Unique Light-gated Ion Channel Properties of a Novel Modular Cation Channelrhodopsin from an Evolutionary Important Terrestrial Green Alga

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Article

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Abstract

Channelrhodopsins are a family of microbial rhodopsins that function as a light-gated ion channel. We report the molecular properties of a novel channelrhodopsin KnRh3 from an evolutionary important filamentous terrestrial alga *Klebsormidium nitens*. KnRh3 is constituted of a 7-transmembrane domain, followed by a long C-terminus moiety that encodes a peptidoglycan binding domain (FimV). When functionally expressed in mammalian cells, KnRh3 showed light-induced cation channel currents. The maximum action spectrum exhibited was at 430 nm and 460 nm, the former making KnRh3 one of the most blue-shifted channelrhodopsins characterized thus far. The channel closure rate was relatively fast ($\tau_{\text{off}} = 10$ ms). Surprisingly, photocurrent kinetics were affected by the C-terminus moiety of KnRh3. When this moiety was truncated to various lengths, this prolonged the channel open lifetime by more than 10-fold. We identified two arginine residues, R287 and R291, those are crucial for altering the kinetics. We propose that electrostatic interaction between the 7-TM domain and the C-terminus domain accelerates the photocycle. The most blue-shifted action spectrum of KnRh3 serves as a novel prototype of channelrhodopsin for studying the molecular mechanism of color tuning. In addition, KnRh3 would expand the optogenetics tool kit, especially for when short wavelength excitation is required.

Introduction

Microbial-type rhodopsins are made up of 7-transmembrane helices with a covalently bound all-*trans* retinal as a chromophore¹. These are found in archaea, bacteria, eukaryotes (such as fungi and algae) and viruses and are physiologically responsible for energy production and phototaxis responses. Molecular functions of microbial rhodopsins involve ion transporters, sensors and light-regulated enzymes^{2,3}. Ion-transporting rhodopsins are divided into pumps and channels. Bacteriorhodopsin was the first identified outward directed proton-pumping rhodopsin⁴. The Cl⁻ pump, the Na⁺ pump, and inward-directed light-gated proton pumps have also been characterized⁵⁻⁸. Detailed structure-function studies together with electrophysiology and molecular dynamics studies revealed the detailed molecular mechanisms of bacteriorhodopsin and other ion-pumping rhodopsins. Channelrhodopsin-1 and -2 (CrChR1 and CrChR2) from *Chlamydomonas reinhardtii* were the first light-gated ion channels to be discovered and characterized^{9,10}. These proteins permeate cations in which the permeability ratio of H⁺, Na⁺ and K⁺ is 10⁶, 1, and 0.5, respectively. High-resolution X-ray structures revealed details of the molecular architecture and provided insight into the photoactivation and ion conduction pathway^{11,12}. Successful expression of CrChR2 in neurons allowed the action potential to be manipulated by light, which initiated a new research field, optogenetics^{13,14}. Channelrhodopsin variants have been engineered to improve the functionality of ChR, and homologous ChRs were also reported accordingly¹⁵. Color-tuning variants of ChRs cover almost the entire visible range of the light spectrum. CrChR2 from *C. reinhardtii* exhibits an action spectrum maximum at 470 nm¹⁰. ChR variants such as C1V1, which is the chimeric version of ChR1 from *C. reinhardtii* and *Volvox carteri*, or C1C2 (green light receiver) absorb around 530 ~ 545 nm light^{16,17}. A further red-shifted ChR, Chrimson from *Chlamydomonas noctigama*, exhibits an

absorption maximum at 590 nm which allows reliable neuronal stimulation over 600 nm light when applied to neurons¹⁸. On the other hand, TsChR or PsChR absorb shorter wavelengths, making it possible to excite neurons at 440 nm¹⁹. The open channel lifetime can be extended by mutations at C128 and D156 (DC pair) which form a hydrogen bond bridge in CrChR2. A mutation at C128 to T, A and S slowed channel closure kinetics by 1000-fold²⁰. The CrChR2 D156C has an even stronger effect, with high light sensitivity and a prolonged open channel lifetime, up to 30 min²¹. Recent genomic database research reveals that various lower eukaryotes contain opsin-related genes. Consequently, a number of ChR genes were identified and their molecular properties, such as ion selectivity and absorption maxima, were shown to be different.

Recently, the genome of an evolutionary important terrestrial green alga (*Klebsormidium flaccidum*) was sequenced, and analysis of the genome delineated the molecular basis of the adaptation of land plants²⁸. *K. flaccidum* was renamed as *Klebsormidium nitens* (http://www.plantmorphogenesis.bio.titech.ac.jp/~algae_genome_project/klebsormidium/). *K. nitens* adapts to diverse environmental conditions such as desiccation, light, and temperature gradients²⁹. However, the sensory photoreceptor landscape of *K. nitens* has not yet been established. For the first time, we identified a ChR homologue that encodes a novel modular channelrhodopsin from an evolutionary important filamentous terrestrial alga, *K. nitens*. A unique feature of KnRh3 is that it includes the FimV domain at the C-terminus of rhodopsin. FimV is involved in peptidoglycan-binding protein which is responsible for twitching motility in *Pseudomonas aeruginosa* and other microbes³⁰. Thus, it is anticipated that light signals are transduced via ChR into motility in *K. nitens*.

In this report, we performed an electrophysiological experiment using KnRh3 to reveal its ion channel function. Its photocurrent properties such as channel kinetics, absorption maximum, light sensitivity and ion selectivity was compared to those of CrChR2. For the first time, we show an unexpected modulation of the photocurrents of KnRh3 by its extended C-terminus region. Novel KnRh3 has the potential to expand the optogenetics tool kit, especially for dual light (two photon) applications when short wavelength excitation is required. Moreover, functional characterization of channelrhodopsin from an evolutionary terrestrial alga indicates the existence of channelrhodopsin-mediated physiological responses beyond aquatic habitats (marine and freshwater systems).

Materials And Methods

Expression plasmids

Synthesized genes encoding KnRh3 and CrChR2 (1-315 amino acids) were subcloned into a pHKR2-3.0-eYFP plasmid (gifted by Dr. H. Yawo (University of Tokyo, Japan)) using an In-Fusion HD cloning kit (Takara Bio, Shiga, Japan). Length variants of KnRh3 (amino acid length of 272, 280, 290, 300, 310, 317, 321, 397, 697, and 831) were created by using appropriate PCR primers. For immunostaining experiment, the plasmid pKnRh3(697 amino acids)-3.0-eYFP was inserted N-QKLISEEDL-C (10 amino acids, c-Myc

epitope tag) in the C-terminal of eYFP using inverse PCR. Site-directed mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Agilent, CA, USA). All the constructs were verified by DNA sequencing (Fasmac Co., Ltd. Kanagawa, Japan).

Mammalian cell culture

Electrophysiological assays and immunostaining of KnRh3 and CrChR2 were performed on ND7/23 cells, which are hybrid cell lines derived from neonatal rat dorsal root ganglia neurons fused with mouse neuroblastoma. ND7/23 cells were grown on a coverslip in Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan) supplemented with 2.0 mM of all-*trans* retinal and 5% fetal bovine serum, and under a 5% CO₂ atmosphere at 37°C. The expression plasmids were transiently transfected by using the FuGENE HD transfection Reagent (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions. Electrophysiological recordings were then conducted 24-36 h after transfection. Successfully transfected cells were identified by eYFP fluorescence under a microscope prior to measurements.

Immunostaining of mammalian cells

ND7/23 cells were cultured on glass coverslips. Cells expressing KnRh3 or KnRh3 bearing the c-Myc epitope tag at the C-terminus were fixed in 4% paraformaldehyde phosphate buffer solution for 15 min at room temperature. Cells were then washed with phosphate-buffered saline (PBS) for three times. When necessary, cells were permeabilized with 0.5% Triton X-100 for 15 min at room temperature. Cells were treated with blocking buffer consisting of 3% goat serum for 60 min at room temperature. After blocking, the cells were incubated with rabbit anti-c-Myc primary antibody (C3956; Sigma-Aldrich, St. Louis, MO, USA) at a 1:500 dilution for 60 min at room temperature, then washed with PBS for three times and labeled with goat anti-rabbit IgG secondary antibody Alexa Fluor 594 (A-11037; Thermo Fisher Scientific, Waltham, MA, USA) at a 1:200 dilution for 2 h at room temperature. After a final wash with PBS, the coverslips were mounted on glass slides with ProLong Diamond Antifade Mountant (Thermo Fisher Scientific).

Live cultured KnRh3 bearing the c-Myc epitope tag at the C-terminus expressing-cells were washed with PBS. The cells were moved from the culture medium to serum-free DMEM and incubated with rabbit anti-c-Myc primary antibody at a 1:500 dilution for 60 min under a 5% CO₂ atmosphere at 37°C. The cells were washed with PBS twice before fixing with 4% paraformaldehyde phosphate buffer solution for 15 min at room temperature. The cells were washed with PBS three times before labeling with goat anti-rabbit IgG secondary antibody Alexa Fluor 594 at a 1:200 dilution for 2 h at room temperature. After a final wash with PBS, the coverslips were mounted on glass slides with ProLong Diamond Antifade Mountant.

Fluorescent images were acquired with a confocal microscopy (LSM880, CarlZeiss, Jena, Germany) equipped with ×40 objective lens.

Electrophysiology

All experiments were carried out at room temperature ($22 \pm 2^{\circ}\text{C}$). Photocurrents were recorded by an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) under a whole-cell patch clamp configuration¹⁴. Data were filtered at 5 kHz and sampled at 20 kHz (Digdata1550, Molecular Devices, Sunnyvale, CA, USA) and stored in a computer (pClamp10.7, Molecular Devices). Pipette resistance was 3–6 M Ω . The standard internal pipette solution for the whole-cell voltage clamp contained (in mM) 126 NaAsp, 0.5 CaCl₂, 2 MgCl₂, 5 EGTA, 25 HEPES, 12.2 NMG, and adjusted to pH 7.4 by citric acid. The standard extracellular solution for the whole-cell voltage clamp contained (in mM) 150 NaCl, 1.8 CaCl₂, 1 MgCl₂, and 10 HEPES, 10 NMG, 5 glucose, and adjusted to pH 7.4 by NMG. Internal and external solutions for ion selectivity are shown in Table 1. The liquid junction potential was calculated and compensated by pClamp 10.7 software. Time constants were determined by a single exponential fit unless indicated otherwise.

Table 1

Measurment solution component for ion selectivity

		NaCl	KCl	CaCl ₂	MgCl ₂	NMG	EGTA	CHES	MES
Pipette	NMG pH9.0	1	1	2	2	140	10	10	-
Bath	NMG pH9.0	1	1	2	2	140	-	10	-
	NMG pH6.85	1	1	2	2	-	-	-	10
	Na ⁺ pH9.0	140	1	2	2	-	-	10	-
	K ⁺ pH9.0	1	140	2	2	-	-	10	-
	Ca ²⁺ pH9.0	1	1	70	2	-	-	10	-
	Mg ²⁺ pH9.0	1	1	2	70	-	-	10	-

Optics

For whole-cell voltage clamp, irradiation at 480 nm was carried out using collimated LED (parts No. LCS-0470-03-22, Mightex, Toronto, Canada) controlled by computer software (pCLAMP10.7, Molecular Devices). Light power was measured directly by an objective lens of a microscope by a power meter (LP1, Sanwa Electric Instruments Co., Ltd., Tokyo, Japan). All action spectra were measured at the same light intensity in the range of 410 nm to 650 nm by a xenon light source OSG (Hamamatsu photonics, Hamamatsu, Japan).

Statistical validation of the data

Data in the text and figures are expressed as mean $\pm$ SEM and were evaluated with the Mann-Whitney *U* test for statistical significance, unless noted otherwise. Means were judged as statistically insignificant when $P > 0.05$.

Results

Genome of terrestrial alga encodes a novel modular channelrhodopsin (KnRh3)

Extensive and targeted mining of the genomic database of *K. nitens* revealed the existence of several rhodopsin-encoding genes (http://www.plantmorphogenesis.bio.titech.ac.jp/cgibin/blast/blast_www_klebsormidium.cgi). Among them, we found one gene that is highly homologous to chlorophyte cation channelrhodopsins such as CrChR2. We named it KnRh3 in this study. KnRh3 possesses 831 amino acids and harbors a membrane-embedded 7-TM rhodopsin domain (amino acids 1-272) followed by a long C-terminus region (273–831 amino acids) (Fig. S1). Sequence comparison of the rhodopsin domain revealed 34.5% identity with and 69.3% homology to CrChR2. Several important amino acid residues were aligned when compared to four ChRs and bacteriorhodopsin (BR) (Fig. 1A). Three (Glu82, Glu90, Glu97) out of five glutamate residues at positions 82, 83, 90, 97 and 101 in CrChR2 that are important for channel activity were conserved in KnRh3. Glu83 was replaced by Val. Glu101 was substituted by Pro in KnRh3 whereas in GtACR1 (light-gated anion channel), Gln was observed at this position. His instead of Asp at position 96 was regarded as a characteristic of channelrhodopsins, but in KnRh3, this position was occupied by Ala, by D156 in GtACR1, while C128 in CrChR2 formed a hydrogen bridge (D-C pair or D-C gate) which altered the channel open lifetime^{20,21}. This pair is also conserved in KnRh3 whereas chlorophyte cation channel rhodopsin (GtCCR4) lacks it. Thus, we predicted that KnRh3 might function as a cation channelrhodopsin when functionally expressed in a suitable system. Notably, KnRh3 has a long C-term domain that encodes a putative peptidoglycan binding domain (FimV) between 410–690 amino acids (Fig. S1). In general, the peptidoglycan layer is located outside of the cytoplasmic membrane in bacterial cell walls. In fact, the homologous region of KnRh3 is predicted to be located on the extracellular side, whereas the C-terminus region is normally located on the cytoplasmic side in the case of microbial-type rhodopsin (Fig. S2). Thus, we considered why the FimV domain is connected to the C-terminus of the 7-TM domain. We anticipated that the membrane topology of KnRh3 is inverted, as in the case of heliorhodopsin (HeR), which is a recently identified subfamily in microbial rhodopsins³¹. We synthesized a codon-adapted full-length gene of KnRh3 (831 amino acids) for functional expression in a mammalian system and fused it with the p3.0-eYFP vector with eYFP at the C-terminus of KnRh3. After transfection of the fusion expression construct, we observed weak YFP fluorescence on the cellular membrane, indicating poor expression in ND7/23 cells. A voltage-clamp measurement was then performed. However, no photocurrent was observed (data not shown). We anticipated that low expression was due to a long C-terminus domain (~ 540 amino acids) after the 7-TM domain. Thus, we

systematically truncated the C-terminus to various lengths. We created five variants with different lengths, carrying 697, 397, 310, 290, and 272 amino acids. Note that only the variant with 697 amino acids included the FimV domain. In addition, we fused a cMyc epitope tag at the C-terminus of the 697 amino acids variant to detect immune fluorescence. Figure 1B-D shows fluorescent images of the transfected cells. eYFP fluorescence indicates successful expression of KnRh3. Although no alexa595 signal was observed in the absence of detergent (Fig. 1B), treatment with detergent to permeabilize the membrane generated a signal (Fig. 1C). No alexa595 signal was observed when KnRh3 without cMyc-tag was expressed even after the detergent treatment (Fig. 1D). These results indicate that the C-terminus domain is on the cytoplasmic side. Thus, KnRh3 exhibits typical membrane topology of a channelrhodopsin.

The C-terminus of KnRh3 modulates photocurrents and photocycle of the channelrhodopsin domain

We then performed patch clamp recordings of each variant. All the constructs having various amino acid lengths are depicted in Fig. 2A. As shown in Fig. 2B-F, large photocurrents were observed in all the five KnRh3 variants upon blue light illumination for 1.0 second. Photo-current amplitudes were comparable to that of CrChR2 (Fig. 2G). Current direction reversed as clamped voltage shifted from -90 to $+50$ mV in 20 mV steps, which is a typical ion channel property. Illumination induced a large peak current (I_p) which slowly decayed into smaller amplitudes during illumination. However, the current did not reach a plateau within 1 sec, whereas the photocurrent of CrChR2 rapidly dropped to a steady state level (Fig. 2G). When light was shut off, this diminished the photocurrent into the baseline, indicating channel closure. Notably, the phases of current decay were significantly different among the five variants, two of which, those with 697 and 397 amino acids, rapidly decayed into the baseline whereas the other three (300, 290 and 272 amino acids) showed a relatively slow decay (Fig. 2B-F). This indicates that the channel kinetics and the photocycle are affected by the cytoplasmic domain of KnRh3. To further investigate the observation above, we created in addition four variants with different lengths of amino acids: 321, 317, 310 and 280. Current decay kinetics (τ -off) at -70 mV from all nine variants were compared (Fig. 2H). The variants longer than 317 amino acids showed a fast off-kinetics (~ 10 ms). The variant with 310 amino acids showed an off-kinetics of about 20 ms. Furthermore, the time constants of the shorter variants became even larger and the shortest variant with 272 amino acids reached 130 ms, more than 10 times slower than the original construct. This result clearly shows a correlation between the length of amino acids and the time constant of channel kinetics in KnRh3, indicating that the C-terminal cytoplasmic domain affects both channel kinetics and the photocycle. In particular, amino acids between 272 and 310 largely contribute to the modulation of light-gated ion channel activities in KnRh3.

Interestingly, the photocurrent amplitudes also differed among the nine variants (Fig. 2I). The variants shorter than 300 amino acids reached about 3 nA at -70 mV, while the longer variants > 310 amino acids showed smaller currents under the same condition in which only about 1 nA was observed from cells expressing the 397 and 697 amino acids variants. We reasoned that current amplitude depends on the open-life time of the channels. As shown in Fig. 2H, the shorter variants exhibited a larger time constant.

This implies that these variants have a stable M-intermediate that corresponds to the channel's open state, according to a previous study on CrChR2³². Thus, the apparent current amplitude of the short variants was elevated as the M-state accumulated during illumination.

The current-voltage relations (I-V plots) of KnRh3 and CrChR2 are depicted in Fig. 2J (peak component) and Fig. 2K (steady state component). Current direction and amplitude depend on the applied voltage. The reversal potential of KnRh3 is about -10 mV while that of CrChR2 is 0 mV under the same condition. As is already known, ion conductance of CrChR2 is inwardly-rectified and thus the outward current was largely suppressed (Fig. 2J and K, black). The I-V plots of KnRh3 show a rather weak rectification and relatively large outward currents were observed (see +50 mV in Fig. 2J and K). The I-V plots of all the variants are summarized in Fig. S3. The photocurrent properties of KnRh3 397 amino acids (excluding FimV) and 697 amino acids (including FimV) are shown in Fig. S4. The current shape, I-V relation, and current amplitudes of these two variants were identical. These results indicate that FimV did not alter channel function.

All nine variants that were tested were fused to eYFP at the C-terminal. Thus, one might argue whether the length-dependent effect in Fig. 2I might have originated from eYFP, i.e. if an interaction between rhodopsin and eYFP may have altered the channel kinetics for an unknown reason. To exclude this possibility, we created 272 amino acids and 397 amino acids KnRh3 variants without eYFP. The variants were transfected, and the photocurrents were recorded to compare the channel-off kinetics and amplitude (Fig. 3). The time constants (t-off) were almost identical between eYFP-tagged and untagged 272 amino acids and 397 amino acids variants (Fig. 3A). This clearly indicates that a length-dependent effect was observed, regardless of the presence of eYFP at the C-terminus of KnRh3. Photocurrent amplitudes of untagged variants were smaller than those of eYFP-tagged constructs (Fig. 3B). This could be due to a lower expression level of the former. In fact, eYFP constructs contain the ER-exporting signal peptide and the membrane trafficking signal peptide to improve membrane localization, whereas no additional sequence was tagged in the untagged variants.

Positively charged residues in the C-terminus domain of KnRh3 regulated intramolecular interactions

Based on the result in which the cytoplasmic domain altered the channel kinetics in KnRh3 (Fig. 2H), it could be hypothesized that the cytoplasmic domain interacts with the rhodopsin domain (7-TM domain). Since no significant change in t-off was observed among variants longer than 317 amino acids, the region from 317 amino acids onwards is not important for the interaction. Thus, we anticipated that one or several amino acids in the cytoplasmic region between 272 and 317 amino acids would be crucial for the interaction. We noticed two characteristic negatively charged residues, E285 and E293, and three positively charged residues, R287, K289 and R291, some of which might form an electrostatic interaction with the 7-TM domain (Fig. 4A and Fig. S1). Thus, we replaced each residue with alanine in the KnRh3-397 amino acids variant which exhibits fast kinetics, similar to the longest variant (697 amino acids). As shown in Fig. 4B-D, photocurrent decay after switching off light apparently became slower after mutating

R287A and R291A. The decay was further slowed in the double mutant R287A/R291A (Fig. 4E). Figure 4F summarizes the time constants of all the mutants tested. The R287A mutant shows a t_{off} of 50 ms which is five-fold slower than that of the KnRh3-397 variant, while R291A was slowed down to 25 ms. The double mutant R287A/R291A reached about 100 ms which is more than 10 times slower than KnRh3-397 amino acids (no amino acid substitution) and is close to the 272 amino acids (no amino acid substitution) variant (130 ms). The three remaining mutants (E285A, K289A and E293A) showed no effect on kinetics. These results clearly indicate that the positively charged residue, R287 and R291, interact with the 7-TM domain and contribute to altered channel kinetics and the photocycle (Fig. 4I). These three mutants exhibited significantly larger photocurrents than KnRh3-397 (Fig. 4G) without any change in their reversal potential (Fig. 4H).

Action spectrum and light sensitivity of KnRh3

The action spectrum, a wavelength dependency of photocurrent amplitude, was measured with a multicolor light source (Fig. 5). The 272 amino acids and 397 amino acids variants showed a spectrum with two λ_{max} at 430 and 460 nm which are shorter than that of CrChR2 ($\lambda_{\text{max}} = 470$ nm). To our knowledge, the λ_{max} of KnRh3 is one of the shortest among naturally occurring ChRs.

We then measured the light-power dependency of two KnRh3 variants (272 amino acids and 397 amino acids), comparing them to CrChR2 (Fig. 6). Photocurrent amplitude, including I_p (peak component) and I_s (steady state component), grew as light intensity increased. The I_s of the 272 variant became saturated at about 0.09 mW/mm² (Fig. 6A empty circle), while I_p increased further and reached a plateau at about 0.2 ~ 0.3 mW/mm² (Fig. 6A filled circle). On the other hand, light sensitivity of the 397 amino acids variant was markedly lower than that of the 272 amino acids variant. Both I_p and I_s became saturated at about 3–4 mW/mm² (Fig. 6B). Note that the x-axis of each panel differs by a single order of magnitude. The light-power dependency of CrChR2 falls in the same range as KnRh3 397 amino acids (Fig. 6C). The half-saturation light intensity (EC_{50}) was determined to be 0.051 mW/mm² (KnRh3-272), 0.712 mW/mm² (KnRh3-397) and 0.731 mW/mm² (CrChR2). This indicates that the C-terminal length affected the light sensitivity of KnRh3.

KnRh3 permeates monovalent and divalent cations

We predicted that KnRh3 functions as a cation channelrhodopsin considering its sequence similarity to chlorophyte channelrhodopsins (e.g. CrChR2). Ion selectivity was tested by patch-clamp recording. We used the KnRh3 272 amino acids variant for all experiments. The extracellular solution was systematically replaced with various cations, while the intracellular solution was fixed (refer to Table 1 for more details). Figure 7A-F shows representative photocurrent traces under various ionic conditions. In the presence of NMG, we assumed that H⁺ was the conducting ion. The I-V plot under several ionic conditions is depicted in Fig. 7G (KnRh3) and Fig. 7H (CrChR2). Note that the peak component (I_p) of the photocurrent was plotted for all the relevant measurements, and essentially the same result was obtained

from the analysis of I_s , which is summarized in Fig. S5 and S6. When Fig. 7A and 7B are compared, voltage dependency of current direction was obviously different. The reversal potential was shifted from -30 mV to $+20$ mV (Fig. 7G red and blue), indicating H^+ conductance across KnRh3 (Fig. 7G, I, J and K). Large Na^+ conductance was observed, as shown in Fig. 7C, with a reversal potential of $+20$ mV (Fig. 7G green, J and K). The shift in reversal potential of the K^+ solution indicates that KnRh3 permeates K^+ (Fig. 7I, J and K Fig. S5). We then tested divalent cations, Ca^{2+} and Mg^{2+} . We observed an inward photocurrent in the Ca^{2+} solution (Fig. 7E) and reversal potential shifted significantly from -30 mV (NMG pH 9.0) to -5 mV (Ca^{2+} pH 9.0) (Fig. 7G red and black, J, K). A similar observation was observed in the Mg^{2+} solution (Fig. 7F, I, J and 7K, Fig. S5). Altogether, we demonstrated that KnRh3 is a cation channelrhodopsin that permeates monovalent and divalent cations. Figure 7H shows the I-V plot of Cr_ChR2 under the same condition as Fig. 7G. Reversal potentials and a shift in reversal potential (ΔE_{rev}) of KnRh3 and CrChR2 are depicted in Fig. 7J, 7K and 7L, respectively. These values allow the permeability ratio of each cation to be estimated. The ΔE_{rev} of H^+ , Na^+ , K^+ , Ca^{2+} and Mg^{2+} from CrChR2 were determined as 90, 70, 65, 40 and 35 mV, respectively (Fig. 7L). This indicates that the rank in permeability ratio is $H^+ > Na^+ > K^+ > Ca^{2+} > Mg^{2+}$. On the other hand, KnRh3 showed no significant difference in ΔE_{rev} among H^+ , Na^+ and K^+ ($45 \sim 48$ mV), indicating that permeability of these cations was almost the same (Fig. 7K). The ΔE_{rev} of Ca^{2+} and Mg^{2+} were about 30 and 25 mV, respectively, indicating lower permeability for divalent cations than monovalent cations (Fig. 7K). As mentioned above, we analyzed the peak component of each photocurrent. In addition, I_s was analyzed. Essentially the same results were found (Fig. S6).

Discussion

In this study, we report on the electrophysiological characterization of a newly identified multi-domain cation channelrhodopsin from *Klebsormidium nitens* (KnRh3). KnRh3 possesses a large cytoplasmic domain made of about 540 amino acids which encode a peptidoglycan binding moiety (FimV). Several ChRs also have a long C-terminus tail. Full-length ChR1 and ChR2 from *Chlamydomonas reinhardtii* contain 712 and 737 amino acids in total (837 and 747 in *Volvox carter*). Among them, the 7-TM rhodopsin domain is made up of only about 300 amino acids. Thus, 400–500 amino acids are located mainly on the cytoplasmic side. However, no significant homology was found among the long cytoplasmic extension of the characterized ChRs from these different organisms. Since the discovery of ChRs, the role of cytoplasmic domains remains barely understood. The initial reports of CrChR1 and CrChR2 described that truncation of the cytoplasmic C-terminal domain did not alter the ion channel function^{9,10}. Thus, researchers investigated their molecular properties by using truncated proteins carrying only the 7-TM domain. However, an extensive study of the phosphoproteome of the eyespot fraction of *C. reinhardtii* showed three phosphorylation sites for CrChR1 and one for CrChR2³³. Thus, a kinase-dependent regulation of function was also proposed. Recently, it has been shown that phosphorylation of CrChR1 is connected to photomotility and calcium signaling of green alga³⁴. Predicting the phosphorylation of KnRh3 suggested that several motifs are involved in phosphorylation

(Fig. S7). It would be interesting to delineate the functional role of these predicted phosphorylation patterns of KnRh3 in a native system.

We demonstrated that the C-terminus region affects the light-gated ion channel function. The channel closure rate (off-kinetics) slowed down from 10 ms to 130 ms as the cytoplasmic domain was shortened (Fig. 2H). In other words, the C-terminus domain accelerates channel closure and the photocycle. Positively charged residues, R287 and R291, in particular, are crucial for the acceleration, because the double mutant R287A/R291A reduced the fast kinetics (Fig. 4F). Furthermore, light sensitivity was altered following systematic truncation of the C-terminus (Fig. 6A and B). Therefore, we propose that an electrostatic interaction exists between the residues of the C-terminus and the 7-TM domain of KnRh3 (Fig. 4I). Although we did not identify the counterparts of R287 and R291 in this study, the candidates could be negatively charged residues such as Asp and Glu in the TM domain, which should be investigated in the future.

Modulation of photocycle kinetics by the cytoplasmic domain has never been proven in ion-transporting rhodopsin thus far. However, in the case of enzyme rhodopsin (HKR or 2C-cyclops), the full-length HKR has been shown to exhibit a normal photocycle which reverts to the initial dark state in 20 sec, whereas truncation of the enzyme domain at the C-terminus region resulted in production of a bi-stable photocycle in which rhodopsin possesses two dark states (400 nm and 380 nm), which exhibit photochromism^{35,36}. Detailed functional characterization of KnRh3 with spectroscopy and a structural study together with mutation analysis would be needed to reveal the mechanism of the functional modulation by the cytoplasmic domain.

The action spectrum of KnRh3, assessed from a voltage clamp experiment, revealed its absorption maxima ($\lambda_{\max}$) at 430 and 460 nm. To the best of our knowledge, this is one of the most blue-shifted $\lambda_{\max}$ in naturally occurring ChRs. Govorunova et al. previously reported a blue-shifted ChR from *Platymonias subcordiformis* (PsChR) that displayed an action spectrum of $\lambda_{\max} = 445$ nm, and an absorption maximum of 437 nm after purification (in solution)¹⁹. They ascribed the blue-shift to amino acid residues G142 and A146 in 4-TM (T159 and G163 in CrChR2). In fact, replacement of these residues resulted in a blue shift when tested with C1C2 and iC + +^{37,38}. KnRh3 conserves A146 (as A161) while G142 is replaced by A157. Thus, A161 would be one of the color determinants in KnRh3, which could be applied to optogenetics. Usually, for optogenetics tools, long-wavelength absorption is ideal for minimizing light scattering by biological tissues. No photocurrent was observed in KnRh3 after illumination with 550 nm light (Fig. 5). The blue-shifted spectrum of KnRh3 is ideal for combinatorial applications with long wavelength-absorbing channelrhodopsin or fluorescent indicators to separate the stimulation or excitations.

Given its high homology to CrChR2, KnRh3 exhibits a non-selective cation channel, and permeating monovalent and divalent cations including H⁺, Na⁺, K⁺, Mg²⁺ and Ca²⁺. The permeation ratio was similar to that of CrChR2. The cation channelrhodopsin from Cryptophyte algae (DTD channelrhodopsin) exhibited different ratio of cation permeation, in which monovalent metal cations were mainly

transported with minimum H⁺ and Ca²⁺ permeation^{39,40}. KnRh3 conducts Ca²⁺ at a relatively high amount (Fig. 7 and Fig. S6). There is the potential for engineering highly Ca²⁺ permeable channels, similar to the calcium-permeable ChR2 variant (Catch)²². The fast off-kinetics of KnRh3 397 amino acids has great potential for optical neuron excitation with high frequency.

Abbreviations

ChR, channelrhodopsin

CrChR2, *Chlamydomonas reinhardtii* Channelrhodopsin-2

KnRh3, *Klebsormidium nitens* channelrhodopsin

TM, trans-membrane

Declarations

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Conflicts of Interest

The authors declare no conflicts of interest.

Author contribution

S.K., S.P.T and H.K. devised the initial idea for the project. K.S and S.S. performed bioinformatic analyses. S.H. performed immunostaining experiments. R.T. performed electrophysiological experiments. S.P.T. prepared the manuscript with contributions from all of the authors to the data analysis, figure generation and the final manuscript.

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Figures

A

BR number	40	41	48	55	56	59	85	89	90	94	96	118	212	216
CrChR2 number	82	83	90	97	98	101	123	127	128	132	134	159	253	257
PsChR number	65	66	73	80	81	84	106	110	111	115	117	142	236	240
KnRh3 number	79	80	87	94	95	98	121	125	126	130	132	157	250	254
GtCCR4 number	67	68	75	89	90	93	116	120	121	125	127	147	242	246
TM number in CrChR2	2	2	2	2	2	2	3	3	3	3	3	4	7	7
BR	K	K	L	T	M	S	D	T	T	L	D	M	D	K
CrChR2	E	E	E	E	F	E	E	T	C	L	H	T	D	K
PsChR	E	E	E	E	L	E	E	T	C	L	H	G	D	K
KnRh3	E	V	E	E	I	P	E	T	C	L	A	A	D	K
GtCCR4	Q	E	E	I	T	G	D	T	C	T	D	T	D	K

B

C

D

Figure 1

A, Amino acid alignments of bacteriorhodopsin (BR), CrChR2, KnRh3 and GtCCR4. The characteristic amino acids in BR and CrChR2 were selected. In addition, amino acid numbers of each protein and transmembrane helix (TM) number are indicated. See Fig. S1 for an alignment of the whole protein sequences. B, Immunostaining of KnRh3. Expression of KnRh3-3.0-eYFP-cMyc in cultured ND7-23 cells, with KnRh3 bearing the c-Myc epitope tag at the C-terminus. eYFP fluorescence (left, green), probed with a

c-Myc antibody under non-permeabilized conditions for immunofluorescent staining with Alexa Fluor 594 (middle, magenta) and merge (right). Scale bar, 20 μm . C, Expression of KnRh3-3.0-eYFP-cMyc in cultured ND7-23 cells. eYFP fluorescence (left, green), probed with a c-Myc antibody under permeabilized conditions (0.5% Triton X-100) for immunofluorescent staining with Alexa Fluor 594 (middle, magenta) and merge (right). D, Expression of KfR-3.0-eYFP in cultured ND7-23 cells. eGYP fluorescence (left, green), probed with a c-Myc antibody under permeabilized conditions (0.5% Triton X-100) for immunofluorescent staining with Alexa Fluor 594 (middle, magenta) and merge (right).

Figure 2

Electrophysiological measurements of various C-terminal length constructs of KnRh3. A, Schematic drawing of truncated constructs. Amino acid positions truncated are indicated. B~G, Photocurrent traces of various C-terminal length constructs and CrChR2. Membrane voltage was clamped at from -90 mV to +50 mV by +20 mV step. H and I, Comparison of time constants and photocurrent amplitude from KnRh3 variants. J and K, I-V relationship of KnRh3 and CrChR2. Filled and empty symbols indicate peak and steady current, respectively. Red: KnRh3-272, Blue: KnRh3-290, Green: KnRh3-397, Black: CrChR2.

Figure 3

eYFP does not alter the channel kinetics. Time constants (A) and peak current amplitude (B) of KnRh3-272 amino acids and -397 amino acids variants with or without fluorescent protein (N=6-7).

Figure 4

Electrophysiological measurements of variants in the C-terminal region. A, Schematic drawing of KnR3 with amino acid sequence between 284 and 294. B-E, Photocurrent traces of KnRh3-397 (no mutation), mutant variants-R287A, -R291A and -R287A/R291A respectively. Membrane voltage was clamped at from -90 mV to +50 mV by +20 mV step. F and G, Comparison of time constants and peak current amplitude.

H, I-V relationship of KnRh3-397 (red), -R287A (blue), -R291A (green), and -R287A/R291A (yellow). I, Schematic drawing of interaction between the 7-TM domain and the cytoplasmic region in KnRh3.

Figure 5

Action spectrum of KnRh3-272 and KnRh3-397. Wavelength dependency of the photocurrent. The photocurrents were measured in standard solutions. The membrane voltage was clamped at -40 mV. Red, KnRh3-272, Black, KnRh3-397. N=5.

Figure 6

Light intensity dependence of KnRh3-272 (A), KnRh3-397 (B) and CrChR2 (C). Photocurrent were measured under standard solutions with various light intensities. Membrane voltage was clamped at -60 mV. Black circle, peak current; empty circle, steady state current (N=3-4).

Figure 7

Ion selectivity of KnRh3 and CrChR2. A-F, Representative photocurrent traces of KnRh3-397 under various cation conditions. Membrane voltage was clamped from at -80 to +60 mV by 20 mV steps. I-V relationship of KnRh3 (G) and CrChR2 (H). Peak component of photocurrent were plotted. Red: Ex NMG pH 9.0, Blue: Ex NMG pH 6.85, Green: Ex Na⁺ pH 9.0, Black: Ex Ca²⁺ pH 9.0. I, Comparison of peak current amplitude under various ionic conditions at -60 mV. light blue, KnRh3; green CrChR2. J, Reversal potential

Erev under various solutions. K, Reversal potential shift (ΔE_{rev}) of KnRh3. ΔE_{rev} of each cation was calculated by subtracting Erev of each ionic condition in panel J by Erev of NMG+ at pHe 9.0. e.g. ΔE_{rev} of H+ is determined as Erev (NMG+ pHe 6.85) - Erev (NMG+ pHe 9.0). L, Reversal potential shift (ΔE_{rev}) of CrChR2.

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