A unique choanoflagellate enzyme rhodopsin exhibits light-dependent cyclic nucleotide phosphodiesterase activity

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Phototransduction constitute a major route to manipulate cell functions. Although microbial rhodopsins are being widely studied as light-gated ion channels, their ability to modulate cellular metabolism has been largely overlooked. Here, we report the discovery of a novel light-activated cyclic nucleotide phosphodiesterase (PDE) from the choanoflagellate Salpingoeca rosetta. This enzyme consists of a C-terminal PDE domain and a N-terminal rhodopsin domain. Light illumination activated the PDE domain and increased intracellular cAMP concentrations in HEK293 cells. This PDE activation was specific and light-dependent, and its activation was not observed in wild-type cells. Furthermore, the rhodopsin domain retained full photoreceptor activity, and the PDE activity was stable and reproducible. These findings suggest that this enzyme has potential applications in optogenetics, and it may serve as a model for understanding the evolution of light-activated enzymes.

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lowering cyclic nucleotide concentration has not been known until now. It should be noted that engineered light-activated cyclic nucleotide phosphodiesterase (PDE) (26) and Gi-coupled animal opsin (27) are able to lower cyclic nucleotide concentration upon illumination. The former combines a GAF (cGMP-binding PDEs, adenylyl cyclase, and FhlA) domain and PDE domain, which allows control of the PDE activity by light (26). However, because of the photochromic nature of the GAF domain, its light-induced enzymatic activity has to be stopped by light of a different color. Gi-coupled animal opsin from mosquitoes (Opn3) decreases cAMP concentration under illumination, because Gi inhibits the activity of adenylyl cyclase (AC) (27). On the other hand, enzymatic activity of mosquito Opn3 persists for several tens of minutes (27), being much longer than that of Rh-GC (12, 13), presumably because of the AC-related signaling network. In addition, GPCRs regulate other signaling cascades through G proteins and arrestin, triggering complex intracellular events. Therefore, proteins composed of a light-sensor domain and a PDE domain, with fast enzymatic turnover induced by single light excitation, are highly desirable.

In this paper, we report on a novel type of microbial rhodopsin with a C-terminal PDE domain, found in the choanoflagellate Salpingoeca rosetta, a unicellular and colonial single flagellate eukaryote, considered to be one of the closest living relatives of metazoans. We successfully expressed and purified the full-length Rh-PDE from mammalian cells, and its enzymatic functions were studied in cells, in membranes, and in detergent, both biochemically and spectroscopically. We discuss a putative molecular mechanism of Rh-PDE activation based on the presented studies of the full-length protein and its applicability to optogenetics.

Results

A new microbial rhodopsin containing a PDE domain

The genome of the choanoflagellate S. rosetta (sequenced by the Broad Institute, NCBI accession number PRJNA37927) contains an unusual gene (NCBI Gene ID 16078606) encoding a rhodopsin fused to a C-terminal PDE domain (Rh-PDE). Fig. 1A shows a schematic drawing of the Rh-PDE architecture, which is composed of the seven-transmembrane helical rhodopsin domain and the cytoplasmic PDE domain. A phylogenetic tree of selected microbial rhodopsins based on the sequences of the transmembrane domains. Conservation of important residues of the Rh domain. Red and blue colors represent acidic and basic amino acids, respectively. Residue numbers in Rh-PDE and BR are shown. D, conservation of important residues of the PDE domain. Residue numbers in Rh-PDE and PDE10A are shown, and sequences are grouped according to the substrate specificity.

Figure 1. Architecture and amino acid composition of Rh-PDE. A, Rh-PDE is composed of the seven-transmembrane helical rhodopsin domain and the cytoplasmic PDE domain. B, phylogenetic tree of selected microbial rhodopsins based on the sequences of the transmembrane domains. C, conservation of important residues of the Rh domain. Red and blue colors represent acidic and basic amino acids, respectively. Residue numbers in Rh-PDE and BR are shown. D, conservation of important residues of the PDE domain. Residue numbers in Rh-PDE and PDE10A are shown, and sequences are grouped according to the substrate specificity.

Rhodopsin-PDE
of BR (Trp-86, Thr-89, Trp-182, and Tyr-185) (1) are also conserved as Trp-165, Thr-168, Trp-264, and Phe-267, respectively, in Rh-PDE. The PDE domain contains many conserved residues typical for the active centers of soluble PDEs (Fig. 1D), but it is not possible to predict the substrate specificity of Rh-PDE (cAMP, cGMP, or both) from the sequence.

Enzymatic activity of Rh-PDE in mammalian cells

To investigate enzyme rhodopsins of lower eukaryotes, it is important to find a suitable expression system. Previous studies on Rh-GC used yeast expression, where the full-length protein could not be obtained (12). In this study, we first measured enzymatic activity of Rh-PDE using HEK293 cells, from which the full-length protein was subsequently purified and characterized in vitro. To measure the in-cell enzymatic activity of Rh-PDE, we used the GloSensor assay, which is based on a cyclic nucleotide-dependent luciferase. In this assay, the Rh-PDE (cAMP, cGMP, or both) activity was measured in HEK293 cells with the empty vector (top), Rh-PDE (middle), and the PDE-inactive D605A mutant (bottom). HEK293 cells were preincubated in the culture medium with all-trans-retinal in the dark.

Figure 2. Enzymatic activity of Rh-PDE in HEK293 cells. A, luminescence signals representing cGMP levels, as observed in HEK293 cells with the empty vector (top), Rh-PDE (middle), and the PDE-inactive D605A mutant (bottom). HEK293 cells were preincubated in the culture medium with all-trans-retinal in the dark. The arrows and vertical light-blue line indicate sodium nitroprusside treatments and irradiation at 510 nm with 2.28 μW mm⁻² intensity, respectively, and the luminescence signals of non-irradiated cells are shown as a control (black lines). B, luminescence signals representing cAMP level, as observed in HEK293 cells with the empty vector (top), Rh-PDE (middle), and the PDE-inactive D605A mutant (bottom). HEK293 cells were preincubated in the culture medium with all-trans-retinal in the dark. The arrows and vertical light-blue line indicate forskolin treatments and irradiation at 510 nm with 2.28 μW mm⁻² intensity, respectively, and the luminescence signals of non-irradiated cells are shown as a control (black lines). Only HEK293 cells expressing Rh-PDE (red line in the middle panel) exhibited a decrease of the luminescence signal, indicating that cAMP concentration is lowered by light. C, changes of luminescence signals upon 2-min 510-nm irradiation (light-blue line) of HEK293 cells with the empty vector (top), Rh-PDE (middle), the PDE-inactive D605A mutant (bottom), expanded from B. Colored and black lines represent the data with and without irradiation, respectively. D, intensity dependence of the light-induced cAMP concentration decrease by Rh-PDE (cells were irradiated for 2 min with 510-nm light with 0–2.28 μW mm⁻² intensities). E, peak amplitudes of luminescence decrease plotted against the light intensity (n = 5 cells). Error bars, S.D. The half-maximal light intensity (EC₅₀) was determined to be 0.244 μW mm⁻² by a single exponential decay fit (red dashed line).

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The luminescence intensity gradually increased after the addition of sodium nitroprusside and reached a plateau after 40 min for HEK293 cells expressing GloSensor only (empty vector). As expected, there was no effect of light on the level of cGMP in this system (Fig. 2A, top). In contrast, such a gradual increase of luminescence after addition of sodium nitroprusside has not been observed upon expression of Rh-PDE (Fig. 2A, middle), which suggests constitutive activity of Rh-PDE toward cGMP, both in the dark and upon illumination. Consistent with this idea, light-independent gradual increase of luminescence occurred for the D605A mutant (Fig. 2A, bottom), with inactivating replacement in the active center of the PDE domain.

Fig. 2, B and C, shows the activity of Rh-PDE toward cAMP. The luminescence intensity gradually increased after the addition of forskolin and reached a plateau after 40 min, similar to the cGMP assay (Fig. 2B). At that time point, illumination at 510 nm with 2.28 μW mm⁻² intensity caused a decrease of the luminescence in cells expressing Rh-PDE (red line) but not in those with the empty vector (blue line) (Fig. 2, B and C). 28 ± 2.3% (S.D.) reduction in the luminescence intensity was reproducibly observed under these experimental conditions (n = 6). Thus, it can be concluded that the decrease of cAMP concentration must originate from the light-induced Rh-PDE activity, which is further supported by the lack of signal for the catalytically inactive D605A mutant (Fig. 2, B and C, yellow line). It should be noted that after the light was switched off, the light-
induced luminescence decrease persisted for a significant amount of time (~10 s) and then decayed with \( \tau_{1/e} \) of 195 s (Fig. 2C, red line). This kinetics involves at least two components; deactivation of Rh-PDE and production of cAMP by endogenous adenylyl cyclases. Based on the exponential fitting, we estimate the lifetime of the light-activated state of Rh-PDE as about 71 s (supplemental Fig. S3). Another notable feature is that the stationary luminescence intensity in the dark was smaller for Rh-PDE (Fig. 2B, middle) than for the empty vector and the D605A mutant (Fig. 2B, top and bottom), suggesting constitutive dark activity toward cAMP, although smaller than toward cGMP. Fig. 2D shows that the light-induced reduction of luminescence increased with the applied light intensity, and saturated with an EC\textsubscript{50} of 0.244 \( \mu \text{W/mm}^2 \) (Fig. 2E). One could expect that the intensity increase was a sigmoidal function if any two-photon processes were involved (e.g., both protein molecules in a dimer need to be activated). Nearly linear increase at lower light intensities in Fig. 2E suggests that Rh-PDE activation is a single-photon event, and activation of one molecule is sufficient even if Rh-PDE is likely to form a dimer. In summary, the in-cell assays demonstrated that Rh-PDE shows strong constitutive activity toward cGMP, whereas the activity toward cAMP is light-dependent.

**Suitability of Rh-PDE for optogenetic applications**

The observed light-dependent decrease of intracellular cAMP concentration suggests that Rh-PDE may be useful as an optogenetic tool. Together with PAC and G\textsubscript{i}-coupled opsins capable of increasing intracellular cAMP concentration, Rh-PDE increases the diversity of the optogenetics toolkit. Unlike the engineered light-activated PDE (26), Rh-PDE is switched off by its photocycle, without the need for an additional illumination. On the other hand, G\textsubscript{i}-opsin, which can decrease intracellular cAMP concentration (27), also switches off in the dark, warranting a comparison of the properties of Rh-PDE and G\textsubscript{i}-opsin.

Thus, we expressed mosquito Opn3 in HEK cells with the addition of all-trans-retinal, using the same protocol as for Rh-PDE. Although the native chromophore of mosquito Opn3 is 11-cis-retinal, it is known that this opsin binds 13-cis-retinal, which can be easily formed from all-trans-retinal, and that the regenerated pigment shows an absorption maximum at 500 nm (27). Upon application of the 510-nm light, the cells expressing mosquito Opn3 show a strong decrease in the intracellular cAMP concentration, which continues to decrease after the light is switched off, reaching a minimum in ~5 min, and then recovers with \( \tau_{1/e} \) of 16 min (Fig. 3A, green line). These data reproduce the data reported previously (27), and it is obvious that the luminescence signal is smaller and faster (both in the rise and the decay) in the case of Rh-PDE under the same illumination conditions (Fig. 3A, red line). In the case of mosquito Opn3, intermolecular protein-protein interactions between opsin and G\textsubscript{i} and between G\textsubscript{i} and AC should amplify the signal. Thus, as an optogenetic tool, Rh-PDE exhibits a less sensitive, but about 5 times faster, response than mosquito Opn3.

**Figure 3. Rh-PDE as a potential optogenetic tool.** A, comparison of in-cell hydrolytic activities of Rh-PDE (top) and mosquito Opn3 (bottom) toward cAMP. The top panel is reproduced from Fig. 2C. Mosquito Opn3 was expressed in HEK293 cells similar to Rh-PDE. During the time period shown in light blue, 510-nm light with 2.28 \( \mu \text{W/mm}^2 \) intensity was applied to the cells. B, effect of the addition of all-trans-retinal on the Rh-PDE activity. C, repeatability of the in-cell Rh-PDE activation. 2-min irradiation at 510 nm with 2.28 \( \mu \text{W/mm}^2 \) intensity was repeated every 15 min, and other conditions were as in Fig. 2C. D, irradiation wavelength dependence of the in-cell activity of Rh-PDE toward cAMP. All of the data were obtained from experiments at the same light intensity (0.4 \( \mu \text{W/mm}^2 \)).
addition, Rh-PDE only acts on cyclic nucleotides, whereas additional intracellular effects of mosquito Opn3 are unclear.

Another important parameter for optogenetic applications is chromophore availability. We compared the activity of Rh-PDE toward cAMP with and without the addition of all-trans-retinal in the cell culture medium after transfection (Fig. 3B). Although the luminescence decrease is smaller without the addition of all-trans-retinal, it still retains robust activity at 70% of the amplitude upon its addition, which is explained by the fact that all-trans-retinal is commonly present in cells. Although mosquito Opn3 11-cis-retinal chromophore is not abundant in cells, Koyanagi et al. (27) found that mosquito Opn3 can bind 13-cis-retinal, a ubiquitous present retinal isomer. Because 13-cis- and all-trans-retinals exist in photoequilibrium, mosquito Opn3 is usable in general tissues, although its activity without the addition of 11-cis-retinal was reduced >2-fold (27). In contrast, Rh-PDE binds naturally abundant all-trans-retinal, and the active state is rapidly reverted to the resting state as a result of the photocycle. This comes along with the high repeatability, another important property for optogenetics. Fig. 3C shows that activation of Rh-PDE can be repeated for longer than 2 h, showing the suitability of Rh-PDE as an optogenetic tool. To define the usable spectral range of Rh-PDE, we systematically changed the irradiation wavelength at the same light intensity (Fig. 3D) and found that the luminescence is maximally decreased by the blue-green light (480–510 nm).

HPLC analysis of the enzymatic activity of Rh-PDE

We further characterized the enzymatic activity of Rh-PDE by HPLC analysis using crude membranes of HEK293 cells. In these experiments, cGMP or cAMP was mixed with the crude cell membranes expressing Rh-PDE, and time- and light-dependent hydrolysis of cyclic nucleotides into 5’-GMP or 5’-AMP was monitored by HPLC. Fig. 4A shows the HPLC profile for cGMP, where Rh-PDE decreases the amount of cGMP and concomitantly increases that of 5’-GMP. Importantly, a 10-min incubation under illumination results in an 85% decrease of cGMP concentration, compared with only 60% in the dark (Fig. 4B). This indicates that Rh-PDE has light-dependent enzymatic activity toward cGMP, although its dark constitutive activity is significant. The hydrolysis rates were calculated from the slopes of the curves (0–10 min) in Fig. 4B as 353 nmol/mg total protein/min in the dark and 511 nmol/mg total protein/min under illumination, giving 142% activation by light.

Fig. 4D shows the HPLC profiles for cAMP in the dark (top) and under illumination (bottom). Rh-PDE decreases cAMP concentration in both cases, but the activity is smaller compared with that for cGMP. It should be noted that a 10-fold higher amount of the membranes was used for the cAMP hydrolysis assay (see “Experimental procedures”). A clear light-dependent activation can be seen for the cAMP hydrolysis, as was observed in the cGMP assay above. The hydrolysis rates were calculated as 32.6 nmol/mg total protein/min in the dark and
53.6 nmol/mg total protein/min under illumination, giving ~164% stimulation by light (Fig. 4, E and F). The activity toward cGMP is ~10-fold higher than toward cAMP (Fig. 4), consistent with the in-cell results.

Hydrolysis of cGMP and cAMP was entirely abolished in the D605A mutant, with inactivating replacement in the active center of the PDE domain (data not shown). Although crude cell membranes contain many other proteins, the comparison between the wild-type and the D605A mutant Rh-PDE indicates that the observed hydrolysis of both nucleotides originates exclusively from Rh-PDE, consistent with the in-cell results (Fig. 2).

**Molecular properties of purified full-length Rh-PDE**

The in-cell and in-membrane studies described above showed that the enzymatic activity of Rh-PDE is stimulated by light toward both cAMP and cGMP. To gain insight into the molecular mechanism of photoactivation, it is necessary to purify and characterize the full-length protein. Although expression levels of rhodopsins are usually much smaller in HEK293T cells than in yeast and *Escherichia coli*, previously, we have been able to conduct infrared spectroscopic studies on monkey red- and green-sensitive pigments based on HEK293 cell expression (31, 32). We thus applied the sample preparation procedure as used for monkey red- and green-sensitive pigments to Rh-PDE, using HEK293T cell expression, solubilization by dodecyl-β-D-maltopyranoside (DDM), and purification by monoclonal antibody column chromatography, with all-trans-retinal being added into the culture medium. As a result, we were able to obtain purified full-length protein (Fig. 5A, inset) with yields comparable with those of monkey color pigments (0.4 mg of Rh-PDE versus 0.3 mg of monkey color pigments from 300 culture plates of HEK293 cells with a 10-cm diameter (31, 32)).

We characterized the photoreactions of the purified full-length Rh-PDE using UV-visible spectroscopy. Fig. 5A shows the absorption spectrum of Rh-PDE in the dark (black) and after irradiation with 510-nm light (red). The activity toward cGMP is ~10-fold higher than toward cAMP (Fig. 4), consistent with the in-cell results (Fig. 2).

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**Figure 5. Molecular properties of purified full-length Rh-PDE.** A, absorption spectra of full-length Rh-PDE in the dark (black) and after irradiation with 510-nm light (red) at 4 °C. Inset, Coomassie Brilliant Blue-stained purified Rh-PDE on 12% SDS-PAGE. The arrow corresponds to about 70 kDa. B, absorption spectrum of purified full-length Rh-PDE (dotted line; reproduced from the black line in A) superimposed with the action spectrum of the light-induced enzymatic activity toward cAMP in HEK293T cells (the peak amplitudes in Fig. 3D are plotted versus irradiation wavelength with 0.4 μW mm⁻² intensity). C, difference absorption spectra of the photoactivation. D, M decay and the parent state recovery kinetics at 4 °C. E, Arrhenius plot for the M-decay kinetics. From the linear fit (dotted line), the decay time constant at 27 °C was estimated to be 6.78 s (red point). Error bars, S.D.
from instability of the oligomer in the heterologous expression. Rh-PDE is derived from the background activity of the PDE in the dark (12). Whether or not the high constitutive activity of cyclase activity, in which the enzyme has only 1% basal activity, is expected to contribute to the tight regulation of the enzymatic activity remains a question. In fact, the Rh-GC exhibits a very tight light-dependent activity (Fig. 1A). The data in Fig. 4 show that Rh-PDE exhibits light-dependent hydrolysis mechanism (Fig. 3C). To estimate the M decay kinetics under the conditions of the enzyme activity experiments (27 °C), we measured its temperature dependence. As expected, the M decay becomes faster at higher temperatures (Fig. 5E), and the temperature dependence follows the Arrhenius behavior well (Fig. 5F), with an activation energy of 57.8 kJ/mol, and activation enthalpy and entropy of 55.3 kJ/mol and −76.5 J/mol·K, respectively. We estimated the M decay time constant at 27 °C to be 6.78 s, which suggests that the enzymatic activity persists well after its decay.

We co-expressed the 1D4-tagged Rh-PDE and its eGFP-tagged version in HEK293 cells followed by solubilization and purification by 1D4-agarose resin. The eluate contained not only the 1D4-tagged protein but also the eGFP-tagged version, judging from the fluorescence spectrum and SDS-PAGE (supplemental Fig. S4). This result indicates that Rh-PDE forms stable oligomers that survive solubilization.

Discussion

We report on a novel type of microbial rhodopsin from the choanoflagellate S. rosetta containing a C-terminal PDE domain. Animal rhodopsins and sensory microbial rhodopsins have their respective transducers (G proteins, Htr membrane transducers, and others) as separate proteins (1–8). In contrast, enzyme rhodopsins such as Rh-HK, Rh-GC, and Rh-PDE contain covalently bound enzymatic domains, whose activation mechanism is unknown and intriguing. Above, we presented data showing that Rh-PDE exhibits light-dependent hydrolysis activity both toward cGMP and cAMP while retaining some levels of constitutive activity in the dark. The data in Fig. 4 show that Rh-PDE has higher activity toward cGMP than toward cAMP, both in the dark and in the light-activated form. It is known that substrate specificity differs among PDE families, as shown in Fig. 1D (28). The observed dark activity was not anticipated, because the light-sensitive rhodopsin domain was expected to contribute to the tight regulation of the enzymatic activity. In fact, the Rh-GC exhibits a very tight light-dependent cyclase activity, in which the enzyme has only 1% basal activity in the dark (12). Whether or not the high constitutive activity of Rh-PDE is derived from the background activity of the PDE domain, from a specific conformation of the rhodopsin part, or from instability of the oligomer in the heterologous expression system will have to be addressed in future studies. At this point, we believe that it is logical to suggest that the membrane and cellular environments differ between S. rosetta and HEK293 cells, preventing hydrolytic activity in the dark in the former. For example, it is possible that the PDE domain is post-translationally modified (phosphorylated), or other regulatory protein(s) associate with it and keep Rh-PDE inactive in S. rosetta in the dark.

During the in-cell cyclic nucleotide concentration measurements by the GloSensor assay, a light-dependent cAMP decrease was observed (Fig. 2), consistent with the results obtained with crude membranes shown in Fig. 4. In contrast, we did not observe any light-dependent change of the intracellular cGMP level in the GloSensor assay in Fig. 2A but observed an enhancement of the PDE-cGMP activity in crude membranes by light. This can be explained, taking into account that the cGMP hydrolysis rate by Rh-PDE is about 10-fold faster than that for cAMP (Fig. 4, C and F). Thus, the dark activity toward cGMP is high enough to completely degrade cellular cGMP, preventing observation of the effects of light. In addition, the concentration of endogenous cGMP is about 10-fold lower than that of cAMP, which is another plausible reason for our observations.

Based on the presented results, we propose the activation mechanism of Rh-PDE shown in Fig. 6. We suggest that Rh-PDE exists in a putative dimeric form, consistent with the reported PDE structures (21, 22). The crystal structure of the full-length PDE-2A, where the elongated C-terminal helix of the GAF domain directly connects to the catalytic region of the PDE domain (30), may offer some insights into the architecture of Rh-PDE. In Rh-PDE, the linker region between the end of transmembrane helix 7 of the rhodopsin domain and the beginning of the catalytic region contains 50–60 residues (supplemental Fig. S1), which corresponds to 7–8 nm in length, assuming a single helix. On the other hand, it is well known that the HAMP (histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases) domain, which connects transmembrane and cytosolic signaling domains in many sensory proteins, forms a coiled-coil bundle of two amphipathic helices (34, 35). Indeed, two such helical amphipathic sequences can be seen in the linker region of Rh-PDE (supplemental Fig. S1). Therefore, we suggest that transmembrane helix 7 of the Rh domain elongates and forms the HAMP-like domain, which connects to the catalytic region of the PDE domain (Fig. 6). Dimer formation of the PDE domain makes the catalytic region inaccessible to the cytoplasmic bulk, so that the PDE activity remains shut off in the dark (a condition which may not be fully reproduced in experiments with HEK293 cells).

The resting state of Rh-PDE shows the A492 max at 492 nm, which coincides well with the action spectrum of the light-induced enzymatic activity (Fig. 5B). The absorption in the visible region indicates that the chromophore Schiff base is protonated in Rh-PDE. In light-driven proton pump BR, the protonated Schiff base, Arg-82, Asp-85, and Asp-212 constitute a quadrupole inside the protein, where Asp-85 acts as a primary counterion of the Schiff base (1). Similarly, the Schiff base, Arg-161, Glu-164, and Asp-292 probably form a quadrupole in Rh-PDE (Fig.
1C). The absorption spectrum is considerably blue-shifted compared with other microbial rhodopsins and is close to those of SRII and channelrhodopsin-2 (ChR2). The position corresponding to Ala-215 in BR is Thr in SRII, whose side chain OH group contributes to the blue spectral shift (36, 37). ChR2 has O-H bearing Ser at the homologous position as well, but Rh-PDE has Ala (Fig. 1C), so that the mechanism of its blue-shifted absorption is not clear.

After the photoexcitation, the M intermediate with deprotonated retinal Schiff base forms in the Rh-PDE photocycle (Fig. 5, A and C), similar to many other microbial and animal rhodopsins. In many cases, proton transfer from the Schiff base induces large structural changes, leading to the respective functional processes (1). In Rh-PDE, the M formation probably triggers protein structural changes at the cytoplasmic surface of the rhodopsin domain, which results in the light-induced increase in PDE activity. The observed increase in the in-cell enzymatic activity toward cAMP for ~10 s after the light was switched off (Fig. 2C) coincides with the time scale of the M decay (7 s; Fig. 5F), suggesting that the enzymatic activity is not yet induced in the PDE domain in the M intermediate. In other words, structural changes of the Rh domain in the M intermediate lead to those of the PDE domain at the later times after photoactivation, presumably altering the structure of helix 7 and the HAMP-like domain (Fig. 6). Linear light intensity dependence of the activity toward cAMP (Fig. 2E) suggests that activation of just one Rh domain in a putative dimer is sufficient to trigger an increase in the enzymatic activity. An alternative (although less plausible) explanation for the linear intensity dependence is that Rh-PDE is monomeric in the mammalian membranes, which could also explain its significant dark activity, although this contradicts our results with eGFP-tagged Rh-PDE, which suggest oligomerization (supplemental Fig. S4).

The recovery of the original spectral state occurs parallel to the M decay (Fig. 5D), suggesting that the photocycle completes in ~7 s at 27 °C (~45 s at 4 °C). This fact appears to contradict the long-lived enzymatic activity (71 s) of PDE in cells (Fig. 2C and supplemental Fig. S3). Because the light absorption originates from the retinal chromophore in the membrane core of the Rh domain, it may be not sensitive to the conformation of the PDE domain, which probably switches on upon the transition from the M intermediate to the initial spectral state in the Rh domain. Structural changes of the Rh domain, particularly in helix 7 (and ensuing changes in the HAMP-like domain) possibly dissociate the PDE dimer, leading to the two catalytic domains being exposed to the aqueous phase (Fig. 6). Photocycles of microbial rhodopsins are generally too short to keep sustained enzymatic activity, so that a special mechanism is needed to achieve this. The presence of the two domains, Rh and PDE, connected through the HAMP-like linker could allow for 10 times longer enzymatic activity than the photocycle of the Rh domain alone.

The molecular mechanism of the light-activated sensory function has been extensively studied for SRII, which forms a 2:2 complex with two transmembrane helical Htr transducer (38, 39). The linker region of the transducer contains a HAMP-like domain. The M-intermediate structure of the receptor-transducer complex (40) and numerous EPR studies (41, 42) revealed significant outward tilting of the cytoplasmic half of helix F in SRII, which causes rotational motion of the second
Rhodopsin-PDE

transmembrane helix of the transducer. Outward motion of helix F on the cytoplasmic side is common for activation of microbial and animal rhodopsins and also causes motion of helix G. Presently, there is no structural information on Rh-PDE, and further studies will be needed to reveal the activation mechanism of the PDE domain by light absorption in the Rh domain.

Light control of second messengers is in high demand in optogenetics, and PAC (22–25) and Rh-GC (12, 13) are promising tools for increasing intracellular concentration of cyclic nucleotides by light. In addition to the engineered light-activated PDE (26) and mosquito Opn3 (27), Rh-PDE may become a promising optogenetics tool, but all of them have their specific drawbacks. The enzymatic activity of the engineered light-activated PDE has to be stopped by additional irradiation of a different color due to the photochromic nature of the GAF domain. Although the chromophore of mosquito Opn3 is 11-cis-retinal, not abundant in mammalian cells, it can be used for optogenetics because it can bind 13-cis-retinal. Mosquito Opn3 is more sensitive and has a longer activation time than Rh-PDE, because of the amplification processes. In addition, mosquito Opn3 regulates other signaling cascades, but all of them have their specific drawbacks. The enzymatic activity of the engineered light-activated PDE has to be stopped by additional irradiation of a different color due to the photochromic nature of the GAF domain. Whereas Rh-PDE lowers cAMP concentration by light, its constitutive activity would be problematic for optogenetic applications, because Rh-PDE maintains very low intracellular cGMP levels. This requires further efforts to make it suitable for optogenetic applications, and additional engineering of the active center of the PDE domain is possibly needed.

**Experimental procedures**

**Molecular biology**

A full-length gene encoding Rh-PDE (NCBI Gene ID 16078606) was synthesized after a human codon optimization (GenScript) and cloned into peGFP-N1 vector between HindIII and BamHI sites. We amplified the Rh-PDE gene by PCR with the forward primer encoding the first 20 bp and the reverse primer encoding the last 15 bp of the Rh-PDE gene followed by the 1D4 epitope tag (TETSQVAPA) sequence. The amplified fragment was inserted into pCDNA3.1 vector (Invitrogen) between HindIII and XbaI sites. The plasmid for D605A Rh-PDE was generated by QuickChange site-directed mutagenesis kit (Agilent Technologies). The MosOpn3 gene, which lacks 99 C-terminal amino acids (27), was synthesized after a human codon optimization (GenScript) and fused to the cDNA encoding a Venus fluorescent protein and cloned into pCDNA3.1 vector between HindIII and XbaI sites. All constructs were verified by the DNA sequencing. Expression of Rh-PDE with the 1D4 epitope tag was confirmed by Western blotting.

**Assay of the enzymatic activity of Rh-PDE in mammalian cells**

HEK293 cells were transfected with plasmid pCDNA3.1-Rh-PDE by the calcium phosphate method. DMEM/F-12 medium contained 0.5 μM all-trans-retinal and penicillin and streptomycin. The cells were harvested after 24 h and washed in buffer A (140 mM NaCl, 3 mM MgCl₂, 50 mM HEPES-NaOH, pH 6.5). The cells were resuspended with buffer A and homogenized using a Potter–Elvehjem grinder (Wheaton) and a syringe with a 27-gauge needle. The syringe was filled and drained five times while stirring the homogenate. The protein amount was measured using a BCA protein assay (Thermo Fisher Scientific). Catalytic activity was measured at room temperature in 100 μl of buffer A with 1.6–1.8 μg (in the case of cGMP) or 16–18 μg (in the case of cAMP) of total protein in a 1.5-ml sample tube. The sample was illuminated with a xenon lamp (MAX-303, Asahi Spectra Co.) through a Y52 filter (7 mW mm⁻²). Reaction was initiated by adding cyclic nucleotides (final concentration 100 μM). Aliquots were taken out at different time points, and the reactions were immediately terminated by adding 100 μl of 0.1 N HCl and frozen in liquid nitrogen. After thawing, the samples were centrifuged to remove the membranes and denatured proteins. Nucleotides (20 μl of aliquot) were separated by HPLC (Shimadzu Corp., Kyoto, Japan) with a C18 reversed-phase column (Waters) and 100 mM potassium phosphate (pH 5.9), 4 mM tetrabutylammonium iodide, and 10% (v/v) methanol as eluent (12, 26). Nucleotides were monitored at 254 nm. Data were evaluated with LabSolutions (Shimadzu). Peak areas were integrated and assigned to the educt cyclic nucleotide based on retention times of corresponding standard compound.
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Purification of Rh-PDE

HEK293T cells expressing Rh-PDE, described above, were harvested after 24 h and homogenized by a Dounce tissue grinder (Wheaton). Membrane proteins were then extracted with 2% (w/v) DDM in buffer A, followed by centrifugation (6,500 × g, 1 h, 4 °C) to remove unsolubilized fractions. The extract was mixed with 1D4-agarose resin and incubated at room temperature for 15–30 min. After washing the resin with buffer A containing 0.02% (w/v) DDM (buffer B), proteins were eluted with buffer B containing 0.11–0.18 mg/ml 1D4 peptide. Protein fractions were concentrated with Amicon Ultra 30K centrifugal devices (Millipore).

Spectroscopy

The samples were illuminated with a 1-kW tungsten-halogen projector lamp (Master HILUX-HR, Rikagaku) through a Y52 filter (>500 nm), and the absorption spectra of Rh-PDE were recorded at various temperatures by using a JASCO V-650 spectrometer.

Estimation of chromophore regeneration

The extinction coefficient of the full-length Rh-PDE protein was determined as 106,340 m⁻¹ cm⁻¹ based on the protein sequence by using the ProtParam tool of Expasy (43). The absorption change, representing the bleaching of a purified Rh-PDE by hydroxylamine (HA), was measured with a UV-visible spectrometer (Shimadzu) equipped with an integrating sphere. The extinction coefficient of the full-length Rh-PDE protein is 1 cm⁻¹ from the ratio between the absorbance of Rh-PDE at 492 nm and retinal oxime (ε = 33,600 m⁻¹ cm⁻¹ at 360 nm (44)) produced by the reaction between retinal Schiff base and HA. From the values above and the absorptions at 280 and 492 nm in Fig. 5A (black spectrum), the extent of chromophore regeneration was estimated.

Phylogenetic analysis of rhodopsin genes

The amino acid sequences of transmembrane domains of selected rhodopsins (corresponding to residues 9–234 of bacterial rhodopsin) were aligned using ClustalO (45), and the resulting phylogenetic trees were visualized with TreeView (46).

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