

## Structural mechanisms underlying anion selectivity and high-speed gating in anion channelrhodopsins

Light is one of the most useful resources for energy and information, and most animals capture light using rhodopsin family proteins. Rhodopsin family proteins are mainly classified into two groups: microbial (type I) and animal (type II). They are both characterized by seven transmembrane (TM) helices that bind a chromophore retinal, but their functions are very different. Animal rhodopsins primarily work as G-protein-coupled receptors, whereas microbial rhodopsins have more divergent functions such as ion pumps, ion channels, sensors, adenylyl/guanylyl cyclases, and phosphodiesterases. Recently, these rhodopsin family proteins have attracted broad attention as powerful tools to control intracellular signalings, ion concentrations, and cyclic nucleotide concentrations in a light-dependent manner (termed optogenetics) [1]. Among these proteins, ion-pump and ion-channel rhodopsins are the most well-established optogenetics tools. Because the inward flow of cations or the outward flow of anions across the cell membrane depolarizes the membrane potential and vice versa, the light-mediated activation of these pumps and channels modulates neuronal excitability. As membrane-depolarizing, excitatory optogenetics tools, the most common proteins currently used are cation channelrhodopsins (CCRs). Since the first discovery of *Chlamydomonas reinhardtii* ChR1 (*CrChR1*) and ChR2 (*CrChR2*) in 2002-3, many variants have been engineered and isolated. The currently available CCRs offer a wide choice of absorption spectra, conductances, light sensitivities, channel kinetics, and so on. As compared with excitatory optogenetics tools, the development of membrane-hyperpolarizing, inhibitory optogenetics tools has been lagging behind. Light-induced neuronal inhibition was first achieved by inward  $\text{Cl}^-$  pumps and outward  $\text{H}^+$  pumps such as *Natronomonas pharaonis* halorhodopsin (*NpHR*) and archaerhodopsin-3 (*AR3*). However, the application of these inhibitory optogenetics tools has been limited because of their low conductance and low light sensitivity. In 2014-5, CCR-based artificial anion channelrhodopsins (ACRs), such as *iC++* and *iChloC*, were engineered, and naturally occurring anion channelrhodopsins, including *GtACR1* and *GtACR2*, were isolated from chlorophyte algae [2]. These ACRs can translocate  $10^4$ - $10^5$  ions per second and have a  $10^2$ - $10^4$ -fold higher light sensitivity than previously used inhibitory tools such as

*NpHR* and *AR3*, and now they are widely applied to neuroscience research in a wide range of animals including mice, flies, and fish. Both designed and natural anion-conducting channelrhodopsins (dACRs and nACRs) have since been applied as inhibitory optogenetic tools, but each also exhibits performance tradeoffs that underscore their limitations. For example, dACRs offer a much wider range of kinetics than nACRs; on the other hand, nACRs exhibit larger photocurrents. Therefore, molecular and structural understanding of both dACRs and nACRs will be critical not only to understand the mechanisms underlying fundamental channel properties including channel kinetics and conductance, but also to enable the creation of new optogenetics tools.

To identify dACRs and nACRs that are suitable for structural studies, we screened all reported dACRs and nACRs, and found that *iC++* (dACR) and *GtACR1* (nACR) show excellent expression and thermostability, which are important for crystallization. The crystals of both *iC++* and *GtACR1* were obtained using the lipidic cubic phase (LCP) crystallization method, and diffraction datasets were collected at SPRING-8 **BL32XU** and the GM/CA-CAT beamlines 23ID-B and 23ID-B of APS. Finally, the structures of *iC++* at two pHs (8.5 and 6.5) and *GtACR1* were determined at 2.9, 3.2, and 2.9 Å resolutions, respectively [3] (The *GtACR1* structure was reported in detail in an accompanying paper [4]).

Both *iC++* and *GtACR1* form a dimer, and each monomer is composed of an N-terminal extracellular domain and a 7-TM domain. The overall architectures of *iC++* and *GtACR1* are similar, including the shape of their ion-conducting pathways (Fig. 1). Both ACRs have two extracellular vestibules, extracellular vestibules

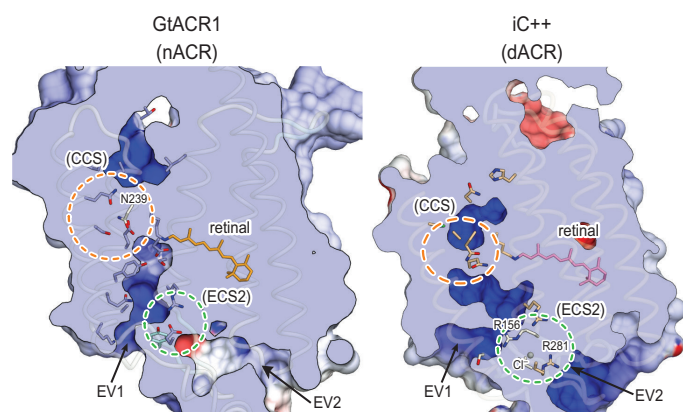


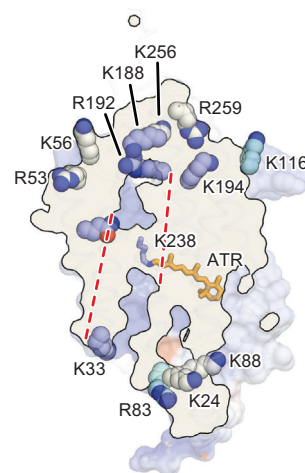
Fig. 1. Structural comparison of ion-conducting pathways between *GtACR1* and *iC++*. Green and orange circles represent ECS2 and CCS, respectively.

1 and 2 (EV1 and EV2), and only EV1 extends to the central constriction site (CCS) of the ion-conducting pathway. EV2 is occluded at extracellular constriction site 2 (ECS2) in both *iC++* and *GtACR1*. To analyze whether these structurally similar motifs (ECS2 and CCS) have the same functions, we performed an all-atom molecular dynamics (MD) simulation and patch clamp analyses of *iC++* and *GtACR1*. The extensive computational and electrophysiological analyses revealed that the functions of these constriction sites are indeed very different. In *iC++*, ECS2 is formed by a Cl<sup>-</sup>-mediated hydrogen bond between Arg 156 and Arg 281. However, the interaction is often broken, and Arg 281 is oriented towards an extracellular bulk solvent during the simulation. It is observed that Arg 281 sometimes catches Cl<sup>-</sup> floating in the solution, drags it into the extracellular vestibule, and forms a transient interaction with Arg 156. These results suggest that the residues at ECS2 of *iC++* are involved in anion conduction, and the electrophysiological analysis of Arg 156 and Arg 281 supports the idea. In contrast, the interaction at ECS2 of *GtACR1* is more stable, and mutational analysis suggests that they are important to regulate the closing of the channel. The stability and functions of CCS are also different between *iC++* and *GtACR1*. The interactions at CCS of *iC++* are weaker than those of *GtACR1*, and mutational analysis suggests that they work as a main determinant of ion selectivity. However, the mutations introduced to the residues at CCS of *GtACR1* do not significantly affect ion selectivity but accelerate channel closing, indicating that they are more involved in the regulation of channel kinetics. Further electrophysiological analyses revealed that, unlike *iC++*, the anion selectivity of *GtACR1* is mainly determined by the positively charged amino acid residues near the ion-conducting pathway; there are 12 positively charged amino acids positioned close to the ion-conducting pathway, and 3 of these 12 mutants show a leakage current of cations (Fig. 2). This suggests that these positively charged residues cooperatively contribute to anion selectivity in *GtACR1*.

Notably, one of the CCS mutants of *GtACR1* tested above (N239Q) has a powerful effect on current decay kinetics while it maintains a photocurrent amplitude comparable to that of wild-type *GtACR1*. It is assumed that this mutant protein can be used as an inhibitory optogenetic channel with unprecedented speed and temporal resolution for single-spike inhibition in neurons. Thus, we combined N239Q and the R83Q mutation, which we had found to be effective for increasing the photocurrent amplitude, and named the R83Q/N239Q mutant as FLASH (Fast, Light-activated Anion-Selective rHodopsin). Since a recently described nACR (ZipACR) showed the fastest reported anion channel kinetics thus far [5], we compared FLASH

and ZipACR, first using HEK293 cells and cultured neurons, and later using acute slices of mouse hippocampus, a living mouse, and a living worm. In all systems, more efficient inhibition was observed from FLASH-expressing cells and neurons, suggesting that FLASH is the ACR of choice for inhibitory optogenetic experiments.

In summary, the current study reported the first crystal structures of a dACR (*iC++*) and a comparison of the *iC++* structure with that of an nACR (*GtACR1*), not only to provide insight into their ion conductance, channel gating, and anion selectivity, but also to enable engineering of the first ACR integrating all the key features of a large photocurrent magnitude and fast kinetics alongside exclusive anion selectivity.



**Fig. 2.** Positively charged residues positioned close to the ion-conducting pathway of *GtACR1*. The mutants of K188, R192, and K256 show significantly lower anion selectivities.

Hideaki E. Kato<sup>a,b,†</sup>

<sup>a</sup>Department of Molecular and Cellular Physiology,  
School of Medicine, Stanford University, USA

<sup>b</sup>JST / PRESTO

Email: hekato@bio.c.u-tokyo.ac.jp

<sup>†</sup>Present address: Komaba Institute for Science,  
The University of Tokyo

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