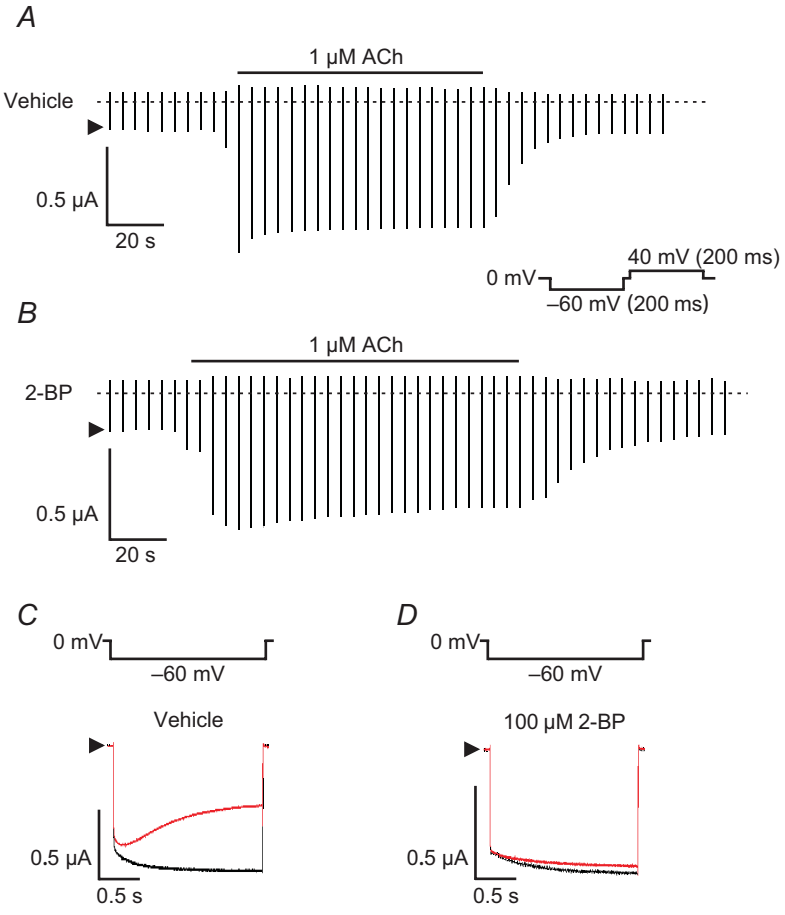

Figure 5. Effects of a palmitoylation inhibitor 2–BP on agonist-evoked K_G currents
ACh (1 μM)-evoked K_G currents were recorded in RGS4-expressing oocytes in vehicle (contained ≤0.1% ethanol) (*A*) or pretreatment with 2–BP (100 μM) for ≥2 h (*B*) with a continuous test pulse as showed on the right side of current traces. The interval of each pulse is 3 s. Relative K_G currents of pilocarpine (100 μM) to ACh (1 μM) were recorded in the same oocyte at −60 mV in the presence of vehicle (*C*) or 2–BP (*D*). Basal currents were subtracted.

Discussion

The novel findings in the present study are as follows: (1) RGS4 is essential for partial agonism of M2R-activated K_G currents; (2) functional coupling within the ligand–M2R complex/G-protein/RGS4 system controls the partial agonism; (3) pilocarpine suppresses G-protein-mediated signalling via promoting the RGS-mediated inhibition.

In the heart, ACh increases K_G currents and thus induces bradycardia (Hibino *et al.* 2010). The muscarinic partial agonist pilocarpine, which is widely used in glaucoma and xerostomia treatments, also decelerates the heart rate via upregulating K^+ flow (Wang *et al.* 1999). Here we demonstrated that RGS4 is essential for reconstituting partial agonism of cardiac M2R-activated K_G currents. Pilocarpine-evoked K_G currents showed a different

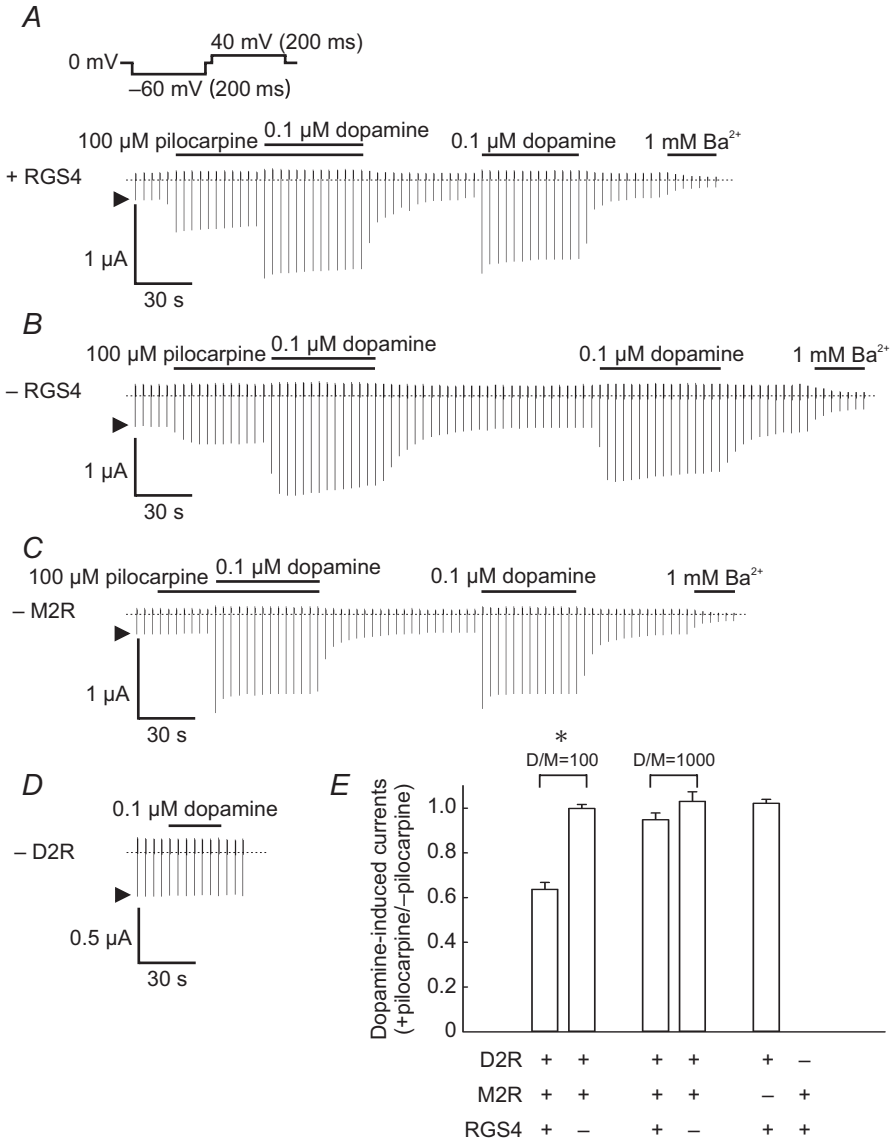

Figure 6. Suppression of dopamine-evoked K_G currents by pilocarpine via RGS4 and M2R
Agonist-evoked K_G currents were recorded in oocytes expressing D2R and M2R (injected cRNA ratio of D2R to M2R = 100, D/M = 100) with (*A*) or without (*B*) RGS4 by a continuous test pulse as shown above the current traces. The bars above each trace indicate the application of dopamine, pilocarpine and K^+ channel blocker Ba^{2+}. *C*, the current recorded in an oocyte that did not express M2R. *D*, the current recorded in an oocyte that did not express D2R. *E*, the ratio of dopamine-evoked currents in the presence of pilocarpine (100 μM) to dopamine-evoked currents in the absence of pilcarpine at −60 mV. Basal and pilocarpine-evoked currents were subtracted. D/M = 100 indicates the high M2R-expressing group and D/M = 1000 indicates the low M2R-expressing group (injected cRNA ratio of D2R to M2R = 1000). Data show the mean ± SEM, $n = 6$. * indicates a significant difference ($P < 0.05$).

time-dependent current change from ACh-evoked K_G currents in rat atrial myocytes (Fig. 1). This current characteristic resembles the hyperpolarization-elicited K_G currents in cat atrial myocytes (Moreno-Galindo *et al.* 2011). The study suggested that the time-dependent decrease of pilocarpine-evoked K_G current is caused by the decreased affinity of pilocarpine for M2R when the membrane becomes hyperpolarized. However, we did not observe a similar decrease in oocytes lacking RGS4, suggesting the decrease of pilocarpine-evoked K_G currents under hyperpolarization is determined by RGS4 and not by agonist binding affinity. The divergent characteristic and efficacy between pilocarpine and ACh should be due to the different level of RGS4-mediated inhibition on K_G currents when different agonists bind to M2R.

We showed that pilocarpine became almost as efficacious as ACh in oocytes lacking RGS4 (Fig. 2). In pancreatic β-cells, RGS4 deficiency can also be found to promote the M3 muscarinic receptor agonist-induced calcium and insulin release (Ruiz de Azua *et al.* 2010). In RGS-insensitive C6μ-Gα_oPTXi cells, the agonist-mediated inhibition of adenylyl cyclase became particularly marked for the partial μ–agonist morphine when compared with C6μ-Gα_oRGS/PTXi cells (Clark *et al.* 2003). These results strongly suggested that the efficacy of GPCR agonists can be modulated by RGS proteins.

We had demonstrated that RGS4 determined the diverse response upon the binding of partial agonists or full agonists as described above. However, the underlying mechanism between the agonist–receptor complex and RGS4 when different agonists bind to M2R remains an enigma. It has been reported that conformational changes of M2R are induced by agonist binding and membrane voltage (Ben-Chaim *et al.* 2006; Navarro-Polanco *et al.* 2011). The binding of pilocarpine induced a different movement of the M2R voltage sensor from that induced by ACh (Navarro-Polanco *et al.* 2011). Diverse conformational changes in the receptor may alter the interactions between the agonist–receptor complex and its direct downstream signal molecules.

Furthermore, we found that the relative efficacy of pilocarpine to ACh was decreased at more negative potentials in the presence of RGS4 but not in the absence of RGS4 (Fig. 3), suggesting RGS4 inhibits pilocarpine-evoked K_G currents in a voltage-dependent manner. The voltage dependence of RGS4-mediated inhibition probably reflects the alteration of agonist–receptor complex–RGS4 interaction when agonist–M2R conformation are changed upon membrane potentials.

Figures 4 and 5 show that the functional coupling between the pilocarpine–M2R complex, Gα subunits and RGS4 controls the partial agonism of M2R-activated K_G currents. Several residues in the RGS domain help to stabilize the RGS4–Gα association and maintain the RGS function of modulating K_G currents (Srinivasa *et al.* 1998; Inanobe *et al.* 2001). The regulatory function of RGS4 is also impaired by 2–BP, which caused marked redistribution of RGS4 from the plasma membrane to cytosol in HEK293 cells (Bastin *et al.* 2012). These findings suggest the normal function of RGS4 is supported by RGS–Gα coupling and RGS4–membrane target coupling.

Recent studies indicate that GPCRs interact with RGS proteins, such as M2R/RGS4, M1 muscarinic receptor/RGS2 and β2 adrenergic receptors/RGS2 (Abramow-Newerly *et al.* 2006; Jaén & Doupnik, 2006). The coupling of receptors and RGS proteins regulates the affinity of RGS proteins for their G-protein targets and the selectivity of RGS activity at the plasma membrane (Abramow-Newerly *et al.* 2006; Jaén & Doupnik, 2006; Croft *et al.* 2013). Therefore, the agonist–M2R complex may not only alter their interaction with RGS4 but also directly or indirectly influence the RGS4–Gα association in a ligand-dependent manner. We found that the pilocarpine–M2R complex suppressed the G-protein-mediated activation of K_G currents via promoted RGS4-mediated inhibition (Fig. 6 and Supporting information Fig. S3). Therefore, the submaximal efficacy of pilocarpine on K_G currents may be explained as follows: (1) decreased GEF activity, which is caused by reducing the affinity of the pilocarpine–M2R complex to Gα subunits; (2) promoted GAP activity, which is caused by enhancing the interaction between the agonist–receptor complex, Gα subunits and RGS.

This idea deserves further research because RGS proteins play important roles in both physiology and disease. RGS proteins not only modulate the heart rate as described previously but are also responsible for embryonic development, neurosecretion, etc. (Wang *et al.* 1999; Hollinger & Hepler, 2002; Cifelli *et al.* 2008). As a result of their negative regulatory effects in GPCR signalling, RGS proteins are potential targets for treating several diseases, such as Alzheimer's disease (Neubig & Siderovski, 2002). Although there are no useful inhibitors of RGS that have been confirmed to suppress RGS4 function in native systems so far, we still need to clarify the role of RGS proteins in partial agonism in physiological conditions by further experimental processes and designs in our future studies.

In rat atrial myocytes, GTPγS-induced irreversible activation of K_G channels eliminated the current character and efficacy difference of agonist-evoked K_G currents (Supporting information Fig. S2). Therefore, the GTP hydrolysis in the G_α subunits is crucial for partial agonism in native GPCR signalling. An emerging paradigm is that GAPs, such as RGS4, are major players in partial agonism in cooperation with GEFs. By regulating the GTP hydrolysis, RGS4 could control the G-proteins and thus determine the effector activation.

In summary, the partial agonism of M2R-activated K_G currents represents a merged outcome of the decreased efficacy of GEFs and the enhanced efficacy of GAPs when partial agonists bind to receptors. This finding helps us to understand the molecular components and mechanisms underlying the partial agonism of M2R-mediated physiological responses and may be applicable to other RGS-coupled GPCR signalling.

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Additional information

Competing interests

We declare no competing interests.

Author contributions

All experiments were performed at Osaka University. I.-S.C. performed all experiments, data collection, analysis and drafting the article; I.-S.C. and K.F. were involved in the conception and design of the study as well as the interpretation of data; I.-S.C., K.F., A.I. and Y.K. were involved in revising the article. All the authors discussed the results and commented on the manuscript. All the authors approved the final version of the manuscript.

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