Structural determinants of the inhibition of M2R by Sigma-1R and the direct inhibition of GIRK channels by Sigma-1R antagonist

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Introduction

Sigma-1 receptor (S1R) is a multimodal chaperone protein located chiefly at the mitochondrion-associated endoplasmic reticulum (ER) membrane (MAM) at rest (Hayashi & Su, 2007; Chu & Ruoho, 2016; Su *et al.*, 2016). S1R can also translocate to various regions of the cell such as plasma membrane (PM) or ER-PM junction, under cellular stress or the application of its ligands (Mavlyutov & Ruoho, 2007).

It has been implicated in a diverse array of pathophysiological conditions including drug addiction, Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (ALS). One of the well-known disease associated mutants of S1R, called ALS-linked E102Q mutation, is reported to cause ER stress-mediated defects in protein homeostasis and dysregulation of RNA-binding proteins, eventually leading to the occurrence of ALS (Dreser *et al.*, 2017).

The crystal structure analysis of human S1R revealed a trimeric architecture of S1R and each protomer contains only one transmembrane domain (TM) with a short N-terminal (N-ter) on the luminal side and a bulky C-terminal (C-ter) including a β-barrel structure on the cytosolic side. It also showed the site of the E102Q mutation locates on the C-ter of S1R (Schmidt *et al.*, 2016). Based on the preceding pharmacological studies, S1R is presumed to exist in various forms from monomer to octamer, and the functional forms of S1R are monomer and dimer. Agonist bound S1R favors monomeric or dimeric forms, while antagonist bound S1R favors tetrameric form which loses its chaperone function (Chu & Ruoho, 2016). BD1047 dihydrobromide (BD1047) is one of the representative antagonists of S1R, however, the endogenous ligands of S1R in physiological processes remain unidentified (Su *et al.*, 2010).

S1R has been reported to directly interact with various proteins such as NMDAR, STIM1, BiP, IP_3R, voltage-gated K^+ and Na^+ channels (Sabeti *et al.*, 2007; Su *et al.*, 2016). It was also identified that S1R colocalizes with muscarinic acetylcholine receptor M2 (M2R) on the soma of motoneurons (Mavlyutov *et al.*, 2010). M2R is one of the Gi/o coupled receptors expressed on PM of such as cardiac muscle cells and neurons, and it plays key roles in wide-ranged physiological

responses. Despite the observation that M2R and S1R colocalize together, there is no study so far focusing on the functional aspect. If S1R could regulate the function of M2R, it is also necessary to reveal the underlying molecular mechanisms, i.e. where and how do they interact with each other.

In the analysis to examine the functional interaction between M2R and S1R using GIRK channel as an effector of M2R activation, I used the known antagonist of S1R, BD1047, and unexpected found that BD1047 directly inhibits GIRK channel.

Thus, the present study was performed with two aims. One, presented in Part 1, is to elucidate the functional interaction between M2R and S1R, as well as the underlying molecular mechanisms and the structural determinants. Another, presented in Part 2, is to characterize the direct inhibition of GIRK channel by BD1047 and to identify the structural determinants.

Materials and Methods

Ethical approval

All animal experiments in this study were approved by the Animal Care Committee of the National Institutes of Natural Sciences (an umbrella institution of National Institute for Physiological Sciences, Japan), and were performed in accordance with its guidelines.

Cell culture and transfection

Plasmids of GIRK1/2 (1μg), M2R (1μg) and S1R (1μg) were transfected to human embryonic kidney 293T (HEK293T) cells or Hela cells using Lipofectamine 2000 reagent (Invitrogen) following the protocol by suppliers for patch-clamp experiments and immunohistochemical staining. Plasmids of M2R (2μg) and S1R (2μg) were transfected to HEK293T cells using Avalanche-Everyday Transfection Reagent (Avalanche®- Everyday) for co-immunoprecipitation following the protocol by suppliers. After 36-48 h of the transfection, cells were re-seeded onto Poly-L-Lysine (PLL) coated glasses for patch-clamp recording, or fixed for immunohistochemical staining.

Preparation of *Xenopus laevis* oocytes

Oocytes were isolated from *Xenopus laevis* (purchased from Hamamatsu Seibutsu Kyouzai, Hamamatsu, Japan) under anesthesia of 0.15% tricaine (Sigma-Aldrich) by surgery. Surgical operation of frog was performed on ice and an incision was made in the frog's abdomen to take out the oocytes. The isolated oocytes were incubated in 2 mg mL^{-1} collagenase type I (Sigma-Aldrich) for 6 hours to remove the follicular membrane and stored in frog Ringer's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$ and 15 mM Hepes with 0.1% penicillin-streptomycin at 17 °C. After the injection of the 50 nL of cRNA, oocytes were incubated in frog Ringer's solution for 3-4 days for electrophysiological recordings.

Mutagenesis and cDNA and cRNA preparations

In the experiments in HEK293T cells, the cRNAs of wild type (WT) human S1R and S1R E102Q cDNA with V5- and histidine-tag, S1R with EGFP-tag in pcDNA3.1(-), provided by Dr Ruth Murrell-Lagnado (University of Sussex, Brighton, UK), were used. The cDNA of porcine M2R, porcine M2R-CFP, rat M4R, $GABA_BR$ and mGluR2 and GIRK1/2 were all subcloned into pcDNA3.1(-). Mutations in M2R and M4R were introduced using the PfuUltra II Fusion HS DNA Polymerase kit (Agilent Technologies) and verified by DNA sequencing. M2R-EGFP construct was made by incorporating the EGFP at the end of M2R WT using the PfuUltra II Fusion HS DNA Polymerase kit (Agilent Technologies), and was subcloned into NotI and EcoRI site of pcDNA3.1(-) and then verified by DNA sequencing.

In the experiments in Xenopus oocytes, cDNAs of WT mouse GIRK2 and WT rat GIRK4 subcloned into pGEMHE were used. Mutations in mouse GIKR2 and rat GIRK4 were introduced using the PfuUltra II Fusion HS DNA Polymerase kit (Agilent Technologies) and verified by DNA sequencing. After linearization of cDNA by restriction enzymes, complementary RNAs were transcribed using mMessage mMachine kit (Ambion). The cRNAs of mouse GIRK2 (4.15 ng $oocyte^{-1}$), rat GIRK4 (4.15 ng $oocyte^{-1}$), bovine G-protein β1 (4.15 ng $oocyte^{-1}$) and bovine G-protein γ2 (4.15 ng $oocyte^{-1}$) were injected to the oocytes.

Electrophysiological recording

In the whole cell patch clamp recording from HEK293T cells, data were acquired using patch clamp amplifier (AXOPATCH 200B), a digital analogue converter (Digidata 1440A, Molecular Devices) and pCLAMP 10.7 software (Molecular Devices). HEK293T cells were attached to PLL coated glass 4-6 hours before recording and placed in the recording chamber, and membrane current were recorded under whole cell patch clamp using a glass micropippette with the access resistance of 3-5MΩ when filled with 140 mM KCl, 5 mM Na_2-ATP, 3 mM EGTA, 0.1 mM $CaCl_2$, 10 mM HEPES, 5 mM $MgCl_2$ (PH 7.3 with KOH). 140K^+ solution (140 mM KCl, 4 mM NaCl,

1mM $CaCl_2$, 0.3 mM $MgCl_2$ and 10 mM Hepes, pH 7.4 with KOH) or 140Na^+ solution (140 mM NaCl, 4 mM KCl, 0.3 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM Hepes, pH 7.4 with NaOH) were used as an extracellular solution for recordings. All experiments were performed at 25-28 °C. Ligands, 50 µM OXO-M, 100 µM GABA, 200 µM Glutamate and 50 µM BD1047 with 50 µM OXO-M in 140K^+ were delivered and wash out by gravity flow using a muti-valve controller system (VC-8 VALVE CONTROLLAER, warner instruments).

In the two electrodes voltage clamp recording from Xenopus oocytes, data were acquired by Oocyte clamp amplifier (OC-725C, Warner Instruments), a digital analogue converter (Digidata 1550A, Molecular Devices) and pCLAMP 10.5 software (Molecular Devices). An oocyte was placed in the recording chamber and membrane current were recorded under voltage clamp using two glass microelectrodes with the resistance 0.1-0.5 MΩ when filled with 3 M potassium acetate with 10 mM KCl. 96K^+ solution (96 mM KCl, 3 mM $MgCl_2$ and 5 mM Hepes, pH 7.5 with KOH) or ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 5 mM Hepes, pH 7.5 with NaOH) were used as an extracellular solution for recordings. All experiments were performed at 25-28 °C. Various concentrations of BD1047 were applied and wash out by perfusion using a peristaltic pump (AC-2110 II, ATTA).

Immunohistochemical staining

HEK293T or Hela cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, blocked in 2% skim milk with 0.1% Triton X-100 in PBS, and then incubated with the primary antibody diluted in PBS. A rabbit polyclonal anti-EGFP antibody (Invitrogen, 1:200 dilution) and a mouse monoclonal anti-V5 tag antibody (Invitrogen, 1:500 dilution) were used. After the primary antibody, the sample were incubated with the secondary antibody conjugated with Alexa Fluor-488 (Invitrogen, dilution 1:500) or Alexa Fluor-568 (Invitrogen, 1:500 dilution).

Co-immunoprecipitation

HEK293T cells were cultured in 60mm dishes until cells reach 80-90% confluency and then were co-transfected with M2R-EGFP and S1R-V5-His using the Avalanche-Everyday Transfection Reagent (Avalanche®- Everyday) following the protocol of the supplier. Cells were lysed after 48 hours of incubation. Cell lysate was preincubated with G-Sepharose (Protein G Sepharose™ 4 Fast Flow, GE Healthcare) in a rotating wheel for 1hr at 4°C and then were centrifuged to remove the G-Sepharose and the supernatant was collected. Then, mouse monoclonal anti-EGFP antibody (Sigma-Aldrich, 2µg) for immunoprecipitating M2R or mouse monoclonal anti-V5 tag antibody (Invitrogen, 2µg) for immunoprecipitating M2R was added to the supernatant with G-Sepharose and incubated in a rotating wheel for 2 hours at 4°C. After 2 hours of rotating incubation, samples were washed by wash buffer and centrifuged 3 times repeatedly. Wash buffer was completely removed following the final centrifuge, and the sample buffer containing SDS was added to the tubes and heated for 5 mins at 95°C. Proteins were finally analyzed by western blot.

Western blot analysis

Total protein lysate and IP samples were separated by SDS/PAGE (SuperSep^{TM}Ace, 10-20%, 13 or 17well, FUJIFILM) and blotted to PVDF membranes (Sigma-Aldrich) by using wet transfer system. Membranes were blotted by Blocking One (Nacalai Tesque Inc.) and then incubated with monoclonal rabbit anti-M2R antibody (Abacm, dilution 1:1000) or polyclonal rabbit anti-His tag antibody (Bethyl Laboratories, dilution 1:1000) diluted in Blocking One solution for 1 hour at room temperature. Membranes were then washed by PBST for 3 times, and samples were incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase (GE Healthcare, dilution 1:5000) to visualize and detected by chemiluminescence (ImmunoStar zeta kit, FUJIFILM).

Molecular docking

Homology model of GIRK4 was built based on the structure of GIRK2 (6XIT, RCSB protein data bank) using SWISS-MODEL (https://swissmodel.expasy.org/). The dimer form of GIRK4 was made from the homology model of GIRK4 using PyMOL software. Chemical structure of BD1047 was downloaded from the website. The dimer form of GIRK4 and the structure of BD1047 were uploaded to SwissDock (http://www.swissdock.ch/) to obtain the docking results. The computational docking results was presented with color marks using Chimera 1.16 software.

Chemicals

OXO-M was purchased from Sigma-Aldrich and dissolved in distilled water to make a 50 mM stock solution and further diluted in the bath solution for the final concentration 50 µM. BD1047 was purchased from Tocris Bioscience and dissolved in distilled water to make a 50 mM stock solution and further diluted in the bath solution for the final concentrations from 0.1µM up to 500 µM. Sources of other materials are described in the relevant methods.

Data analyses

In the patch-clamp experiments, all data were analyzed by Clampfit 10.7 (Molecular Devices) and Igor 5 (WaveMetrics) and are shown as mean ± SD from n single HEK293T cells. The current recorded in 140 Na^+ was subtracted to remove the endogenous current from HEK293T cells. The current amplitude of GIRK1/2 channel was recorded at -120mV before and after the application of the agonists to various $G_{i/o}$ coupled receptors. Activation ratio of GIRK1/2 channel by various agonists was calculated by the current amplitude in the presence and absence of the ligand after subtraction of endogenous current in 140 Na^+ ($I_{GIRK\ agonist\ (+)}$ /$I_{GIRK\ agonist\ (-)}$). Activation ratio of 1 means there was no change in the current amplitude after the application of agonists.

In the two-electrode voltage clamp experiments, all data were analyzed by Clampfit 10.7 (Molecular Devices) and Igor 5 (WaveMetrics) and are shown as mean ± SD from n single oocytes.

The current amplitude of GIRK channel was recorded at -100mV before and after the application of BD1047. The inhibition percentage of GIRK current by BD1047 was calculated by the decrease in the current amplitude in the presence of 100 μM BD1047 from the current before its application at -100 mV ($I_{GIRK\ basal}$ -$I_{GIRK\ BD1047\ (+)}$ / $I_{GIRK\ basal}$), and the basal current amplitude after subtraction of the current in ND96 solution was normalized as 100%. The dose-inhibition relationship was analyzed by sequential application of various concentrations of BD1047. By using Igor, the data were fitted to a Hill equation: y = min + (max − min) / (1 + (x/IC_{50}) ^ (Hillslope)).

All experiments were performed with the number of $n \geq 4$ for each group. Statistical significances of differences were evaluated using Tukey's multiple comparison tests following one-way ANOVA or Student's *t*-test (unpaired). The values of $P<0.05$ were judged to be statistically significant.

Results

Part I

Regulation of M2R by S1R and S1R disease mutant E102Q

It has been demonstrated that M2R is colocalized with S1R at the postsynaptic site of motoneurons in the ventral horn in the mouse brainstem and spinal cord (Mavlyutov *et al.*, 2010), but the functional relationship between them has not been explored yet. As the initial step, I examined whether S1R regulates the function of M2R or not. M2R is one of the Gi/o coupled receptors and G protein-gated inward rectifier potassium channel (GIRK) is an effector of the Gi/o coupled receptor, therefore the function of M2R could be measured by GIRK current. By patch-clamp recordings of HEK293T cells coexpressing GIRK channel and M2R with or without S1R, it was observed that S1R inhibits the function of M2R.

A disease associated mutant of S1R, called ALS-linked S1R E102Q mutation which related to the occurrence of ALS (Dreser *et al.*, 2017). It is interesting to examine whether or not this S1R E102Q could affect the function of M2R. By patch-clamp recordings of HEK293T cells coexpressing GIRK channel and M2R with or without S1R E102Q. It was observed that the inhibition effect on M2R is lost in S1R E102Q and the function of M2R cannot be regulated by the S1R disease mutant.

Regulation of M2R and other types of $G_{i/o}$ coupled receptors by S1R

Besides M2R, there are various $G_{i/o}$ coupled receptors, for example, muscarinic acetylcholine receptor M4 (M4R), gamma-aminobutyric acid type B receptor ($GABA_BR$), metabotropic glutamate receptor 2 (mGluR2). They share a similar downstream signaling pathway after stimulation by their ligands (Fredriksson *et al.*, 2003). In order to examine the effect of S1R on other types of $G_{i/o}$ coupled receptors, experiments to co-transfect various $G_{i/o}$ coupled receptors such as M2R, M4R, $GABA_BR$ or mGluR2 with or without S1R in HEK293T cells were conducted.

It was observed that S1R has no inhibition effect on M4R, $GABA_BR$ and mGluR2, and the inhibition effect of S1R on M2R is unique among other types of Gi/o coupled receptors.

Immunohistochemical staining of M2R and S1R in HEK293T and Hela cells

There are two possibilities about how S1R inhibits the function of M2R. One possibility is that S1R retains M2R within the cell and thereby reduces the surface expression of M2R on PM. The other possibility is that S1R directly binds to M2R on PM and regulates its function. To examine these possibilities, it is important to confirm the surface expression level of M2R. Towards this aim, immunohistochemical staining (IHC) of M2R and S1R were performed using HEK293T cells as they were used for the electrophysiological analyses. For better special discrimination of PM and ER, Hela cells which are flatter and wider were also used. EGFP tagged M2R was transfected alone or co-transfected with V5 tagged S1R in HEK293T cells or Hela cells. All images were obtained by confocal microscope. It was observed that the expression level of M2R on PM is not clearly down regulated by S1R in HEK293T cells, and that of M2R on PM is slightly decreased by S1R in Hela cells. In both HEK293T and Hela cells, S1R is expressed on ER and localizes close to M2R, suggesting a possible interaction with M2R presumably at the ER-PM junction.

Co-immunoprecipitation of M2R and S1R

As a next step, the protein-protein interaction between M2R and S1R was examined by co-immunoprecipitation analysis. M2R was tagged with EGFP (M2R-EGFP), and S1R was tagged with V5 and His tags (S1R-V5-His). The predicted molecular weights of M2R and S1R including tags are 78.6kDa and 27.2kDa, respectively. Lysate sample and immunoprecipitation sample were collected and prepared from the HEK293T cells which M2R-EGFP and S1R-V5-His were co-transfected. It was observed that S1R co-precipitates with M2R and that M2R co-precipitates with S1R, demonstrating the protein-protein interaction between M2R and S1R.

Regulation of M2R/M4R chimeras and M2R/M4R mutants by S1R

Although it was confirmed that S1R inhibits the function of M2R and that they indeed directly interact with each other, the underlying mechanism remains unclear. Thus, it is needed to reveal the structural determinants of the inhibition of M2R by S1R. M2R and M4R both belong to the muscarinic cholinergic receptor family, and share a high similarity of the amino acid sequences, whereas the inhibition of S1R on them is distinguishable. Taking advantage of the different inhibition effects of S1R on them, chimeras between M2R and M4R and their mutants were constructed in order to identify the structural determinants for the interaction between M2R and S1R. By analyzing various chimeras and mutants between M2R and M4R, it was identified that the extracellular loop 2 region of M2R, as well as the transmembrane domain 6 (TM6), are critical for the inhibition by S1R.

Part II

Inhibition of GIRK channels by BD1047

In the initial stage of the study to examine whether S1R can regulate the function of M2R, I also tried to inhibit the function of S1R using the known antagonist of S1R, BD1047. In the HEK293T cells transfected with GIRK1/2, M2R and S1R, the application of BD1047 suppressed the OXO-M induced GIRK1/2 current. The application of BD1047 also suppressed the OXO-M induced GIRK1/2 current even in the absence of S1R. The results show that BD1047 might has a direct inhibition effect on GIRK channel. For the sake of high throughput experiments, Xenopus oocytes were used as an in vitro expression system and two-electrodes voltage clamp experiments were performed to examine this possibility. The effect of BD1047 on the heteromeric GIRK1/2 and GIRK1/4, the homomeric GIRK2 and GIRK4 were examined without co-expression of M2R and S1R. Since homo GIRK4 channel shows a small basal current (Hibino *et al.*, 2010), its physiological activator $G_{\beta\gamma}$ subunits were co-expressed together with GIRK4 in order to clearly

observe the GIRK4 current. It was observed that the application of BD1047 decreased the current amplitude of GIRK1/2, GIRK2, GIRK1/4 and GIRK4 to various degrees, especially that of GIRK4. Therefore, it was confirmed that BD1047 directly inhibits the current amplitude of GIRK channels.

Inhibition of GIRK2/4 chimeras and GIRK2/4 mutants by BD1047

It was found that BD1047 has a weak inhibition effect on GIRK2 channel and a strong inhibition effect on GIRK4 channel. As a next step, the dose inhibition relationship of BD1047 on GIRK2 or GIRK4 current was analyzed. The results confirmed that GIRK4 is more sensitive to the block by BD1047 than GIRK2. Thus, taking advantage of the obvious differences of the effect of BD1047 on GIRK2 and GIRK4, chimeras between them and their mutants were constructed and analyzed electrophysiologically to identify the binding site between GIRK channel and BD1047. The results showed that the proximal cytoplasmic region of N-ter of GIRK4 is critical for the inhibition of BD1047.

Molecular docking of GIRK4 channel and BD1047

Molecular docking is a useful approach to computationally predict the preferred binding site and docking orientation of a small molecule with a target protein. Thus, computational docking using the software named SwissDock online was conducted to approach the possible binding sites of BD1047 with GIRK4 channel. As the structure of GIRK4 has not been solved yet, a homology structural model of GIRK4 was made based on the available structure of GIRK2 (6XIT) using SWISS-MODEL online. Then, the obtained structure of GIRK4 and the chemical structure of BD1047 were uploaded to the SwissDock site. The computational docking data showed that multiple amino acid residues in the proximal N-ter cytoplasmic region of GIRK4 have a high chance to interact with BD1047. The inhibition effect of BD1047 was remarkably decreased in the mutants of these amino acid residues, conforming the validity of the computational docking results.

Conclusion

The first aim of this study was to clarify whether M2R can be regulated by S1R and to identify the structural determinants. I used HEK293T cells as an in vitro expression system and observed followings: (1) By patch-clamp, it was confirmed that S1R inhibits the function of only M2R among other types of $G_{i/o}$ coupled receptors. The disease associated mutant S1R E102Q does not inhibit the function of M2R. (2) By immunohistochemical staining and confocal imaging, it was observed that the expression level of M2R on the PM is not clearly down regulated by S1R in HEK293T cells, and that of M2R on PM is slightly decreased by S1R in Hela cells. In both HEK293T and Hela cells, S1R localizes close to M2R, suggesting a possible interaction with M2R presumably at the ER-PM junction. (3) By co-immunoprecipitation analysis, the binding of M2R and S1R was confirmed. (4) By analyzing various chimeras and mutants of M2R and M4R, it was shown that the extracellular loop 2 region of M2R, as well as the TM6, are essential for the inhibition by S1R. Taken together, the data shows that S1R partly decreases the expression of M2R on the PM and also inhibits its function, via the extracellular loop 2 and TM6.

Furthermore, I happened to find that the BD1047, an antagonist of S1R, directly inhibits the current of GIRK channel. In order to confirm the direct inhibition of BD1047 on GIRK channels, I used Xenopus oocytes as an in vitro expression system and performed two-electrodes voltage clamp and observed followings: (1) BD1047 directly inhibits GIRK channels in the absence of S1R. (2) BD1047 has a weak inhibition effect on GIRK2 channel and a strong inhibition effect on GIRK4 channel. (3) The chimera which contains the proximal N-ter of GIRK4 showed strong inhibition effects by BD1047. (4) Molecular docking analysis confirmed the importance of multiple amino acid residues in the proximal N-ter of GIRK4 for the binding. Taken together, the data showed that BD1047 directly inhibits GIRK channels and that the N-ter of GIRK4 is critical for the inhibition by BD1047.

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