Chimeric microbial rhodopsins for optical activation of Gs-proteins

Kazuho Yoshida¹, Takahiro Yamashita², Kengo Sasaki¹, Keiichi Inoue¹,³,⁴, Yoshinori Shichida² and Hideki Kandori¹,³

¹Department of Life Science and Applied Chemistry, Nagoya Institute of Technology, Nagoya, Aichi 466-8555, Japan
²Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan
³OptoBioTechnology Research Center, Nagoya Institute of Technology, Nagoya, Aichi 466-8555, Japan
⁴PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan

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We previously showed that the chimeric proteins of microbial rhodopsins, such as light-driven proton pump bacteriorhodopsin (BR) and Gloeobacter rhodopsin (GR) that contain cytoplasmic loops of bovine rhodopsin, are able to activate Gt protein upon light absorption. These facts suggest similar protein structural changes in both the light-driven proton pump and animal rhodopsin. Here we report two trials to engineer chimeric rhodopsins, one for the inserted loop, and another for the microbial rhodopsin template. For the former, we successfully activated Gs protein by light through the incorporation of the cytoplasmic loop of β²-adrenergic receptor (β²AR). For the latter, we did not observe any G-protein activation for the light-driven sodium pump IndiR2 or chloride pump NpHR, whereas the light-driven proton pump GR showed light-dependent G-protein activation. This fact suggests that a helix opening motion is common to G protein coupled receptor (GPCR) and GR, but not to IndiR2 and NpHR. Light-induced difference FTIR spectroscopy revealed similar structural changes between WT and the third loop chimera for each light-driven pump. A helical structural perturbation, which was largest for GR, was further enhanced in the chimera. We conclude that similar structural dynamics that occur on the cytoplasmic side of GPCR are needed to design chimeric microbial rhodopsins.

Key words: microbial rhodopsin, GPCR, G-protein activation, retinal, FTIR

Animal and microbial rhodopsins convert light into signals and energy by employing the photochemical reaction of retinal [1]. Animal rhodopsins contain 11-cis retinal as the chromophore, and photoisomerization from the 11-cis to the all-trans form initiates protein structural changes, leading to activation of the trimeric G protein transducin (Gt) [1–4]. Microbial rhodopsins contain all-trans retinal as the chromophore, and the initiation of protein structural changes, which are caused by the photoisomerization from the all-trans to the 13-cis form, lead to various functions such as light-driven pumps, light-gated channels, photosensors and light-activated enzymes [1,5–9]. There are no sequence homologies between animal and microbial rhodopsins, but both possess similar chromophore (retinal) and protein structural changes.

Chimeric proteins of a light-driven proton pump GR containing the cytoplasmic loop of β₂-adrenergic receptor (β₂AR) activate Gs protein by light. In contrast, chimeric proteins of light-driven sodium pump IndiR2 or chloride pump NpHR containing the same loop of β₂AR do not activate Gs protein at all. Light-induced difference FTIR spectroscopy showed largest helical structural perturbation for GR, which was further enhanced in the chimera. Similar structural dynamics that occur on the cytoplasmic side of GPCR are needed to design chimeric microbial rhodopsins.
Microbial rhodopsins have been used as tools in optogenetics, a field of study in which animal behavior is controlled by light [10–12]. In optogenetics, animal brain functions are studied by incorporating microbial rhodopsins, but not animal rhodopsins, into the animal brain. There are two reasons for this. One is the isomeric structure of the chromophore. Whereas 11-cis retinal is not abundant in animal cells, endogenous all-trans retinal is sufficient for optogenetics in animal cells. The second reason is the cyclic behavior of the chromophore in the photoreaction. In animal rhodopsins, isomerized all-trans retinal does not return to the 11-cis form, a process that is termed “photobleaching.” This is not a problem in visual cells because enzymatically isomerized 11-cis retinal is newly supplied, which is not the case in other cells. In contrast, the 13-cis form is thermally reisomerized into the all-trans form, and the spontaneous return leads to the “photocycle” in microbial rhodopsins. This is highly advantageous in optogenetics.

For these reasons, animal rhodopsins have not been actively used in optogenetics. Although Arian et al. engineered ‘optoXRs’ [13], in which a bovine rhodopsin chimera containing the cytoplasmic loop of other G-protein coupled receptors (GPCRs) was used to respond to light, problems with 11-cis retinal and photobleaching limit broad applications. Thus, the optogenetic application of GPCR signaling requires that these two problems be resolved. One approach is to use bistable animal rhodopsins whose photointermediate does not bleach and is thermally stable [14,15]. By photo-isomerizing the intermediate (normally in an active state) into the original state (normally in an inactive state), activation of GPCR signaling is switchable by light. Some bistable rhodopsins can bind a 13-cis retinal, which exists in normal cells in thermal equilibrium with an all-trans form. In addition, it was recently reported that a ciliary opsin from Platynereis dumerilii can bind all-trans retinal directly and exhibit bistability [16].

Another approach is to use chimeric proteins of animal and microbial rhodopsins. We have engineered chimeric proteins of microbial rhodopsins containing cytoplasmic loops of animal rhodopsin [17,18]. These chimera contain all-trans retinal, display a photocycle (no bleaching), and activate Gt. So far, the second and third cytoplasmic loops of bovine rhodopsin have been replaced by Gt, in which the third loop is essential for the activation of Gt [18]. As templates of microbial rhodopsin, we attempted light-driven proton pumps bacteriorhodopsin (BR), Gloeobacter rhodopsin (GR), proteorhodopsin (PR), and sensory rhodopsin II (SRII). Among these, BR, GR and SRII chimera activated Gt, but PR chimera did not.

Gt activation by these chimera suggest a common activation mechanism between animal and microbial rhodopsins, in which helix opening occurs at the cytoplasmic surface [18]. These chimera are potential candidates of new optogenetic tools for GPCR signaling. In this paper, we report two trials that advance the engineering of chimeric rhodopsins, one for the inserted loop, and another for the microbial rhodopsin template. Regarding the first trial, we have so far only tested the activation of Gt, which is localized in the retina. Here we examined the activation of Gs protein by light, for which we incorporated the cytoplasmic loop of β2-adrenergic receptor (β2AR). Similar structural changes for G-protein activation have been suggested for bovine rhodopsin and β2AR [19–21], and indeed we successfully activated Gs protein by using a microbial rhodopsin chimera with β2AR. Regarding the second trial, we have so far only tested proton-pump proteins as the template of microbial rhodopsins. SRII is a phototaxis sensor but functions as a light-driven proton pump without its transducer protein [22]. Here we examined two light-driven pumps, the sodium pump [23,24] and the chloride pump [24–26]. Interestingly, we did not observe any G-protein activation for the light-driven sodium pump from Indi bacter alkaliphilus (IndiR2) and the light-driven chloride pump halorhodopsin from Natronomonas pharaonis (NpHR), although a light-driven proton pump GR showed light-dependent G-protein activation. The molecular mechanism of G-protein activation by chimeric proteins will be discussed.

Materials and Methods

Sample Preparation

The chimeric constructions were designed based on the wild-type (WT) GR (GenBank accession number: BAC88139), IndiR2 (BAV92787) and NpHR (P15647) (Fig. 1), and the DNA template of the human β2AR loop was exchanged by the following three-step PCR. First, three PCR products were constructed and purified: the front side for the inserted loop, and another for the microbial rhodopsin, into the animal brain. There are two reasons why we incorporated the cytoplasmic loop of β2-adrenergic receptor (β2AR). Similar structural changes for G-protein activation have been suggested for bovine rhodopsin and β2AR [19–21], and indeed we successfully activated Gs protein by using a microbial rhodopsin chimera with β2AR. Regarding the second trial, we have so far only tested proton-pump proteins as the template of microbial rhodopsins. SRII is a phototaxis sensor but functions as a light-driven proton pump without its transducer protein [22]. Here we examined two light-driven pumps, the sodium pump [23,24] and the chloride pump [24–26]. Interestingly, we did not observe any G-protein activation for the light-driven sodium pump from Indi bacter alkaliphilus (IndiR2) and the light-driven chloride pump halorhodopsin from Natronomonas pharaonis (NpHR), although a light-driven proton pump GR showed light-dependent G-protein activation. The molecular mechanism of G-protein activation by chimeric proteins will be discussed.
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phosphate, 2 mM NaCl for IndiR2 and NpHR). An 80 μl aliquot of the sample was deposited on a BaF₂ window of 18 mm diameter and dried in a glass vessel that was evacuated by an aspirator. The film sample was hydrated with 1 μL of H₂O before measurements. Although the salt concentration cannot be precisely measured for hydrated IndiR2 and NpHR films, we roughly estimated it to be >100 mM. Then, the sample was placed in a cryostat (Oxford DN-1704, UK) mounted in the FTIR spectrometer (Bio-Rad FTS-7000, USA). The cryostat was equipped with a temperature controller (Oxford ITC-4, UK), and the temperature was regulated with 0.1 K precision.

Long-lived intermediates must be responsible for G-protein activation of chimeric microbial rhodopsins, such as metarhodopsin-II in the case of bovine rhodopsin. Therefore, we attempted to capture late intermediates that accumulate at the last stage of the photocycle. In GR [18] and IndiR2 [32], the late intermediate that forms is the red-shifted O intermediate. We illuminated GR, IndiR2 and their chimeras with 520±5 nm light (an interference filter) at 250 K for 2 min. On the other hand, the O intermediate does not accumulate in NpHR under high salt conditions [33], and our previous FTIR study showed that the L2 (or N) intermediate is formed at 250 K [34]. Thus, we illuminated NpHR and its chimeras with >500 nm light at 250 K for 2 min. The difference spectra were obtained with 2 cm⁻¹ resolution. We averaged 3–4 independent measurements with 128 scans.

Results

Absorption Properties of the GR, IndiR2 and NpHR Chimera

In the present study, we replaced the second or third cytoplasmic loop of microbial rhodopsins into those of β₂AR. The schematic structure of the second and third loop of β₂AR inserted into chimeras is shown in Figure 1, together with the removed amino-acid sequences in GR, IndiR2 and NpHR. The crystal structures of NpHR [35] and β₂AR [36] are known, and we designed the amino acids to replace them...
of the binding of GTPγS to Gs-protein, where the light-dependent GDP/GTPγS exchange was monitored by using [35S]GTPγS. At least 200 DDM molecules are needed to solubilize one microbial rhodopsin [39], thus 0.05% DDM was used in our study to fully solubilize the chimeric proteins whose concentration was \(0.2\, \mu\text{M}\) (molecular ratio of chimera: DDM = 1:500). In the case of IndiR2 (Fig. 3b) and NpHR (Fig. 3c) chimeras, all time-courses looked similar. This fact indicates that the amount of light-induced time-dependent GTPγS binding is similar to the level of each chimera in the dark, regardless of whether it is a second or third loop chimera, and this is also the case for WT (dotted lines). This feature is clearly seen in Figure 3d, where the amount of GTPγS binding is similar between dark and light conditions within current experimental accuracy, whose level coincides with the spontaneous incorporation of GTPγS to trimeric Gs without receptors (Gs only in Fig. 3d). Thus, we conclude that chimeric proteins of light-driven sodium (IndiR2) and chloride (NpHR) pumps do not activate G-protein.

In contrast, different features were observed for the GR chimera. Figure 3a shows light-dependent Gs-protein activations of GR, IndiR2 and NpHR chimeras. Figure 3a shows the time-course

**Gs-Protein Activation Properties of the GR, IndiR2 and NpHR Chimera**

We next tested the Gs-protein activation of GR, IndiR2 and NpHR chimeras. Figure 3a–c shows the time-course of the binding of GTPγS to Gs-protein, where the light-dependent GDP/GTPγS exchange was monitored by using [35S]GTPγS. At least 200 DDM molecules are needed to solubilize one microbial rhodopsin [39], thus 0.05% DDM was used in our study to fully solubilize the chimeric proteins whose concentration was \(0.2\, \mu\text{M}\) (molecular ratio of chimera: DDM = 1:500). In the case of IndiR2 (Fig. 3b) and NpHR (Fig. 3c) chimeras, all time-courses looked similar. This fact indicates that the amount of light-induced time-dependent GTPγS binding is similar to the level of each chimera in the dark, regardless of whether it is a second or third loop chimera, and this is also the case for WT (dotted lines). This feature is clearly seen in Figure 3d, where the amount of GTPγS binding is similar between dark and light conditions within current experimental accuracy, whose level coincides with the spontaneous incorporation of GTPγS to trimeric Gs without receptors (Gs only in Fig. 3d). Thus, we conclude that chimeric proteins of light-driven sodium (IndiR2) and chloride (NpHR) pumps do not activate G-protein.

In contrast, different features were observed for the GR chimera. Figure 3a shows light-dependent Gs-protein activations of GR, where G-protein activation was almost identical between light and dark conditions for WT (black circles in Fig. 3a). Unlike WT, clear light-dependent Gs-protein activation was observed for the second (red circles in Fig. 3a) and third (blue circles in Fig. 3a) loop chimeras, where light-dependent activation was more enhanced in the latter. These features are obvious from Figure 3d. Dark activation was higher for WT GR than those for GR chimera and other proteins, but the reason is unclear. However, similar results for light activation of WT GR suggest no difference between light and dark, nor between IndiR2 and NpHR chimeras.

Only the GR chimera activated G-protein, in which the
loop chimeras of GR (Fig. 4a), IndiR2 (Fig. 4b) and NpHR (Fig. 4c). All difference spectra were measured at 250 K, where late intermediates accumulated during their photo-cycles. The spectra of WT and the chimera in each rhodopsin were very similar, particularly the frequency region of the C=C (1550–1500 cm$^{-1}$) and C–C (1250–1150 cm$^{-1}$) stretches of the retinal chromophore. This indicates that similar intermediates formed in both WT and the loop chimera of each rhodopsin. In other words, different properties among ion-pump rhodopsins such as G-protein activation by chimeric proteins essentially originate from the structural dynamics of each ion-pump protein.

The left panel of Figure 4 highlights an amide-I vibration that appears at 1700–1600 cm$^{-1}$. The frequency of that vibration strongly depends on the secondary structure of the protein, where the frequency of the α-helix appears at 1660–1650 cm$^{-1}$. Figure 4a shows the results for GR, where difference spectra correspond to those between the O intermediate and the resting state [18]. Here we also measured the Gs activation ability by β$_2$AR as a positive control. However, we utilized membrane-embedded β$_2$AR, not purified samples, and we could not estimate the amount of β$_2$AR in the sample. Thus, we could not compare the Gs activation ability between β$_2$AR and our chimeras.

We found that the proton pump chimera (GR) possessed the ability to activate Gs-protein and Gt-protein, whereas the sodium pump (IndiR2) and the chloride pump (NpHR) were unable to activate Gs-protein. Similar absorption spectra for all chimeras in Figure 2 show a retained protein structure around their retinal chromophore. To further characterize the molecular properties of these chimeras, we applied light-induced difference FTIR spectroscopy.

Light-Induced Difference FTIR Spectroscopy of the GR, IndiR2 and NpHR Chimera

Figure 4 compares structural changes of WT and the third loop chimera of GR (Fig. 4a), IndiR2 (Fig. 4b) and NpHR (Fig. 4c). All difference spectra were measured at 250 K, where late intermediates accumulated during their photo-cycles. The spectra of WT and the chimera in each rhodopsin were very similar, particularly the frequency region of the C=C (1550–1500 cm$^{-1}$) and C–C (1250–1150 cm$^{-1}$) stretches of the retinal chromophore. This indicates that similar intermediates formed in both WT and the loop chimera of each rhodopsin. In other words, different properties among ion-pump rhodopsins such as G-protein activation by chimeric proteins essentially originate from the structural dynamics of each ion-pump protein.

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Discussion

Microbial rhodopsin chimeras that contain the cytoplasmic loop of GPCR offer potential as optogenetic tools. We previously reported that the chimera of bovine rhodopsin are able to activate Gt-protein [17,18]. It is important to extend this ability to more general G-proteins such as Gs, Gi, Go, and others, and there is interest in studying if these chimeras are able to activate various G-proteins, or not. In this paper, we showed that GR chimeras containing the second and third loops of β<sub>2</sub>AR are able to activate Gs-protein. Gs-activating rhodopsin in jellyfish was reported as a potential optogenetic tool for light-dependent Gs activation [40,41], and the present study provides different kind of Gs activation tool by light using microbial rhodopsin. Previously we suggested similar protein structural changes between bovine rhodopsin and microbial rhodopsins such as BR, SRII and GR [17,18]. The present study further generalizes such structural changes for another GPCR, β<sub>2</sub>AR. This generalization is reasonable because the outward motion of helix 6 was reported upon activation of bovine rhodopsin and β<sub>2</sub>AR, together with microbial rhodopsins [1,3,6,19–21].

GR chimeras show no G-protein activation in the dark,
suggesting that the interaction surface of the receptor is hidden in the dark and is only exposed by light. This concept is also common for GPCR activation [19–21]. A weakened hydrogen bond of the amide-I vibration of the α-helix was observed upon formation of the O intermediate of WT GR, and we infer that this α-helical perturbation is caused by opening of helix 6 on the cytoplasmic side. The GR template was identical for the GR chimera of the third loop of bovine rhodopsin [18] and β2AR (Fig. 1). In addition, the amplitude of the amide-I signal for the positive 1667-cm⁻¹ and negative 1659-cm⁻¹ bands was enhanced 1.67-times for bovine rhodopsin chimera [18] and 1.42-times for β2AR chimera (Fig. 4a) relative to WT. This suggests that inserted third loop enlarges opening motion of helix 6, which is larger in bovine rhodopsin chimeras.

The present study shows that light-driven proton pump (GR) chimeras contain cytoplasmic loops that activate Gs-protein, whereas light-driven sodium (IndiR2) and chloride (NpHR) pump chimeras do not. Thus, we are able to classify a new template of microbial rhodopsins into two classes: BR (proton pump), SRII (sensor, but acts as proton pump without transducer) and GR (proton pump) chimeras that can activate G-proteins (class I), and PR (proton pump), IndiR2 (sodium pump) and NpHR (chloride pump) chimeras that cannot (class II). A remaining question involves the possible mechanism to distinguish the activation of G-proteins. G-protein activation is caused by helix opening at the cytoplasmic side. Thus, no G-protein activation by the chimeras of light-driven sodium and chloride pumps suggests a small helix opening on the cytoplasmic side. This hypothesis is strongly supported by the present FTIR observation, in which the helical structural perturbation was smaller in the sodium-pump IndiR2 and the chloride-pump NpHR than in the proton-pump GR (Fig. 4).

Information on the structural dynamics of light-driven sodium pumps is limited, whereas X-ray structure and computational studies of KR2 suggested a more polar environment at the cytoplasmic region than proton pumps [38,42,43]. Therefore, large conformational changes on the cytoplasmic side might not be needed for the uptake of sodium ions. In the case of NpHR, detailed structural dynamics have been performed based on the crystal structures of intermediates [44]. Although the authors detected a large deformation of helix 6, the motion did not accompany helix opening at the cytoplasmic surface. These structural dynamics in the literature for light-driven sodium and chloride pumps are consistent with the mechanism of G-protein activation by chimeric proteins.

Conclusion

In the present study, chimeric proteins of a light-driven proton pump GR containing the cytoplasmic loop of β2AR successfully activated Gs protein when exposed to light. In contrast, no G-protein activation was observed in chimeric proteins of a light-driven sodium pump IndiR2 and a light-driven chloride pump NpHR carrying the cytoplasmic loop of β2AR. This fact suggests that the helix opening motion, which is common to GPCR and GR, is different for IndiR2 and NpHR. Thus, GR chimera can serve as a potential tool in optogenetics, where the activation of various G-proteins can be initiated by light.

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Conflicts of Interest

All authors declare that they have no conflicts of interest.

Author Contributions

H.K. directed the research, and wrote the manuscript. K.Y. prepared samples with the help of K.S. and K.I. T.Y. performed the G-protein activation assay with the help of K.Y. and Y.S. K.Y. measured light-induced difference FTIR spectra. All authors discussed and commented on the manuscript.

References

[1] Ernst, O. P., Lodowski, D. T., Elstner, M., Hegemann, P., Brown, L. S. & Kandori, H. Microbial and animal rhodopsins: structures, functions, and molecular mechanisms. Chem. Rev. 114, 126–163 (2014).
[2] Shichida, Y. & Matsuyama, T. Evolution of opsins and phototransduction. Philos. Trans. R. Soc. Lond. B Biol. Sci. 364, 2881–2895 (2009).
[3] Hofmann, K., Lanyi, J., Scheerer, P., Hildebrand, P. W., Choe, H. W., Park, J. H., Heck, M., et al. A G protein-coupled receptor at work: the rhodopsin model. Trends Biochem. Sci. 34, 540–552 (2009).
[4] Palczewski, K. Chemistry and biology of vision. J. Biol. Chem. 287, 1612–1619 (2012).
[5] Haupts, U., Titor, J. & Oesterhelt, D. Closing in on bacteriorhodopsin: progress in understanding the molecule. Annu. Rev. Biophys. Biomol. Struct. 28, 367–399 (1999).
[6] Lanyi, J. K. Bacteriorhodopsin. Annu. Rev. Physiol. 66, 665–688 (2004).
[7] Grote, M., Engelhard, M. & Hegemann, P. Of ion pumps, sensors and channels—perspectives on microbial rhodopsins between science and history. Biochim. Biophys. Acta 1837, 533–545 (2014).
[8] Inoue, K., Kato, Y. & Kandori, H. Light-driven ion-translocating rhodopsins in marine bacteria. Trends Microbiol. 23, 91–98 (2015).
[9] Govorunova, E. G., Sineshchekov, O. A., Li, H. & Spadich, J. L. Microbial rhodopsins: diversity, mechanisms, and optogenetic applications. Annu. Rev. Biochem. 86, 845–872 (2017).
[10] Boyden, E. S., Zhang, F., Bamberg, E., Nagel, G. & Deisseroth, K. Millisecond-timescale, genetically targeted optical control
of neural activity, *Nat. Neurosci.* 8, 1263–1268 (2005).

[11] Zhang, F., Wang, L.-P., Brauner, M., Liewald, J. F., Kay, K., Watzke, N., *et al.* Multimodal fast optical interrogation of neural circuitry. *Nature* **446**, 633–639 (2007).

[12] Chow, B. Y., Han, X., Dobry, A. S., Qian, X., Chuong, A. S., Li, M., *et al.* High-performance genetically targetable optical neural silencing by light-driven proton pumps. *Nature* **463**, 98–102 (2010).

[13] Airan, R. D., Thompson, K. R., Fenno, L. E., Bernstein, H. & Deisseroth, K. Temporally precise in vivo control of intracellular signalling. *Nature* **458**, 1025–1029 (2009).

[14] Koyanagi, M., Kawanabe, A., Nakatsuma, A., Yamashita, T., Sasaki, K., Kamo, N., Demura, M., *et al.* Hydrogen-bonding alterations of the protonated Schiff base and water molecule in the chloride pump of *Natronobacterium pharaonis*. *Biochim. Biophys. Acta* **1793**, 2279–2289 (2009).

[15] Zhang, F., Wang, L.-P., Brauner, M., Liewald, J. & Kandori, H. Structural changes of the complex between halorhodopsin and its cognate transducer upon formation of the M photoreceptor. *Biochemistry* **44**, 2900–2915 (2005).

[16] Kajimoto, K., Kikukawa, T., Nakashima, H., Yamaryo, H., Saito, Y., Fujisawa, T., *et al.* Transient resonance Raman spectroscopy of a light-driven sodium-ion-pump rhodopsin from *Indibacter alkalophilus*. *J. Phys. Chem. B* **121**, 4431–4437 (2017).

[17] Váró, G., Brown, L. S., Sasaki, J., Kandori, H., Maeda, A., Needleman, R., *et al.* Light-driven chloride ion transport by halorhodopsin from *Natronobacterium pharaonis*. 1. The photochemical cycle. *Biochemistry* **34**, 14490–14499 (1995).

[18] Shibata, M., Muneda, N., Sasaki, T., Shichida, Y., Kamo, N. & Lanyi, J. Crystal structure of the light-driven chloride pump halorhodopsin from *Natronomonas pharaonis*. *J. Mol. Biol.** 396**, 564–579 (2010).

[19] Rasmussen, S. G., Choi, H. J., Rosenbaum, D. M., Koblika, B. K. The structure and function of G-protein-coupled receptors. *Nature* **459**, 356–363 (2009).

[20] Deupi, X., Standfuss, J. & Schertler, G. Conserved activation pathways in G-protein-coupled receptors. *Biochem. Soc. Trans.** 40**, 383–388 (2012).

[21] Deupi, X. Relevance of rhodopsin studies for GPCR activation. *Biochem. Biophys. Acta* **1837**, 674–682 (2014).

[22] Sudo, Y., Iwamoto, M., Shimono, K., Sumi, M. & Kamo, N. Photo-induced proton transport of phorahaon phorbadorphodin (sensory rhodopsin II) is ceased by association with the transducer. *Biophys. J.* **89**, 916–922 (2001).

[23] Inoue, K., Ono, H., Abe-Yoshizumi, R., Yoshizawa, S., Ito, H., Kogure, K., *et al.* A light-driven sodium ion pump in marine bacteria. *Nat. Commun.* **4**, 1678 (2013).

[24] Kandori, H. Ion-pumping microbial rhodopsins. *Front. Mol. Biosci.* **2**, 52 (2015).

[25] Schobert, B. & Lanyi, J. K. Halorhodopsin is a light-driven chloride pump. *J. Biol. Chem.* **257**, 10306–10313 (1982).

[26] Esseln, L. O. Halorhodopsin: light-driven ion pumping made simple? *Curr. Opin. Struct. Biol.* **12**, 516–522 (2002).

[27] Kandori, H., Shimono, K., Sudo, Y., Iwamoto, M., Shichida, Y. & Kamo, N. Structural changes of *Phorahason phorbadorphodin* upon photoisomerization of the retinal chromophore: infrared spectral comparison with bacteriorhodopsin. *Biochemistry* **40**, 9238–9246 (2001).

[28] Yamashita, T., Terakita, A. & Shichida, Y. Distinct roles of the second and third cytoplasmic loops of bovine rhodopsin in G protein activation. *J. Biol. Chem.* **275**, 34272–34279 (2000).

[29] Hashimoto, K., Choi, A. R., Furutani, Y., Jung, K. H. & Kandori, H. Low-temperature FTIR study of Gloeobacter rhodopsin: presence of strongly hydrogen-bonded water and long-range structural protein perturbation upon retinal photoisomerization. *Biochemistry* **49**, 3343–3350 (2010).

[30] Kandori, H., Yamaizaki, Y., Shichida, Y., Raap, J., Lugtenburg, J., Belenky, M., *et al.* Tight Asp-85--Thr-89 association during the pump switch of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA** 98**, 1571–1576 (2001).

[31] Furutani, Y., Kamada, K., Sudo, Y., Shimono, K., Kamo, N. & Kandori, H. Structural changes of the complex between *Phorahason phorbadorphodin* and its cognate transducer upon formation of the M photoreceptor. *Biochemistry* **44**, 2900–2915 (2005).

[32] Deupi, X., Standfuss, J. & Schertler, G. Conserved activation pathways in G-protein-coupled receptors. *Biochem. Soc. Trans.** 40**, 383–388 (2012).

[33] Koyanagi, M., Takano, K., Tsukamoto, H., Ohtsu, K., Demura, M., *et al.* Hydrogen-bonding alterations of the protonated Schiff base and water molecule in the chloride pump of *Natronobacterium pharaonis*. *Biochemistry* **44**, 12279–12286 (2005).

[34] Shibata, M., Muneda, N., Sasaki, T., Shichida, Y., Kamo, N. & Lanyi, J. Crystal structure of the light-driven chloride pump halorhodopsin from *Natronomonas pharaonis*. *J. Mol. Biol.** 396**, 564–579 (2010).

[35] Rasmussen, S. G., Choi, H. J., Rosenbaum, D. M., Koblika, B. K. The structure and function of G-protein-coupled receptors. *Nature* **459**, 356–363 (2009).

[36] Deupi, X., Standfuss, J. & Schertler, G. Conserved activation pathways in G-protein-coupled receptors. *Biochem. Soc. Trans.** 40**, 383–388 (2012).

[37] Deupi, X. Relevance of rhodopsin studies for GPCR activation. *Biochem. Biophys. Acta* **1837**, 674–682 (2014).

[38] Sudo, Y., Iwamoto, M., Shimono, K., Sumi, M. & Kamo, N. Photo-induced proton transport of phorahaon phorbadorphodin (sensory rhodopsin II) is ceased by association with the transducer. *Biophys. J.* **89**, 916–922 (2001).

[39] Inoue, K., Ono, H., Abe-Yoshizumi, R., Yoshizawa, S., Ito, H., Kogure, K., *et al.* A light-driven sodium ion pump in marine bacteria. *Nat. Commun.* **4**, 1678 (2013).

[40] Kandori, H. Ion-pumping microbial rhodopsins. *Front. Mol. Biosci.* **2**, 52 (2015).

[41] Schobert, B. & Lanyi, J. K. Halorhodopsin is a light-driven chloride pump. *J. Biol. Chem.* **257**, 10306–10313 (1982).

[42] Essen, L. O. Halorhodopsin: light-driven ion pumping made simple? *Curr. Opin. Struct. Biol.* **12**, 516–522 (2002).

[43] Kandori, H., Shimono, K., Sudo, Y., Iwamoto, M., Shichida, Y. & Kamo, N. Structural changes of *Phorahason phorbadorphodin* upon photoisomerization of the retinal chromophore: infrared spectral comparison with bacteriorhodopsin. *Biochemistry* **40**, 9238–9246 (2001).

[44] Yamashita, T., Terakita, A. & Shichida, Y. Distinct roles of the second and third cytoplasmic loops of bovine rhodopsin in G protein activation. *J. Biol. Chem.* **275**, 34272–34279 (2000).

[45] Hashimoto, K., Choi, A. R., Furutani, Y., Jung, K. H. & Kandori, H. Low-temperature FTIR study of Gloeobacter rhodopsin: presence of strongly hydrogen-bonded water and long-range structural protein perturbation upon retinal photoisomerization. *Biochemistry* **49**, 3343–3350 (2010).

[46] Kandori, H., Yamaizaki, Y., Shichida, Y., Raap, J., Lugtenburg, J., Belenky, M., *et al.* Tight Asp-85--Thr-89 association during the pump switch of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA** 98**, 1571–1576 (2001).

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