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REVIEW

Channelrhodopsins: From Phototaxis to Optogenetics

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Abstract—Channelrhodopsins stand out among other retinal proteins because of their capacity to generate passive ionic currents following photoactivation. Owing to that, channelrhodopsins are widely used in neuroscience and cardiology as instruments for optogenetic manipulation of the activity of excitable cells. Photocurrents generated by channelrhodopsins were first discovered in the cells of green algae in the 1970s. In this review we describe this discovery and discuss the current state of research in the field.

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INTRODUCTION

Bacteriorhodopsin, the focus of L. A. Drachev's early research [1-3], was the first retinal protein identified in a microbe rather than in mammalian tissues. The discovery of bacteriorhodopsin by D. Oesterhelt and W. Stoeckenius in the early 1970s [4] was a sensation, and the ease of isolation of this protein in large quantities from the source organism, haloarchaeon *Halobacterium salinarum*, and its remarkable stability have made it a favorable object of many biophysical, biochemical, and structural studies [5-8]. It would not be an exaggeration to say that today we know about bacteriorhodopsin more than not only about any other retinal protein, but also more than about any integral membrane protein, a model of which it represents. In contrast to visual rhodopsins that fulfil their photosensory function by activation of an enzymatic transduction cascade, bacteriorhodopsin is an electrogenic proton pump that transports protons from the cytoplasm to the external medium and thus provides basic means of solar energy utilization in haloarchaeal cells.

When photoelectric activity of bacteriorhodopsin was discovered, it stimulated search for similar processes in other microorganisms, including eukaryotes. Most motile pro- and eukaryotes respond to light with a change in their swimming pattern [9, 10]. The first sensory rhodopsins, SRI [11-13] and SRII [14], have been discovered in *H. salinarum*. Their photoexcitation does not lead to generation of transmembrane electrical currents but activates an enzymatic cascade, eventually causing motor responses. In contrast to haloarchaea that change their reversal frequency in response to light, unicellular green flagellate algae demonstrate genuine phototaxis, i.e., oriented movement along the direction of the light beam [15]. Discussion of this phenomenon with E. N. Kondratieva, a well-known expert on phototroph microorganisms [16], and successful registration of photoinduced changes in the membrane potential of chroloplasts isolated from a higher plant [17, 18], inspired research on photosensing in green algae in the Department of Physico-Chemical Biology of the Moscow State University under the guidance of F. F. Litvin. However, attempts to detect electrical stages of photosensory transduction by insertion of microelectrodes into algal cells, as used for recording electrical processes associated with photosynthesis, have been unsuccessful [19]. This goal could only be achieved by the development of a new method for extracellular recording using a suction pipette by the corresponding author of this review [20, 21].

Using this method, it has been shown that light in the spectral range of phototaxis evokes transmembrane photoreceptor potential in the region overlaying the eyespot – a cellular organoid made of carotenoid globules that acts as a modulator of photoreceptor illumination

Abbreviations: ChR, channel rhodopsin; EPC, early photoreceptor current; LPC, late photoreceptor current.

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during helical swimming of the cell. Isotonic propagation of the depolarizing photoreceptor potential to the flagellar membrane triggers a regenerative electrical response similar to an action potential in neurons. The selection of the object, the green alga *Haematococcus pluvialis* of a relatively large cell size and with the elastic cell wall, was a major factor in this work's success. Subsequently the corresponding author of this review has shown that both stages of this photoelectric sensory cascade could be recorded directly in a cell suspension [22], which enabled its detection in many other green and cryptophyte algae [23-27].

In contrast to bacteriorhodopsin, the main component of purple membranes that constitute ~50% of the cell surface, photoreceptor proteins that guide algal phototaxis are present in their cells in very low concentrations [28], so that their purification by biochemical methods is not feasible. Analysis of phototaxis action spectra by K. Foster led to a hypothesis that these photoreceptors are retinal proteins [29]. A large body of indirect evidences obtained during the 1980s and 1990s, including restoration of phototaxis in "blind" mutants of the green alga *Chlamydomonas reinhardii* deficient in carotenoid biosynthesis upon the addition of exogenous retinal provided the first experimental validation of this hypothesis [30-32]. Identification of *Chlamydomonas* photoreceptor proteins or, rather, genes encoding them, at the molecular level has been achieved only upon the development of high-throughput methods of polynucleotide sequencing. It turned out that the genome of *C. reinhardtii* encodes not just one, but two such proteins [23, 33-35]. Remarkably, protein sequences, isomer composition of the chromophore, and primary photochemical reactions of *C. reinhardtii* rhodopsins turned out to be closer to bacteriorhodopsin than to animal visual rhodopsins. Analysis of photoreceptor currents in *C. reinhardtii* genetic transformants with reduced amounts of rhodopsins showed that these proteins serve as photoreceptors in phototaxis and the photophobic (photoshock) motile response of this alga, although photoreceptor currents generated by them differ in their properties [23, 28].

But only expression of *C. reinhardtii* rhodopsin genes in animal cells, such as *Xenopus* oocytes, carried out by G. Nagel, P. Hegemann, and E. Bamberg, has revealed their unique properties [33, 34] (earlier this approach was successfully used also to study bacteriorhodopsin [36]). It tuned out that unlike the latter, *C. reinhardtii* rhodopsins passively transport cations (not only protons, but also sodium, potassium, and, to a lesser extent, calcium ions) across the cell membrane, i.e., act as light-gated cation channels, the only such channels known so far. This property was highlighted by introducing a new name, "channelrhodopsin (ChR)", to designate these proteins, which rapidly replaced the earlier suggested names, "Chlamydomonas sensory rhodopsins" [23] and "archaeal-type rhodopsins" [35].

ChRs became very popular after they had been shown to stimulate neuronal activity upon illumination [37-39]. This technique, knows as "optogenetics", has revolutionized neuroscience and related fields of research [40-43]. Moreover, it is anticipated that ChRs might be used for gene therapy to cure many neurological, psychiatric, and cardiovascular disorders [44, 45]. The prospect of vision restoration in patients with degenerative retinal conditions looks most promising [46]. Photoactivated proton pumps similar to bacteriorhodopsin are also used in optogenetics as the tools for inhibition of neuronal activity [47], although they transport less than one charge per captured photon, generate smaller currents than ChRs and therefore require illumination of longer duration and/or higher intensity to change the membrane potential.

High-throughput sequencing of genomes and transcriptomes led to identification of ChRs in several other major eukaryotic lineages besides green algae. Moreover, it turned out that phototrophic [48] and even heterotrophic [49] protists possess ChRs that selectively conduct anions. It also looks like cation selectivity independently emerged at least twice in ChR evolution, and in one case the proteins preserved many features of bacteriorhodopsin [50]. Finally, last year ChRs were found that are more permeable for K^+ than for Na^+ [51], and ChR diversity might not yet be exhausted.

In retinal ion pumps like bacteriorhodopsin, all steps of the ion transport are tightly linked to specific photochemical conversions of the photocycle monitored by optical methods in purified proteins or membranes [52]. In contrast to this, in ChRs of different families photocurrents correlate with different photochemical conversions, and this correlation provides information on functional mechanisms of the channels [50, 53-55].

In this review we will discuss methods of recording ChR activity in native cells, the photoelectric sensory cascade in flagellate algae, the photoreceptor role of ChRs in phototaxis, and, briefly, their diversity and optogenetic applications. For more detail of ChR molecular mechanisms we recommend other reviews [56-59]. Also, many reviews cover the history and principles of optogenetics [42, 60-65].

METHODS FOR REGISTRATION OF ChR ACTIVITY IN PROTIST CELLS

Photoinduced changes in the membrane potential in retinal rodes and cones brought about by closing cyclic-nucleotide-gated channels can be recorded with intracellular electrodes [66]. However, the small cellular volume of flagellate algae makes this approach not feasible for the study of ChR activity. Some green flagellates such as *H. pluvialis* possess an elastic cell wall, which makes it possible to suck their cells into a micropipette.

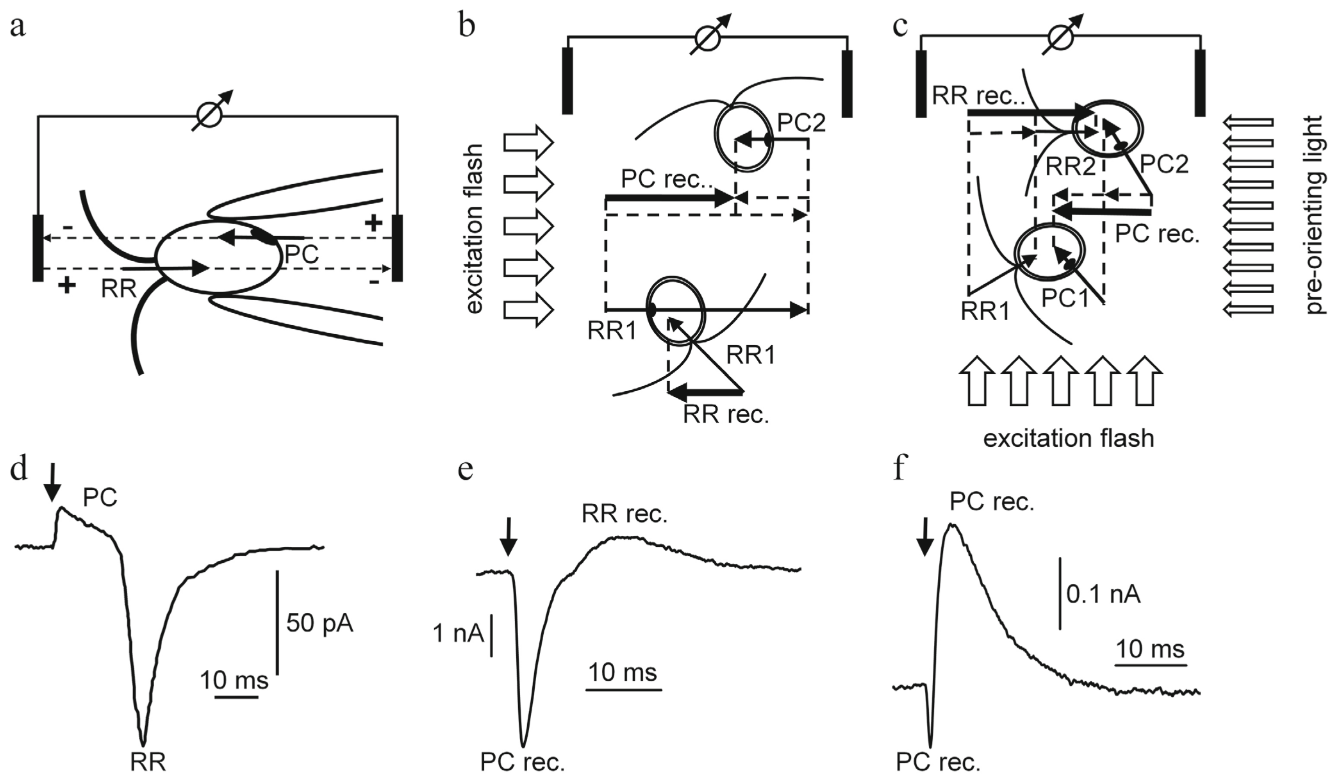

Fig. 1. Principles of recording and examples of photoelectric signals recorded in the cells of green flagellate algae: a-c) principles of the suction pipette technique (a), measurements in non-oriented cell suspensions (b), and measurements in pre-oriented cell suspensions (c); d-f) examples of photocurrent traces recoded from an individual cell (d), a non-oriented cell suspension (e), and a pre-oriented cell suspension (f). The arrows in panels (a-c) show the time of the excitation flash. Designations: PC, photoreceptor current; RR, regenerative response; rec., recorded.

This does not lead to formation of a gigaOhm seal that is necessary for voltage clamp, but asymmetric distribution of ChR molecules in the cell membrane allows registration of their photoinduced responses [20, 21]. Thirteen years later this approach (known as the suction pipette technique, Fig. 1a) was applied to a *C. reinhardtii* mutant lacking outer layers of the cell wall [67], and in eight more years, to a *Volvox carteri* mutant [68]. *C. reinhardtii* is a model object of many biological studies, in the genome of which the first channelrhodospin genes were subsequently identified [23, 33-35].

The development of the suction pipette technique enabled the discovery of photoinduced depolarization of the membrane in green flagellate algae mediated by their phototaxis receptors [20, 21], which laid the foundation for the development of optogenetics twenty-five years later. However, application of the suction pipette technique is not only labor-consuming, but also limited to cells with the elastic cell wall, rare among flagellates. In addition, sucking a cell into a pipette may activate its mechanoreceptors [69] and in any case cannot be considered as a physiological condition. Asymmetric localization of molecular generators of photocurrents in different regions of the cell membrane, revealed by using the suction pipette technique, led to the hypothesis that a total photocurrent of thousands of cells in a suspension can be recorded even without artificial increase of the electrical resistance between different regions of the membrane by the glass of the pipette.

In the first modification of this assay, a photoexcitation flash is directed along the line connecting the electrodes (Fig. 1b). Photoreceptor current generated by the cells oriented with their photoreceptors toward the light source exceeds that by the cells in the opposite orientation, and the difference signal is detected by the electrodes immersed in the cell suspension. Another modification of this assay uses preorientation of the cells with the light that causes phototaxis, or with gravity, directed at the 90° angle to the excitation flash (Fig. 1c). In this case the electrodes register a projection of the photocurrent on the direction of the preorienting factor, which allows instantaneous measurements of the orientation degree [22, 70, 71].

Both modifications of this suspension assay allow recording under fully physiological conditions and are not limited by the cell size and the cell wall structure. Such versatility and technical simplicity allowed using this method to study many *C. reinhardtii* mutants with defects in the eyespot size [72] and photomotility [73, 74]. Moreover, the suspension assay was used to confirm that phototaxis restoration in “blind” carotenoid-deficient mutants upon the addition of exogenous

retinal indeed reflects reconstitution of functional photoreceptor proteins rather than, for instance, retinal incorporation in the eyespot [31]. Furthermore, the suspension assay enabled the discovery and characterization of channelrhodopsin-mediated photoinduced electrical cascades in several other green algae, including members of the genera *Spermatozopsis*, *Hafniomonas*, *Polytomella*, *Mesostigma*, and *Platymonas*, and in the phylogenetically distant cryptophyte alga *Cryptomonas* [24-27]. Finally, the suspension method was used as an indirect approach to study gravitaxis [22] and chemotaxis [75] in *C. reinhardtii*, and provided a foundation for the development of an express bioassay for detection of water pollution with heavy metals and formaldehyde [76, 77]. A more detailed technical description of both methods, the suction pipette technique, and the suspension assay, can be found in the earlier published reviews [25, 70, 71].

Electrophysiological investigation of photoreceptor currents allowed characterization of photoreceptor proteins in flagellate algae even before their genes were cloned. Approximation of the light dependence of photoreceptor current produced the value of 0.8 Å for the product of the quantum yield and the optical cross section, which is close to that of other retinal proteins [78]. The recovery of photoreceptor current after a saturating flash provided an estimate of the duration of the photoreceptor pigment's photocycle (100 ms). Analysis of the dependence of photoreceptor current amplitude on the orientation of the polarization plane of the light stimulus showed that the retinal chromophore is orientated in parallel with the membrane plane [79, 80]. The action spectra of photoreceptor current and photomotility of *H. pluvialis* reveal a complex multiband structure, with the most red-shifted band at 550 nm [20, 21]. This result led to the conclusion that in flagellates the photoreceptor system might consist of several pigments acting as an antenna for a bacteriorhodopsin-type protein. Subsequently, a contribution of two photoreceptor proteins in phototaxis has directly been shown in the model alga *C. reinhardtii* [23], and even more candidates for this role have been found in some other algae. However, all these proteins are light-gated ion channels rather than photoactivated ion pumps like bacteriorhodopsin [33, 34].

PHOTOELECTRIC CASCADE IN PHOTOTAXIS OF GREEN FLAGELLATE ALGAE

The photoelectric signal recorded in *H. pluvialis* cells sucked into a micropipette comprises a gradual primary photoreceptor current and a secondary regenerative response that is driven by the primary current and develops in the "all or nothing" manner. Comparison of the signs of these signal components upon suction of different parts of the cell into the pipette revealed that the primary inward photocurrent flows only across a small patch of the plasma membrane overlaying the eyespot [20, 21, 67]. This intracellular structure originally was thought to contain photoreceptor pigment [81], but later it was found to play only an accessory role as a shading/reflecting device [29, 82, 83].

After cloning of ChR genes, immunofluorescent methods could be used to determine intracellular localization of the encoded proteins. It has been shown that both *C. reinhardtii* ChRs are mostly confined to the eyespot region [35, 84, 85], which is fully consistent with the results of electrophysiological studies. Proteomic analysis of the isolated eyespot preparations also showed the presence of ChRs [86]. According to the current view, ChR molecules are embedded in the plasma membrane that shows specific ultrastructure in the eyespot region [87]. In *C. reinhardtii* cells grown under a light-dark cycle, immunofluorescence microscopy also shows ChRs in the flagella and basal bodies, and their amount in these organelles depends of the phase of the cycle [88, 89]. However, the functional state of ChRs found in these locations is not yet known.

Flagellates rotate around their longitudinal axis during swimming, so that the photoreceptor patch of the plasma membrane experiences a periodic change in illumination [9, 29, 90-94]. These conditions can be modeled in a cell held on a micropipette by periodic illumination at the 1 Hz frequency. At the light intensities eliciting phototaxis in *H. pluvialis*, the beat frequency of the *cis*-flagellum (the one closest to the eyespot) increases, whereas that of the *trans*-flagellum decreases after switching the light on, and the opposite responses are observed after switching the light off [78, 90, 95]. Such asymmetric changes in the flagella beat frequency in a freely swimming cell are expected to steer the cells in the direction of light, i.e., lead to phototaxis. Photoinduced motor responses of *C. reinhardtii* flagella have been studied in more detail and involve changes of not only beat frequency, but also amplitude and velocity [96-99].

The regenerative electrical response occurs in the flagellar membrane and therefore is described in the literature as "the flagellar current" [32, 67, 70]. Parallel registration of flagella beating and photoelectric responses in a cell held on a micropipette has shown that the regenerative response triggers a change in the flagella beating mode from a ciliary stroke to undulation, observed during the photophobic response in a freely swimming cell [20, 21, 78, 95, 100]. The photophobic response consists of a sudden stop and/or a random change in the swimming direction and is observed when the stimulus intensity and/or duration exceeds a certain threshold [9, 10, 91, 101, 102].

The amplitude of the regenerative response is practically independent on the intensity of the light stimulus, but the duration of the lag period from the beginning of illumination till the onset of this response shows an inverse dependence on it. The integral under

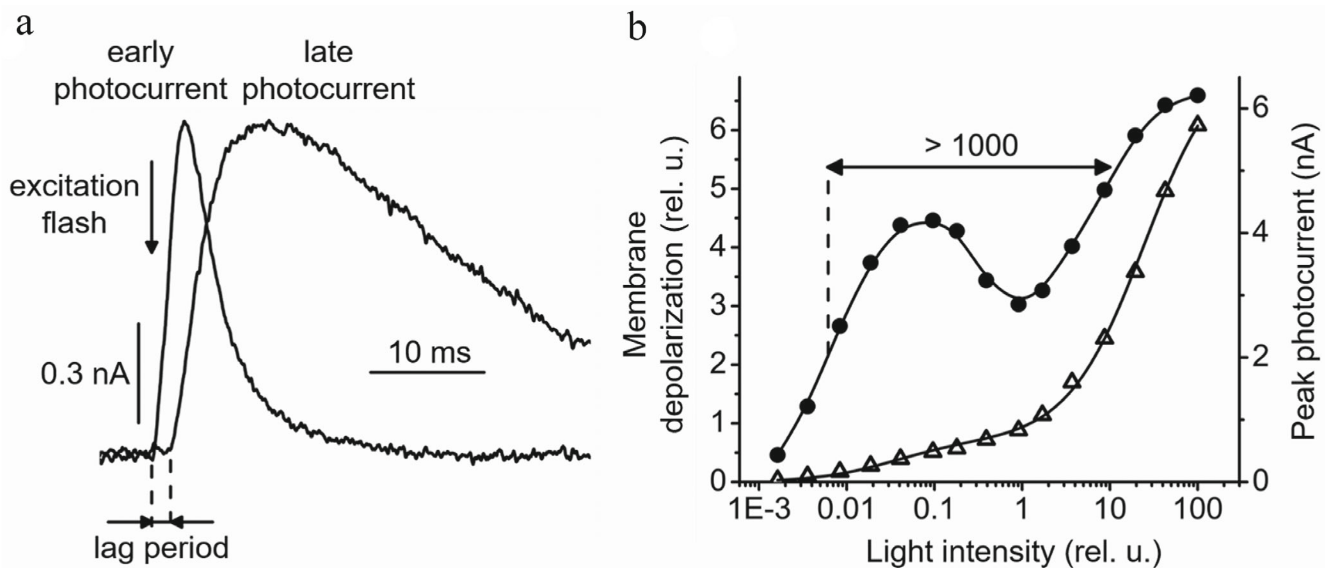

Fig. 2. Properties of the photoreceptor current components in the green flagellate alga *Chlamydomonas reihardtii*: a) the kinetics of the early and late photoreceptor currents, recorded from a cell suspension; b) the light dependence of the peak amplitude of photoreceptor current (right Y axis) and an integral under the photocurrent curve (left Y axis).

the photocurrent curve till the onset of the regenerative response is constant at any light intensity and increases under red background illumination that hyperpolarizes the membrane due to photosynthesis [19, 78, 79, 103]. These results show that the regenerative response is triggered by translocation of a certain number of charges across the membrane that depolarizes it by a few mV.

The strict dependence of the regenerative response on the Ca^{2+} concentration in the medium and its sensitivity to blockers of Ca^{2+} channels suggest that it is mediated by opening of potential-gated Ca^{2+} channels in the flagellar membrane [20, 21, 67]. Analysis of the regenerative response amplitude in *C. reinhardtii* cells during flagella regrowth after their amputation has shown that these channels are distributed over the entire flagella length [104]. Selection of a mutant deficient in the regenerative response [74] enabled cloning of the gene *cav2*, encoding these channels [105]. Immunofluorescent microscopy shows that the CAV2 protein is predominantly located in the proximal part of the flagella.

PHOTORECEPTOR CURRENT COMPONENTS

Experiments with nanosecond laser flash excitation and high acquisition rate have revealed that photoreceptor current comprises two components with different properties [25, 32, 78, 95, 106, 107]. These components are resolved in the current traces recorded from all tested organisms, which indicates common principles of their photoreceptor systems and signal transduction chains. The rise of the early photoreceptor current (EPC) is limited only by the response time of the setup (<30 μs for the suction pipette technique [106] and <3 μs for the suspension assay [31]). In contrast, the late photoreceptor current (LPC) occurs after a delay up to several milliseconds, the duration of which depends on the stimulus intensity. At least two kinetic components can also be resolved in the photocurrent decay. The light saturation level of the EPC is determined only by photochemical processes of photon absorption, whereas that of LPC is observed at ~1000-fold lower intensities. Only LPC but not EPC depends on temperature and red background illumination that hyperpolarizes the membrane by activating photosynthesis [19, 106]. Finally, LPC shows a much stronger dependence on the Ca^{2+} concentration in the medium, as compared to EPC [25]. Because of that, the actual kinetics of EPC can be determined by calculating the difference between the traces recorded before and after these treatments (Fig. 2a).

The maximal amplitudes of EPC and LPC measured at saturation yield the ratio <1 : 10. However, membrane depolarization is proportional not to the maximal photocurrent, but to the number of charges transported across the membrane, i.e., the integral under the photocurrent trace. Owing to slow closing of the LPC channels, the membrane depolarization brought about by LPC at saturation is practically equal to that produced by EPC at saturation, although the levels of their saturation differ 1000 times (Fig. 2b).

Ca^{2+} channels that mediate LPC are located in the plasma membrane near the eyespot region, but not necessarily within it. These secondary Ca^{2+} channels are the primary reason of the very high, nearly "single-quantum" photosensitivity of phototaxis [108]. In contrast to the Ca^{2+} channels in the flagellar membrane, these channels are not voltage-gated, because they generate gradual current (i.e., the current amplitude of which depends

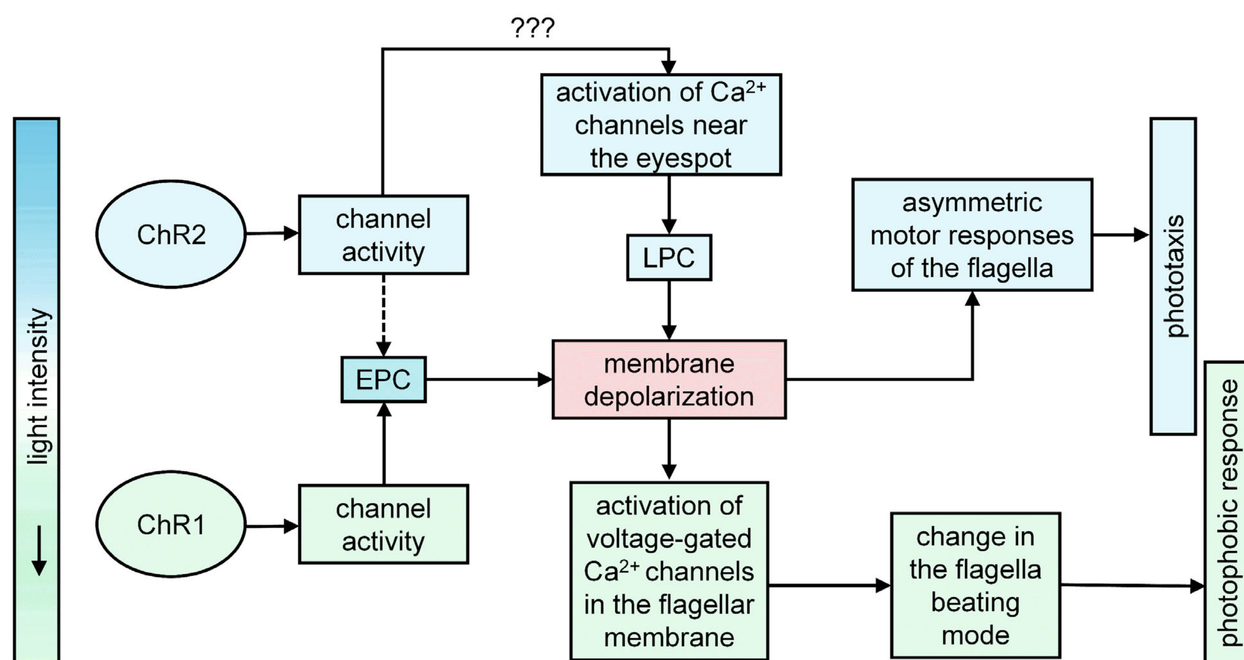

Fig. 3. A schematic depiction of photosensory transduction in phototaxis of flagellate algae. ChR1 and 2, channelrhodopsins 1 and 2, respectively; EPC and LPC, early and late photoreceptor currents, respectively.

on the stimulus intensity). At least 43 genes encoding Ca^{2+} channels have been found in the *C. reinhardtii* genome [109], but functions of only few of them are known. Several Ca^{2+}-binding proteins but no Ca^{2+} channels have been detected in the eyespot preparations [86, 110]. One of possible reasons for this failure is the insufficient sensitivity of proteomics for detection of integral membrane proteins. It is also possible that these presumable channels are not homologous to any other known Ca^{2+} channels and therefore their sequences cannot be recognized as such by bioinformatic analysis.

Mechanisms by which EPC activates the secondary Ca^{2+} channels in the eyespot region are not yet clear. Heterotrimeric GTPases, and Ca^{2+}-dependent protein kinases and phosphatases have been detected in preparation of isolated eyespots [111-113]. However, these proteins can participate in other sensory cascades, such as that activated by photoexcitation of phototropin, also found in the eyespot preparations [86, 110]. Phototropin has been shown to mediate photoregulation of the eyespot size and the cellular content of ChRs [114].

The lack of a lag period and the high level of light saturation led to the conclusion that EPC reflects an ion flow through rhodopsin molecules themselves [32, 103]. Attempts to determine the ionic selectivity of channelrhodopsins in *C. reinhardtii* [115, 116] and *V. carteri* [68] using the suction pipette technique have not been successful, because photocurrent recording in the algae cannot be carried out under voltage clamp, and because of the difficulty to separate EPC and LPC in these experiments. Ionic selectivity of these and tens of other channelrhodopsins has been determined only by patch clamp measurements upon channelrhodopsin expression in cultured animal cells.

Identification of ChRs genes in *C. reinhardtii* and the development of methods for genetic transformation of this model alga allowed verification of the role of ChRs as photoreceptor proteins in phototaxis and the photophobic response. Using the method of RNA interference, K.-H. Jung in the laboratory of J. L. Spudich created transformants with a decreased content of each of the two ChRs, initially named *Chlamydomonas* sensory rhodopsins A and B [23, 28], better known today as ChRs 1 and 2 (ChR1 and ChR2), respectively. Remarkably, knocking down of ChR1 led to stimulation of ChR2 expression, which led to a further increase in the ChR2/ChR1 ratio in the ChR1 transformant. Analysis of photoelectric signals recorded from transformant cells in response to a laser flash showed that kinetics of photoreceptor current generated by ChR1 is limited only by the time response of the measuring system, whereas the current generated by ChR2 appears after a light-dependent delay. In other words, ChR1 generated current with the kinetics of EPC, and ChR2, current with the kinetics of LPC. Moreover, ChR1 current exhibited high light saturation like EPC, whereas ChR2 current, low saturation like LPC. The action spectra of ChR1 and ChR2 photocurrents were also different, which reflected their different absorption spectra: the maximum of ChR1 current was observed at 510 nm, and that of ChR2 current, at 470 nm. These results were corroborated by absorption spectroscopy of purified proteins obtained by heterologous expression of their genes [84, 117, 118]. Measurements of photoorientation by photoelectric recording in

cell suspensions and of photophobic response by motion analysis showed that both ChRs mediate both photomotility responses [23, 28]. A light scattering assay for photoorientation in an independently created transformant with a reduced ChR1 content confirmed photoreceptor role of this protein [84]. The low light saturation of LPC mostly generated by ChR2 suggest that the latter is the primary photoreceptor in phototaxis, whereas ChR1, mostly responsible for EPC, primarily contributes to the photophobic response that is observed at higher light intensities (Fig. 3). It has to be however noted that the ratio of ChR1 and ChR2 is different in different strains [23, 84, 119], which may influence their relative contributions in photomotility responses.

The method of RNA interference allows to reduce the amount of the encoded protein in the cell, but not to prevent its biosynthesis completely. The latter has been achieved first with insertional mutagenesis [85, 120], and later with targeted disruption of ChR genes [119, 121, 122]. In a strain with the ChR1 gene disrupted by zinc finger nuclease, the sensitivity of photoorientation was decreased over 3 orders of magnitude, whereas disruption of ChR2 gene produced a smaller effect, and knocking out both ChR1 and ChR2 genes practically abolished the response [122]. When the CRISPR/Cas9 system was used for genome editing, knockout of either ChR gene had little effect on the sensitivity of photoorientation, but disruption of both genes eliminated the response [119]. The difference between these results is most probably explained by the difference between the amounts of ChR1 and ChR2 in the parental strains and the degree of compensatory upregulation of one rhodopsin upon suppression of the other one.

The CRISPR/Cas9 genome editing method enabled not only knocking out ChR genes, but also creating *C. reinhardtii* strains carrying point mutations of each of them [119]. All tested mutants exhibited reduction of photoorientation sensitivity, although the cellular levels of mutant proteins were also reduced in most cases, as compared to control. A ChR2 mutant with reduced relative permeability for protons compared to Na^+, as determined by patch clamp analysis in model mammalian cells, generated photoreceptor currents only in the presence of Na^+ in the medium, which confirmed the importance of proton currents for phototaxis in the wild type.

THE ROLE OF THE CYTOPLASMIC FRAGMENT

Molecules of all known ChRs consist of the rhodopsin domain, formed by seven transmembrane α-helices, and a cytoplasmic fragment made of an almost equal number of amino acid residues. Heterologous expression studies show that the rhodopsin domain is necessary and sufficient for channel conductance, although at least in one ChR the cytoplasmic fragment has been found to influence the kinetics of channel current [123]. Almost exclusively, only rhodopsin domains are used for optogenetic applications and functional studies in heterologous systems. Functional analysis of the cytoplasmic fragment has only become possible after the development of genome editing methods in *C. reinhardtii* cells [121]. Truncation of the last 78 C-terminal amino acid residues of ChR1 leads to displacement of the eyespot to a more anterior position, and the same effect is observed after disruption of the entire ChR1 gene [122]. Moreover, the attachment of a fluorescent tag to the C-terminus impedes trafficking of ChR1 to the plasma membrane. These observations confirm the earlier proposed hypothesis that the cytoplasmic fragments are responsible for the correct cellular localization of ChRs.

The important role of the cytoplasmic fragment in the function of microbial rhodopsins has been demonstrated earlier in sensory rhodopsins from the blue-green alga *Anabaena* [124]. Modification of the cytoplasmic fragment in a channelrhodopsin has been shown to influence photocurrent kinetics [123]. Phosphorylation of amino acid residues in the cytoplasmic fragment of ChR1 and ChR2 has been detected by mass-spectrometric analysis *C. reinhardtii* eyespot preparations [110]. Rapid changes in the degree of ChR1 phosphorylation correlate with the sign of phototaxis, i.e., swimming towards or away from the light source [125]. Obviously, localization of the photoreceptors, as well as changes in photocurrents they generate determine the phototaxis sign.

Bioinformatic analysis of the cytoplasmic fragments of ChRs from some protists (but not *C. reinhardtii*) has identified various protein domains known to participate in enzymatic cascades in other organisms [126, 127]. However, no evidence of the functional role of these domains as parts of ChR fusion proteins has been obtained so far. In addition, the cytoplasmic fragments of some green algae channelrhodopsins exhibit SUMO (small ubiquitin-like modifier) binding sites, and biochemical studies confirm reversible binding of this protein to ChR1 linked to regulation of its stability in *C. reinhardtii* cells [128]. Finally, immunoprecipitation shows that the cytoplasmic fragments of ChR1 and ChR2 bind the small GTPase *Cr*ARL11. *Cr*ARL11 belongs to the Arf family, other members of which are known to mediate trafficking of proteins to the cilia and flagella in animal cells [128].

ChR DIVERSITY AND OPTOGENETIC APPLICATIONS

Recording of photoreceptor currents in green flagellate algae has revealed a wide distribution of ChRs in members of this taxonomic group [25-27]. Green algae (chlorophytes) are the closest relatives of land plants, which justified genome sequencing in many of their species [129, 130] including those earlier studied by electro-

physiological methods. Bioinformatic analysis of the data obtained has identified >200 individual ChR sequences in these microorganisms (their lists can be found in [127] and [131]), but only few of them have been studied by patch clamp recording upon heterologous expression in model cells. The great majority of the latter are primarily proton channels like *C. reinhardtii* ChRs, and some of them also conduct Na^+, K^+ and, to a lesser extent, Ca^{2+} [26, 27, 33, 132, 133]. Collectively, these proteins are referred to as "cation channelrhodopsins" (CCRs) [58, 131, 134]. These ChRs are widely used for photostimulation of neurons and other excitable cells [40-42].

All tested cryptophytes are capable of photoorientation [135-137], and photoreceptor currents similar to those in green flagellates have been recorded from one fresh-water species [24]. The fully sequenced genome of the marine cryptophyte *Guillardia theta* encodes tens of rhodopsins, among which there are ChRs [48, 138], proton pumps [139] and proteins that do not transport ions [24]. At least five *G. theta* ChRs function as cation channels when their genes are expressed in mammalian cells [138, 140, 141], and many their homologs are found in other cryptophytes [142]. According to their sequences and details of the transport mechanism, these proteins are closer to haloarchaeal proton pumps than chlorophyte ChRs, and therefore have been named "bacteriorhodopsin-like cation channelrhodopsins" (BCCRs) [50]. Their similarity to haloarchaeal proton-pumping rhodopsins is further corroborated by their trimeric structure [143, 144], whereas chlorophyte ChRs form dimers [145-147]. Most BCCRs generate small currents upon expression in mammalian cells, but at least one their representative, known as ChRmine (also known as *Rhodomonas lens* cation channelrhodopsin 1, *Rl*CCR1), is a popular optogenetic tool for neuronal activation using green light [148, 149].

Another group of *G. theta* ChRs exhibit exclusively anion selectivity [48]. Their homologs have been found in many other cryptophytes and are known as "anion channelrhodopsins" (ACRs) [150, 151]. As the Cl^- concentration in the soma of mature neurons is low, opening of these channels leads to Cl^- influx across the membrane and inhibition of spiking. ACRs have successfully been used as optogenetic inhibitors of neuronal activity and behavior in worms, insects, fish, and mammals [48, 152-158]. In addition, they have been applied for optogenetic control of guard cell movements and growth direction in higher plants [159, 160].

Besides cryptophytes, haptophyte [49] and even some chlorophyte [127] algae have been shown to possess ACRs. Moreover, homologous proteins (or rather genes that encode them) have been found in heterotroph organisms from the infrakingdom of stramenopiles [49, 131, 161]. Most if not all of these organisms develop flagella at least at one stage of their life cycle. It is plausible that ACRs, as well as CCRs, acts as photoreceptors in their phototaxis, but this hypothesis requires experimental verification.

Using ACRs, ~200 sequences of which are currently known [127, 131], as optogenetic inhibitors is limited by the direction of their photocurrents in recipient cells. The Cl^- concentration in the axon terminals is typically higher than in the soma, so ACR photoexcitation depolarizes the membrane and may even lead to triggering back-propagating action potentials [162-165]. These side effects can be reduced by the addition of soma-targeting motifs that decrease trafficking of ACRs to the terminals [163, 165, 166], but complete elimination of this undesired trafficking has not yet been possible.

That is why the discovery of ChRs more permeable for K^+ than for Na^+ last year attracted much attention of the optogenetic community. The first two proteins of this functional class, known as "kailum channelrhodopsins" (KCRs), have been identified in the heterotrophic stramenopile *Hyphochytrium catenoides* [51], and later their homologs were found in some other organisms [167, 168]. The results of the first experiments on inhibition of neuronal and cardiomyocyte activity using KCRs look very promising [51, 167, 169], so these proteins are anticipated to become the primary molecular instrument for his purpose.

It is important to note that only one function of channelrhodopsins is currently used in optogenetics – their direct channel activity. Their second function that is dominant in native algal cells, generation of the late (delayed) photoreceptor Ca^{2+} current with 1,000-fold magnification, has not yet found its practical application, because the channels involved have not yet been identified at the molecular level.

CONCLUSION

In the 1970s, when the studies on photoelectric activity of bacteriorhodopsin and yet unidentified ChRs were launched at the Moscow State University, it was impossible to predict the emergence of the entire new field of biomedicine known today as optogenetics and based on application of these proteins to manipulate the membrane potential with light. At present, only one function of phototaxis receptors (their channel activity) is used in optogenetics. A complex and highly sensitive photosensory cascade that mediates phototaxis is still awaiting its applications. Nevertheless, this example serves as a very convincing demonstration of the importance of fundamental studies for scientific and technical progress.

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