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Kinetic profiles of photocurrents in cells expressing two types of channelrhodopsin genes

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Channelrhodopsin-2 (ChR2), a light-activated cation-selective ion channel, has been widely used as a tool in optogenetic research. ChR2 is specifically sensitive to wavelengths less than 550 nm. One of the methods to expand the sensitivity of a channelrhodopsin to a wider range of wavelengths is to express another channelrhodopsin in the cells by the transduction of an additional gene. Here, we report the characteristic features of cells expressing two types of channelrhodopsins, each having different wavelength sensitivities. In HEK293 cells stably expressing ChR2, photocurrents were elicited at stimuli of 400–550 nm, and the wavelength sensitivity range was expanded by the additional transduction of the modified Volvox channelrhodopsin-1 (*mVChR1*) gene, which has broad wavelength sensitivities, ranging from 400 to 600 nm. However, the photocurrent at 550 nm was lower than that of the *mVChR1*-expressing cell; moreover, the turning-on and turning-off constants were delayed, and the deactivation rates were decreased. Meanwhile, the response to lower light intensity was improved by the additional gene. Thus, the transduction of an additional gene is a useful method to improve the light and wavelength sensitivities, as well as photocurrent kinetic profiles, of channelrhodopsins.

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1. Introduction

Optogenetic technologies have been used in diverse types of research, majorly in neuroscience, with an array of applications. In initial optogenetic studies, the channelrhodopsin-2 (*ChR2*) gene [1,2] was transduced into neuronal cells for neuronal excitation [3,4] and silencing [5] by light stimuli. The purpose of such studies was to investigate neuronal networks in neuroscience research [6–9].

In the field of vision research, optogenetic technologies have been developed for treating blindness. Bi et al. [10] and our group [11] reported that the transduction of *ChR2* into the retinal ganglion cells of blind mice and rats could recover their light responses. We also demonstrated the safe use of microbial rhodopsins for vision restoration by gene therapy [12,13]. Currently, gene therapy using optogenetic genes for restoring vision is underway in several ongoing clinical trials.

Recently, various types of optogenetic genes with characteristics different from those of *ChR2* have been identified or designed [14–16]. In 2014, we designed modified Volvox channelrhodopsin-1 (*mVChR1*), which has broader wavelength sensitivities than those of *ChR2* [17]. Optogenetic technologies using various types of channelrhodopsins (ChRs) can be applicable against various diseases. However, there is a gap between the basic research and the clinical application. The application of newly discovered or developed optogenetic genes for human diseases takes a long time because lots of pre clinical studies has to be performed. Therefore, a functionally advanced version of an optogenetic gene might be developed by the time the original gene has been used for curing

Abbreviations: ChR, Channelrhodopsin; ChR2, Channelrhodopsin-2; *mVChR1*, Modified Volvox channelrhodopsin-1; τON, Turning-ON constant; τOFF, Turning-OFF constant.

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some diseases in humans. In future, additional gene therapy using a newly designed gene in a patient who has already received gene therapy with the original optogenetic gene might be performed to compensate for the defect of the former optogenetic gene. However, the characteristics, including kinetic profiles of photocurrents, of cells expressing two types of ChRs remain to be explored.

2. Materials and methods

2.1. Cell preparation

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified minimum essential medium (Thermo Fisher Scientific, Tokyo, Japan) supplemented with 10% fetal bovine serum under a 5% CO_2 atmosphere at 37 °C. The culture medium was changed every 3 days, and cells were passaged using a 0.02% ethylenediaminetetraacetic acid/PBS solution.

2.2. Establishment of ChR2-and mVChR1-expressing cells

The expression plasmids—pChR2-IRES-puro, pmVChR1-IRES-puro, and pAAV-mVChR1-Venus—were previously established by Tomita et al. [17] and Sato et al. [18]. The pChR2 or pmVChR1-IRES-puro vectors were linearized using a restriction enzyme and electroporated into cultured HEK293 cells using the CUY21Pro-vitro system (Nepa Gene, Chiba, Japan). The transformants (HEK-ChR2 or HEK-mVChR1) were selected in culture medium containing puromycin (2 μg/mL) for at least 10 days. For the establishment of transformants stably expressing both genes, the linearized pAAV-mVChR1V vector was electroporated into stable transformant HEK-ChR2 cells. Venus-positive cells were sorted as cells stably expressing *ChR2* and *mVChR1* (HEK-ChR2+mVChR1) using a cell sorter (SH800; SONY, Tokyo, Japan).

2.3. Patch clamp recordings

Photocurrents elicited by stimuli of various wavelengths (400, 450, 500, 550, and 600 nm) of light produced by a xenon lamp were recorded using an EPC-10 amplifier (HEKA Electronik, Lambrecht, Germany) under whole-cell patch clamping of isolated cells. The intensity of light at each wavelength was also adjusted to 1 μW/mm^2 by setting an appropriate density filter for each wavelength into the carousel. The data were collected by filtering at 10 kHz and sampling at 20 kHz. The internal solution contained 130 mM CsCl, 10 mM HEPES, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM NaCl, 2 mM Na_2ATP, 1.1 mM EGTA, with the pH adjusted to 7.2 for whole-cell current recordings. Tyrode's solution contained 138 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 4 mM NaOH, 10 mM HEPES, with the pH adjusted to 7.4 by HCl. The turning-ON (τON) and turning-OFF (τOFF) time constants were analyzed as previously described [19]. In brief, the τON kinetics of each recording was measured by fitting in a single-exponential function between 10% and the maximal photocurrent during the light stimulus. For the τOFF kinetics, the photocurrent was fitted by a single-exponential function for the transition between 90% and 10% of the amplitude at the end of the light stimulus. The deactivation was quantified as the difference between the peak and the steady-state amplitudes divided by the peak amplitude.

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Data are expressed as the means ± SD, and the statistical method used was Tukey's multiple comparison test.

3. Results

3.1. Photocurrent in ChR-expressing cells

The peak wavelengths of HEK-ChR2 and HEK-mVChR1 are shown in Fig. 1A. The peak photocurrents of HEK-ChR2 and HEK-mVChR1 were at 450 and 550 nm, respectively. Cells expressing both genes showed no increase in photocurrents at 400, 450, or 500 nm, mainly corresponding to the ChR2-sensitive wavelength. However, the increase in photocurrents was observed at over 500 nm by the additional expression of the *mVChR1* gene (Fig. 1B). The photocurrent of HEK-ChR2+mVChR1 at 450 nm was significantly larger than that of HEK-mVChR1 but not HEK-ChR2 (Fig. 1C). With a stimulus of 500 nm, no significant increase was observed between HEK-ChR2+mVChR1 and HEK-ChR2; however, we detected significant differences between HEK-ChR2+mVChR1 and HEK-mVChR1 (Fig. 1D). With a stimulus of 550 nm (Fig. 1E), the photocurrent of HEK-ChR2+mVChR1 was significantly higher than that of HEK-ChR2 and lower than that of HEK-mVChR1.

3.2. Kinetic profiles of photocurrents in ChR-expressing cells

As shown in Fig. 2A, typical wave forms of HEK-ChR2 and HEK-mVChR1 were different from that of HEK-ChR2+mVChR1. We evaluated the τON and τOFF constants to clarify the characteristic features in each cell. With a stimulus at 450 nm, to which ChR2 has a higher response than mVChR1 does, HEK-ChR2 showed the shortest τON, and the additional expression of *mVChR1* delayed the τON compared to those of HEK-ChR2 and HEK-mVChR1 (Fig. 2B). The τON at 500 nm corresponding to both *ChR2* and *mVChR1* had high sensitivities and showed a delay by the additional expression (Fig. 2C). In the case of the 550-nm stimulus, to which mVChR1 has high sensitivity, a significant delay of τON was observed compared to that of HEK-mVChR1 (Fig. 2D). The dual gene expression caused the prolongation of τONs at all wavelengths measured compared to those of*ChR2*-and *mVChR1*-expressing cells (Table 1A). Similarly, the τOFFs were prolonged by the dual gene expression (Table 1B). The τOFF of HEK-ChR2+mVChR1 was significantly longer than those of HEK-ChR2 and HEK-mVChR1 (Fig. 2E, F, G), except for HEK-mVChR1 with a stimulus of 450 nm (Fig. 2E). The dual gene expression prolonged the τONs and τOFFs at every wavelength (Fig. 2B–G), and the prolongation increased with the wavelength of the stimulus (Table 1A and B). The rates of deactivation of HEK-ChR2+mVChR1 were smaller than those of HEK-ChR2 (Fig. 2H and I) and HEK-mVChR1 (Fig. 2H, I, J). There was no significant difference in the rate of deactivation of HEK-ChR2 with a stimulus of 550 nm (Fig. 2J). The rate of deactivation was decreased by the dual gene expression except when compared to HEK-ChR2 at a stimulus of 550 nm (Table 1C).

3.3. Response to light intensity

Photocurrents elicited by various light intensities at 500 nm were evaluated. The maximum photocurrent of HEK-ChR2+mVChR1 did not increase compared to that of HEK-ChR2 (Figs. 1A and 3A). However, the response of lower light intensity (0.1 μW/mm^2) was significantly increased (Fig. 3B). The curve of relative sensitivity adjusted at the maximum photocurrent (10 μW/mm^2) was well-fitted with that of HEK-mVChR1 (Fig. 3C).

4. Discussion

Recently, various types of ChRs have been developed all over the world [17,19,20]. Point mutation studies have elucidated the key amino acids and key transmembranes and their specific functions

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