Neuroscience Research 75 (2013) 13–22

Contents lists available at SciVerse ScienceDirect

Neuroscience Research

journal homepage: www.elsevier.com/locate/neures

Involvement of glutamate 97 in ion influx through photo-activated channelrhodopsin-2

Saki Tanimoto[a,b], Yuka Sugiyama[b], Tetsuo Takahashi[c], Toru Ishizuka[a], Hiromu Yawo[a,b,*]

[a] *Department of Developmental Biology and Neuroscience, Tohoku University Graduate School of Life Sciences and JST, CREST, Sendai 980-8577, Japan*
[b] *Tohoku University Basic and Translational Research Center for Global Brain Science, Sendai 980-8575, Japan*
[c] *Faculty of Pharmaceutical Sciences, Toho University, Chiba 274-8510, Japan*

ARTICLE INFO

Article history:
Received 8 May 2012
Received in revised form 17 May 2012
Accepted 18 May 2012
Available online 1 June 2012

Keywords:
Optogenetics
Channelrhodopsin
Ion channel
Permeability
Channel block
Rectification
Gadolinium
Gd^{3+}
Lanthanum
La^{3+}
Channel pore

ABSTRACT

The light absorption of a channelrhodopsin-2 (ChR2) is followed by conformational changes to the molecule, which allows the channel structure to become permeable to cations. Previously, a single point mutation in ChR2, which replaces glutamate residue 97 with a nonpolar alanine (E97A), was found to attenuate the photocurrent, suggesting that the E97 residue is involved in ion flux regulation. Here, the significance of E97 and its counterpart ChR1 (E136) were extensively studied by mutagenesis, whereby we replaced these glutamates with aspartate (D), glutamine (Q) or arginine (R). We found that the charge at this position strongly influences ion permeation and that the photocurrents were attenuated in the order of ChR2 > E97D ≈ E97Q > E97R. We observed similar results with our chimeric/synthetic/artificial construct, ChR-wide receiver (ChRWR), which contains the first to fifth transmembrane helices of ChR1. The E-to-Q or E-to-R mutations, but not the E-to-D mutation, strongly retarded the sensitivity to the Gd^{3+}-dependent blocking of the ChR1 or ChR2 channels. Our results suggest that the glutamate residue at this position lies in the outer pore, where it interacts with a cation to facilitate dehydration, and that this residue is the primary binding target of Gd^{3+}.

© 2012 Elsevier Ireland Ltd and the Japan Neuroscience Society. All rights reserved.

1. Introduction

Earth's living creatures use light for two purposes: for obtaining useful energy and as a carrier of information. For instance, in the case of the unicellular green algae *Chlamydomonas reinhardtii*, light is sensed by two channelrhodopsins (ChRs), ChR1 and ChR2, which are localized in small regions of the plasma membrane covering the eyespot (Nagel et al., 2002, 2003; Sineshchekov et al., 2002; Suzuki et al., 2003). The homologues of these ChRs have also been identified in other species, e.g., *Volvox carteri* (VChR1 and VChR2), *Chlamydomonas augustae* (CaChR1), *Chlamydomonas yellowstonensis* (CyChR1), *Chlamydomonas raudensis* (CraChR2) and *Mesostigma viride* (MChR1) (Ernst et al., 2008; Zhang et al., 2008; Kianianmomeni et al., 2009; Govorunova et al., 2011; Hou et al., 2012). Each ChR is a member of the microbial-type rhodopsin family and is a seven-pass transmembrane (TM) protein with a covalently bound retinal. Light absorption is followed by the photoisomerization of the all-*trans* retinal to a 13-*cis* configuration and subsequent conformational changes of the molecule, which allow the channel structure to become permeable to cations (Bamann et al., 2008; Ernst et al., 2008). This enables very rapid (in the orders of ms) generation of a photocurrent in cell membranes expressing ChRs (Nagel et al., 2002, 2003; Boyden et al., 2005; Ishizuka et al., 2006). Despite extensive studies, researchers have yet to describe how the cations flow through the molecule.

In relation to other archaeal-type rhodopsins, the TM3-7s are assumed to form a core structure in which the amino acid residues lie close to, and interact with, the retinal (Adamian et al., 2006; Pebay-Peyroula et al., 2002). The N-terminal region, including the TM1-2, is rather unique to ChRs (Nagel et al., 2002, 2003, 2005; Sineshchekov et al., 2002; Suzuki et al., 2003; Ernst et al., 2008; Zhang et al., 2008). Given this fact, the TM1-2s may somehow be involved in the ion channel properties. In particular, it has been assumed that the five glutamate (E) residues, which are conserved

Abbreviations: *afu*, arbitrary fluorescent unit; ChR, channelrhodopsin; ChRWR, channelrhodopsin-wide receiver; g_{in}, effective inward conductance; g_{out}, effective outward conductance; I_{peak}, effective peak current amplitude; *I–V*, current–voltage; LED, light-emitting diode; LJP, liquid junction potential; *RI*, rectification index; TM, transmembrane helix.

* Corresponding author at: Department of Developmental Biology and Neuroscience, Tohoku University Graduate School of Life Sciences, 2-1-1 Katahira, Aoba-ku, Sendai 980-8577, Japan. Tel.: +81 22 217 6208; fax: +81 22 217 6211.
E-mail address: yawo-hiromu@m.tohoku.ac.jp (H. Yawo).

0168-0102/$ – see front matter © 2012 Elsevier Ireland Ltd and the Japan Neuroscience Society. All rights reserved.
http://dx.doi.org/10.1016/j.neures.2012.05.008

in the N-terminal region of the known ChRs, are involved in ion permeation (Hegemann, 2008; Sugiyama et al., 2009). Actually, a single point mutation in ChR2 that replaced E97 with a nonpolar alanine (E97A) caused a reduced photocurrent amplitude without influencing other photocurrent properties, suggesting that residue E97 is involved in ion flux regulation rather than the photocycle (Sugiyama et al., 2009). Here, the significance of E97 and its counterpart in ChR1 (E136) were studied extensively. We found that the charge at these positions strongly influenced ion permeation and Gd^{3+}-dependent blocking. Our results suggest that both E97 (ChR2) and E136 (ChR1) interact with hydrated cations to facilitate their permeation and that these residues are the primary binding site of Gd^{3+}.

2. Materials and methods

2.1. Cell culture and molecular biology

A C-terminal fusion construct with *Venus* (Nagai et al., 2001) of the ChR2(1-315) apoprotein, chop2(1-315), and channelrhodopsin/wide receiver (ChRWR), a chimeric molecule that consists of the TM1-5 of ChR1 and the TM6-7 of ChR2, have been described previously (Wang et al., 2009). Amino acid substitutions of ChR2 or ChRWR were introduced by PCR-based site-directed mutagenesis using the KOD-Plus-Mutagenesis Kit (Toyobo, Osaka, Japan). All PCR-derived constructs were verified by sequencing.

Human embryonic kidney (HEK)293 cells were grown in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum under a 5% CO_2 atmosphere at 37 °C. The expression plasmids were transiently transfected in HEK293 cells using Effectene Transfection Reagent (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Twenty-four hours later, the cells were dissociated and replated onto collagen-coated glass coverslips. Electrophysiological recordings were then conducted 72 h after transfection. Successfully transfected cells were identified by the fluorescence.

2.2. Quantification of membrane fluorescence

The cells were prepared similarly to those used for the electrophysiology recordings. Under conventional confocal microscopy (LSM 510 META, Carl Zeiss, Oberkochen, Germany), *Venus* was excited at 488 nm, and the fluorescence was obtained with an LP505 emission filter. All of the images were captured under predetermined conditions, such as the laser power and the photomultiplier gain, to compare the fluorescence distribution among ChR2, ChRWR and their variants. Three parallel lines were drawn across the sampled cell to divide it into four strips of equal width. Then, a series of pixel values was measured along each line. The pixel values of the arbitrary fluorescent unit (*afu*) at the plasma membrane were averaged, background-subtracted and stored as the fluorescence intensity at the plasma membrane of the cell of interest. All of the above image analyses were conducted using ImageJ software (http://rsb.info.nih.gov/ij/).

2.3. Electrophysiology

All experiments were carried out at a room temperature (23 ± 2 °C). Photocurrents were recorded as previously described (Ishizuka et al., 2006) using an EPC-8 amplifier (HEKA Electronic, Lambrecht, Germany) under a whole-cell patch clamp configuration of isolated cells to eliminate any influence of electrical coupling. Series resistance was compensated up to 70% to reduce series resistance errors. The data were filtered at 1 kHz and sampled at 20 kHz.

The internal pipette solution for whole-cell current recordings contained (in mM) 120 CsOH, 100 glutamate, 50 HEPES, 2.5 $MgCl_2$, 2.5 MgATP, 5 Na_2EGTA and 1.2 Leupeptin, with the pH adjusted to 7.2 with CsOH. The extracellular Tyrode's solution contained (in mM) 134 NaCl, 3 KCl, 2.5 $CaCl_2$, 1.25 $MgCl_2$, 4 NaOH, 10 HEPES and 2 g/L glucose, with the pH adjusted to 7.4 with HCl. The liquid junction potential (LJP) was experimentally measured and determined to be −10 mV. All voltage readings were corrected by this value.

Photostimulation was carried out by blue LED illumination (470 ± 25 nm, LXHL-NB98, Philips Lumileds Lighting Inc., San Jose, CA) regulated by a pulse generator (SEN-7203, Nihon Kohden, Tokyo, Japan) and a computer (pCLAMP9, Molecular Devices Co., Sunnyvale, CA). Blue LED illumination at 3-s durations was applied every 20 s. The maximal power density of the LED light was directly measured by a thermopile (MIR-100Q, Mitsubishi Oil Chemicals, Tokyo, Japan) and was 4.4 mW mm^{-2}.

The photocurrent amplitude and kinetics are dependent on the light power density (Ishizuka et al., 2006), the holding potential (Nagel et al., 2002, 2003; Ishizuka et al., 2006) and the pH (Nagel et al., 2002, 2003; Hegemann et al., 2005). Therefore, every photocurrent was measured at the maximal LED power (470 ± 25 nm, 4.4 mW mm^{-2}) with a holding potential of −50 mV and at pH 7.2 and pH 7.4 inside and outside of the membrane, respectively. The current–voltage (*I–V*) relationship was obtained by applying a 1-s voltage ramp (from −60 to 60 mV) during the steady-state of photocurrent with the subtraction of the leak *I–V* curve obtained before light illumination. The photocurrent peak amplitude was expressed as an effective value (I_{peak}) after being divided by the whole-cell capacitance, which is proportional to the cell's surface area. The slope between −60 mV and the reversal potential was divided by the whole-cell capacitance and expressed as an effective inward conductance (g_{in}). Similarly, an effective outward conductance (g_{out}) was calculated from the slope between +60 mV and the reversal potential. The rectification index (*RI*) was defined as $RI = g_{out}/g_{in}$.

For evaluating the magnitude of desensitization, the difference between the peak photocurrent and the steady-state photocurrent at the end of a 1-s light pulse was divided by the peak photocurrent amplitude. The photocurrent OFF kinetics was fitted by either a single or bi-exponential function of the time during the early phase (0–20 ms), although it obviously had deviated from the single exponential function in the later phase.

2.4. Numerical and statistical analyses

Data analysis was performed with Clampfit 9.2 or 10.1 (Molecular Devices Co., Sunnyvale, CA). The reversal potential was measured as the *x*-intersection of a third polynomial function fitted to the *I–V* relationship. The dose–response relationship of a blocker was fitted to the one-to-one binding model of Michaelis–Menten kinetics. That is, $y = b(1 - x/(x + a)) + c$, where x is the concentration of a blocker and y is the relative value to that without the blocker. Parameters a, b and c were determined by a least-squares fitting algorithm (*R*, R Development Core Team, 2005). The parameter, a, was adopted as a K_D value of the blocker.

The Gd^{3+} effect (Y) on each value of photocurrent (X) was estimated as $Y\ (\%) = (X_{cont} - X_{Gd})/X_{cont}$, where X_{cont} is a value in the absence of Gd^{3+} and X_{Gd} is that in the presence of 0.5 mM Gd^{3+}.

All data in the text are presented as the means ± SEM (number of observations). The Kruskal–Wallis test was used for statistical analysis unless otherwise noted. According to this P value, the statistical significance was scored as *ns* if $P > 0.05$, as *1 if $0.05 > P > 0.005$, *2 if $0.005 > P > 0.0005$, *3 if $0.0005 > P > 0.00005$ and *4 if $P < 0.00005$.

Download English Version:

https://daneshyari.com/en/article/4351521

Download Persian Version:

https://daneshyari.com/article/4351521

Daneshyari.com