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Review

Channelrhodopsin unchained: Structure and mechanism of a light-gated cation channel ☆

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ABSTRACT

The new and vibrant field of optogenetics was founded by the seminal discovery of channelrhodopsin, the first light-gated cation channel. Despite the numerous applications that have revolutionised neurophysiology, the functional mechanism is far from understood on the molecular level. An arsenal of biophysical techniques has been established in the last decades of research on microbial rhodopsins. However, application of these techniques is hampered by the duration and the complexity of the photoreaction of channelrhodopsin compared with other microbial rhodopsins. A particular interest in resolving the molecular mechanism lies in the structural changes that lead to channel opening and closure. Here, we review the current structural and mechanistic knowledge that has been accomplished by integrating the static structure provided by X-ray crystallography and electron microscopy with time-resolved spectroscopic and electrophysiological techniques. The dynamical reactions of the chromophore are effectively coupled to structural changes of the protein, as shown by ultrafast spectroscopy. The hierarchical sequence of structural changes in the protein backbone that spans the time range from 10^{-12} s to 10^{-3} s prepares the channel to open and, consequently, cations can pass. Proton transfer reactions that are associated with channel gating have been resolved. In particular, glutamate 253 and aspartic acid 156 were identified as proton acceptor and donor to the retinal Schiff base. The reprotonation of the latter is the critical determinant for channel closure. The proton pathway that eventually leads to proton pumping is also discussed. This article is part of a Special Issue entitled: Retinal Proteins — You can teach an old dog new tricks.

1. Introduction

Ion transport in living organisms is divided into active and passive, and the proteins responsible are known as transporters (pumps, exchanges, carriers, etc.) and channels [1,2]. Ion channels are central to living organisms; they are involved in signal transduction processes and in the conduction of electrical signals. Specifically, they mediate the uncoupled downhill movement of cations and anions across biological membranes by transiently opening an ion conductance pathway, a process known as gating [3]. The ion pathway generally consists of a narrow water-filled pore formed by specific residues in the protein interior, through which ions can diffuse across the otherwise impermeable cell membrane. Channels can be classified by the way the gating process is triggered. Ligand-gated channels open when specific molecules bind to them; voltage-gated channels open in response to changes in the electric field across the membrane; and mechanosensitive channels open following pressure changes [4]. The functionality of ion channels is generally studied by electrophysiology techniques, by measuring the flow of changes through the membrane (current) under variable conditions [5,6].

Channelrhodopsins (ChRs) are the first and so far unique light-gated ion channels known in nature [7]. Cation permeation by ChRs can be triggered fast, repetitively, reproducibly, and non-invasively by light, opening new ways of addressing fundamental aspects of channel on- and off-gating with unprecedented temporal and spatial resolution. The first identified ChRs were ChR1 [7] and ChR2 [8], naturally hosted by the eyespot of the unicellular alga *Chlamydomonas reinhardtii*. More recently, up to thirteen ChR sequences have been identified in other green algae, differing among each other mainly in cation selectivity, kinetics, light wavelength sensitivity, and light intensity sensitivity [9–12]. Upon illumination, ChRs transiently increase their conductance for a variety of monovalent and divalent cations, leading to depolarisation of the cell membrane in milliseconds. Such a property has made ChRs a versatile and valuable optogenetic tool to alter the membrane potential of a host cell, mostly to control neural activity [13].

Abbreviations: ASR, *Anabaena* sensory rhodopsin; BHK cells, baby hamster kidney cells; bR, *H. salinarum* bacteriorhodopsin; CaChR, *Chlamydomonas augustae* channelrhodopsin; ChR, channelrhodopsin; ChR1, ChR1 from *Chlamydomonas reinhardtii*; ChR2, ChR2 from *Chlamydomonas reinhardtii*; C1C2, ChR1–ChR2 chimaera; CyChR, *Chlamydomonas yellowstonensis* channelrhodopsin; DsChR, *Dunaliella salina* channelrhodopsin; FTIR, Fourier transform infrared; HeLa cells, Henrietta Lacks cells; HEK cells, human embryonic kidney cells; hR, *H. salinarum* halorhodopsin; MD, molecular dynamics; MM, molecular mechanics; MvChR, *Mesostigma viride* ChR; QM, quantum mechanics; SB, Schiff base; sRII, sensory rhodopsin II; VcChR, *Volvox carteri* ChR; YFP, yellow fluorescent protein

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In their native host, ChRs are photoreceptors, being part of the poorly understood molecular machinery that directs unicellular algae toward or away from light (phototaxis) to optimise photosynthetic growth [14]. ChR1 and ChR2 are supposed to directly mediate the photocurrents that induce membrane depolarisation of the eye spot of *C. reinhardtii* [14], but the mediation of a secondary channel cannot be discarded [15]. While both ChR1 and ChR2 are responsible for the phototactic responses (each responding to different light intensities), the photophobic response is dominated by ChR1 [16–18].

ChRs lack sequence homology to any other known ion channel. Instead, they belong to the family of microbial rhodopsins, also known as type I rhodopsins, comprising light-driven ion-pumps and photoreceptors in archaea, eubacteria, fungi and algae [19]. Microbial rhodopsins share a similar primary sequence for the apoprotein (known as opsin), and a characteristic seven transmembrane membrane fold (7-TM) with the retinal chromophore covalently linked to a conserved lysine to form a protonated Schiff base (SB) [20]. The primary step in all known microbial rhodopsins, bacteriorhodopsin (bR) being the paradigm, is the photo-induced isomerisation of retinal around the $C_{13}{=}C_{14}$ bond of the retinal chromophore. In ChRs, retinal isomerisation eventually leads to channel opening, presumably via concerted structural and electrostatic changes that are yet to be defined.

This review focuses on the functional and structural dynamics of ChR2 from *C. reinhardtii*. Among all ChRs known so far, ChR2 is most widely used in optogenetic applications [11,13]. The properties of ChR1 or ChRs from organisms other than *C. reinhardtii* are discussed only whenever relevant to ChR2. All along the text, we use ChR2 residue numbering for all ChR sequences, but use the original numbering for other microbial rhodopsins (e.g., bR). Some additional aspects of ChR2 and ChRs in general, such as their physiological role in the phototaxis of algae [14,21,22], the properties of variants and chimaeras [23,24], or applications in optogenetics [13], are not or are only obliquely treated here.

2. Function and general properties of ChR2

2.1. Ion channel activity

In their seminal work, Nagel et al. [8] showed that both full length ChR2 and a fragment (1–315) comprising only the transmembrane region produced identical photocurrents and a very similar current/voltage relationship. The channel function is therefore confined to the 7-TM region. The function of the soluble cytoplasmic domain (approximately 400 amino acids) remains unknown. A truncated ChR2 construct has been used as a de facto wild-type (WT) in posterior studies, with the occasional addition of a yellow fluorescent protein (YFP) sequence at the C-terminus to monitor expression levels and cellular localisation by fluorescence microscopy [25] or a His tag when ChR2 is expressed and purified from *Pichia pastoris* [26].

The channel activity of ChR2 is monitored in electrical measurements [8]. Well-defined conditions require expressing ChR2 in a host cell and holding the membrane potential (voltage-clamp) at a constant ionic composition of the medium. The resulting light-induced current, the photocurrent, gives a measure of the net flow of cations. ChR2 photocurrents have been recorded in many naturally light-insensitive cells, namely *Xenopus* oocytes, human embryonic kidney (HEK) cells, baby hamster kidney (BHK) cells, Henrietta Lacks (HeLa) cells, cultured neurons, etc., and their properties have been shown to be mostly insensitive to the host system used [8,24,27,28]. The photocurrents, usually measured using light pulses of several hundreds of milliseconds in duration, show a reversal potential for symmetrical ion concentrations close to zero volts: they are passive and thus linked to ion permeation. Anions are not conducted [7,8], but protons and a wide range of monovalent and divalent cations are [8]. The permeability for protons is at least 10^6-fold higher than for any other monovalent cation [8,27,29], while the permeability for divalent alkaline cations (e.g., Ca^{2+}) is 10–100 times lower than for monovalent alkali ones (e.g., Na^+) [27,29]. We should note that although ChR1 was initially defined as a selective proton channel [7], it was later shown to be permeable to other cations as well [18]. Despite the much higher proton permeability, at physiological pH and cation concentrations, a significant part of the current is carried by cations other than protons [27,30].

As expected for a channel, the photocurrents modestly increase with temperature (activation energy of ~20 kJ/mol), suggesting that cation permeation in the open state in ChR1 and ChR2 is rate-limited by small barriers for ion diffusion and not by energetic protein conformational fluctuations [7,31]. While the activation energy for channel opening has not been determined yet, it is ~65 kJ/mol for channel closure [7,31], large enough to indicate a coupling with protein conformational changes. The intensity of the photocurrents is inversely related to the atomic radius for alkali and alkaline cations, suggesting that they traverse the narrower region of the pore (the selectivity filter) in a mostly dehydrated state [8]. The dependence of the photocurrent on alkali cations of different sizes was used to estimate an effective cation pore of ChR2, found to be ~6.2 Å in diameter, in between the pore diameter of voltage-activated Na^+ channels and the nicotinic acetylcholine receptor [8,32]. The photocurrents as a function of Na^+ concentration showed no sign of saturation [33], but they did for guanidinium ($K_m = 82$ mM [31]) and Ca^{2+} ($K_m = 18$ mM [33]).

The curved photocurrent/voltage relationship of ChR2 is characteristic for an inward rectified channel [34], i.e., for the same membrane potential, the current is higher (and thus the resistance is lower) when the potential is negative and cations flow to the inside of the cell. Single-molecule properties, indirectly deduced by photocurrent fluctuations, indicate that the inward rectification of ChR2 is intrinsic to the single-channel conductance, and not simply the result of a higher fraction of open channels at negative potentials [31]. The photocurrent-voltage dependence of ChR2 for various conducted cations could be better modelled using a single re-oriented binding site for which all the conducted cations compete (a model akin to those of transporters [35,36]), than by using electro-diffusion models more familiar to those for channels [27,37]. Two putative cation-binding sites have been identified in the dark state of ChR2 by molecular dynamics (MD) simulations [38], one on the extracellular side (close to residues S52, N56, and E97 in helices A and B) and the other on the cytoplasmic side (close to E82, E83, H134, H265, and R268 in helices B, C, and G).

The single-channel conductance of ChR2 was estimated to be 60 fS for Na^+ at −60 mV by analysis of photocurrent fluctuations [31]. Such conductance would translate to a maximum turnover of 2×10^4 Na^+/s per ChR2 molecule (a current of ~15 fA for a single channel), too low for single-molecule electrical recordings [8,31]. Despite its widespread use in optogenetics, ChR2 is arguably one of the less efficient ion channels known. The estimated unitary conductance of ChR2 is lower than archetypical ion channels by a factor of 10–10^4 [3,39]. ChR2 cation turnover is only two times higher than that of the Cl/HCO_3^- exchanger [40], one of the fastest transporters. Thus, the efficiency of ChR2 lies in the grey area between channels and transporters.

It is worth noting that it is not only the microbial rhodopsin family that comprises both ion pumps and channels, but also the family of Cl^- channels and transporters (ClC) [41]. In the latter case, it was even shown that a Cl^-/H^+ exchanger could be converted into a passive channel for Cl^- by the substitution of only two key residues [42]. The resulting Cl^- flow was 4×10^4 ions/s, i.e., 100–1000 times smaller than the most efficient Cl^- channels of the ClC family [39], but in the order of cation permeation by ChR2. This and other examples illustrate the fuzzy barrier between transporters and channels [39,43,44].

2.2. Proton pumping activity

In addition to being a cation channel, ChR2 exhibits light-driven proton pumping activity, as shown from photocurrents in planar