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***Proteomonas sulcata* ACR1: A Fast Anion Channelrhodopsin**

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Abstract

Natural channelrhodopsins with strictly anion selectivity and high unitary conductance have been recently discovered in the cryptophyte alga *Guillardia theta*. These proteins, called anion channelrhodopsins (ACRs), are of interest for their novel function and also because they were shown to be highly efficient tools to inhibit neuronal action potentials with light. We show that a homologous protein from the cryptophyte alga *Proteomonas sulcata* (named here *PsuACR1*) exhibits similar strict anion selectivity as the previously identified *G. theta* ACRs. Like *G. theta* ACRs, *PsuACR1* lacks a protonatable residue at the position of the proton acceptor Asp-85 in bacteriorhodopsin, which may be a key characteristic of ACR family members shared by haloarchaeal chloride pumps. Of importance for its potential use in optogenetics, despite its 10-fold lower channel activity than the *GtACRs*, *PsuACR1* exhibits an ~8-fold more rapid channel closing half-time making it uniquely suitable for silencing the subclass of high-frequency firing neurons when high time resolution is needed. The existence of a rhodopsin with properties similar to *G. theta* ACRs in a different cryptophyte genus indicates that such proteins may be widespread in the phylum of cryptophyte algae.

INTRODUCTION

Genome projects undertaken in recent years combined with physiological studies have greatly expanded the family of microbial rhodopsins and revealed new functions that can be fulfilled by their shared molecular scaffold. One striking example are rhodopsins from the phototactic marine cryptophyte alga *Guillardia theta* that act as light-gated anion channels when expressed in animal cells and therefore have been named anion channelrhodopsins (ACRs) (1). Compared to other known microbial rhodopsins ACRs have the highest sequence similarity to cation-conducting channelrhodopsins (CCRs), although this similarity is slight.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this manuscript:

Figure S1. A ClustalW alignment of the rhodopsin domains of ACRs and *C. reinhardtii* CCRs. The numbers at left show the last residue numbers of each sequence fragment. The carboxylate residues conserved in helices 2 and 3 of CCRs are highlighted red; the corresponding polar and non-polar residues in ACRs are highlighted green and yellow, respectively. For further details see text.

Figure S2. The voltage dependence of the time constants of *PsuACR1* current decay components. The data points are mean values $\pm$ SEM ($n = 8$ cells).

Unlike engineered Cl^- -conducting CCR mutants (2–3), naturally evolved ACRs completely lack permeability for protons and metal cations. Their unitary conductance is 25-fold higher than that of the most so far known CCRs (1), and they hyperpolarized the membrane of cultured animal cells with much faster kinetics at less than one-thousandth of the light intensity than required by the most efficient currently available optogenetic proteins used for neural inhibition. These advantages make *G. theta* ACRs superior optogenetic tools for hyperpolarization of the membrane and inhibition of neuronal firing. As was with CCRs (4–6), search for additional natural variants is desired, in the case of ACRs to expand the neural inhibitor optogenetic tool set.

G. theta is so far the only cryptophyte species the genome of which has been completely sequenced (7), but several other cryptophytes were included in the 1000 plants (oneKP or 1KP) transcriptome sequencing initiative (8). One of the sequences identified by this project in the marine cryptophyte *Proteomonas sulcata* shows a close similarity to *G. theta* ACRs. This sequence, named *PsChR1*, was observed to generate photocurrents when expressed in cultured neurons (6), but these measurements did not distinguish between cation and anion conductance.

In this study we show that the corresponding *P. sulcata* protein exhibits light-gated anion conductance similar to that of the earlier characterized ACRs from *G. theta*. Therefore, we rename it *PsuACR1* (the third letter from the species name was added because the abbreviation *Ps* was used earlier for a CCR from the green alga *Platymonas subcordiformis* (9)). The existence of *PsuACR1* reveals that ACRs are not confined to the one species *G. theta*, but have a broader distribution among cryptophyte algae. The rates of *PsuACR1* channel closing and the dark recovery of the peak current after photoexcitation are very rapid, several times faster than those of *G. theta* ACRs at neutral pH. Therefore, *PsuACR1* is expected to be useful as an inhibitory optogenetic tool for silencing neurons firing at high frequencies when high time resolution is needed.

MATERIALS AND METHODS

Molecular biology

A plasmid carrying the nucleotide sequence encoding a rhodopsin from *Proteomonas sulcata* (Acc. number KF992074) was kindly provided by Drs. Nathan Klapoetke and Edward S. Boyden (Massachusetts Institute of Technology, Boston, MA). The construct was transferred to the pcDNA3.1 expression vector (Life Technologies, Grand Island, NY) in frame with an EYFP tag.

Whole cell patch clamp recording

HEK293 (human embryonic kidney) cells were transfected using the ScreenFectA transfection reagent (Wako Chemicals USA, Richmond, VA). All-*trans*-retinal (Sigma) was added as a stock solution in ethanol at the final concentration of 5 μM . Measurements were performed 48–72 h after transfection with an Axopatch 200B amplifier (Molecular Devices, Union City, CA). The signals were digitized with a Digidata 1440A using pClamp 10 software (both from Molecular Devices). Patch pipettes with resistances of 2–5 M Ω were

fabricated from borosilicate glass and filled with the following standard solution (in mM): KCl 126, MgCl₂ 2, CaCl₂ 0.5, EGTA 5, HEPES 25, pH 7.4. The standard bath solution contained (in mM): NaCl 150, CaCl₂ 1.8, MgCl₂ 1, glucose 5, HEPES or Tris 10; pH was adjusted with N-methyl-D-glucamine to the desired value. The compositions of other solutions used to test ion selectivity were the same as used in experiments with *G. theta* ACRs (1). A 4 M salt bridge was used in all experiments. All current-voltage dependencies were corrected for liquid junction potentials calculated using the ClampEx built-in LJP calculator (1). In experiments aimed to test cation permeability E_{rev} shifts were calculated by subtraction of the reference value measured at 150 mM NMG⁺ in the bath (pH 7.4) from the values measured at 150 mM Na⁺ (pH 5.4), 150 mM Na⁺ (pH 7.4), 150 mM K⁺ (pH 7.4) or 75 mM Ca²⁺ (pH 7.4). The Cl⁻ concentration in the bath was 155.6 mM with all cations. In tests of anion permeability E_{rev} shifts were calculated by subtraction of the reference value measured at 150 mM Asp⁻ from the value measured at 75 mM SO₄²⁻ or 150 mM of F⁻, Br⁻, I⁻ or NO₃⁻. The Na⁺ concentration in the bath was 150 mM with all anions except F⁻, in which it was 155.6 (because of poor solubility of MgF₂ and CaF₂). All measurements were carried out at room temperature (25° C).

Continuous light pulses were provided by a Polychrome IV light source (T.I.L.L. Photonics GMBH, Grafelfing, Germany) in combination with a mechanical shutter (Uniblitz Model LS6, Vincent Associates, Rochester, NY; half-opening time 0.5 ms). The light intensity was attenuated with the built-in Polychrome system or with neutral density filters. Maximal quantum density at the focal plane of the 40× objective lens was 8.5 mW/mm². Laser excitation was provided by a Minilite Nd:YAG laser (532 nm, pulse width 6 ns, energy 12 mJ; Continuum, Santa Clara, CA). A laser artifact measured with a blocked optical path was digitally subtracted from the recorded traces. For further analysis the signals were logarithmically averaged with a custom-created computer algorithm. Numerical data in the text are presented as the mean values ± SEM. Curve fitting was performed using Origin 7 software (OriginLab Corporation, Northampton, MA).

RESULTS

*Psu*ACR1 protein sequence

In addition to high overall similarity, the *Psu*ACR1 protein sequence exhibits several characteristic features previously found in *G. theta* ACRs (1). An alignment of the three ACRs and two CCRs from *Chlamydomonas reinhardtii* is shown in Fig. S1. First, *Psu*ACR1 contains a Glu residue (Glu-64) corresponding to Glu-90 of *CrChR2*. In CCRs Glu-90 is an important residue in the selectivity filter since its substitution with a neutral or positively charged residue is sufficient to shift their dominant selectivity from cations to anions (2–3). Therefore its conservation in the natural anion selective ACRs reveals a fundamental difference in its role in these proteins from that in CCRs. Second, the position of the proton acceptor in bacteriorhodopsin (Asp-85) is occupied by a non-carboxylic residue. However, this residue is Ala (Ala-93) in *Psu*ACR1, whereas it is Ser in both *G. theta* ACRs. Finally, none of the three residues predicted to form a cytoplasmic channel gate according to the crystal structure of cation-conducting C1C2 chimera (Tyr-109, His-173 and His-304; (10)) is conserved in *Psu*ACR1 and *G. theta* ACRs: Tyr-109 is replaced with Met in all three

proteins; His-173 (the position corresponding to Asp-96 in bacteriorhodopsin), with Leu in *Gt*ACR1 and Gln in *Psu*ACR1 and *Gt*ACR2 (and in rhodopsin sodium pumps); His-304 is replaced with Arg in *Psu*ACR1, Ala in *Gt*ACR1 and Ser in *Gt*ACR2.

Anion selectivity

To test electrophysiological properties of *Psu*ACR1 we expressed it in human embryonic kidney (HEK293) cells and measured photocurrents under voltage clamp conditions. A series of current traces recorded with our standard solutions (126 mM KCl in the pipette and 150 mM NaCl in the bath, pH 7.4; for other components see Methods) in response to a light pulse of the saturating intensity are shown in Fig. 1a. The mean peak amplitude at -60 mV at the amplifier output was 707 ± 184 pA (mean $\pm$ SEM, $n = 18$ cells), which was roughly 10 times less than that of the currents generated by ACRs from *G. theta* under the same conditions (1). Another difference was that *Psu*ACR1 currents displayed rapid inactivation under continuous illumination to a plateau level of $56 \pm 2\%$ (mean $\pm$ SEM, $n = 18$ cells), whereas inactivation of photocurrents generated by *G. theta* ACRs was much smaller and slower (1). The sign of *Psu*ACR1 photocurrents reversed when the membrane potential was shifted to more positive values. The dependence of its peak amplitude on the holding potential (E_h) (the IE curve) was linear (Fig. 1d, black symbols and line), as that for *G. theta* ACRs, in contrast to the outward rectification typical of CCRs (11). The reversal potential (E_{rev}) of photocurrents recorded in standard solutions was close to zero (Fig. 1d, black symbols and line).

When most of the Cl^- in the bath was replaced with non-permeable Asp^- (shifting E_{Cl} to 81 mV), the E_{rev} shifted to 80 ± 2 mV (mean $\pm$ SEM, $n = 3$ cells). A typical series of photocurrents measured under these conditions is shown in Fig. 1b, and an IE curve, in Fig. 1d (red symbols and line). Replacing Cl^- with Asp^- in the pipette, but keeping the bath solution standard, caused a shift of the E_{rev} in the opposite direction. Photocurrents and an IE curve for these conditions are shown in Fig. 1c and d (blue symbols and line), respectively. These results indicated that *Psu*ACR1 photocurrents were due to passive Cl^- transport.

Next, we replaced Cl^- in the bath with other anions, measured the IE curves and determined the E_{rev} values. Fig. 2 (left) shows the shifts of the E_{rev} measured with each anion from the value measured with non-permeable Asp^- . As for *G. theta* ACRs, I^- was the most, and F^- , the least permeable halide, whereas divalent sulphate was nearly impermeable. Similarly, no shift beyond experimental error of the E_{rev} was measured with Na^+ , K^+ or Ca^{2+} in the bath instead of non-permeable N-methyl-glucamine (NMG^+), or when the bath pH was decreased from 7.4 to 5.4 (Fig. 2, right). These results confirmed that *Psu*ACR1 is a light-gated anion channel.

Spectral sensitivity

The action spectrum of *Psu*ACR1 photocurrents measured at pH 7.4 was red-shifted compared to those of both *G. theta* ACRs (Fig. 3). In addition to the main peak at 520 nm, a pronounced shoulder was found at 550 nm (Fig. 3, black symbols and line). This shoulder is unlikely to reflect a fraction of a pigment with the protonated counterion, since it showed no

dependence on the bath pH (Fig. 3, black and red solid lines). Although the maximum of the *Psu*ACR1 spectrum was only 5 nm red-shifted compared to that of *Gt*ACR1, the existence of a long-wavelength shoulder makes the integral absorption of wavelengths above the absorption of hemoglobin several folds greater for *Psu*ACR1 than for *G. theta* ACRs. This long-wavelength absorptivity increase partially compensates for the 10-fold lower channel activity of *Psu*ACR1 compared to that of *G. theta* ACRs.

Current kinetics under single-turnover conditions

To characterize the kinetics of *Psu*ACR1 photocurrent under single-turnover conditions, we used laser flashes (6 ns) for photoexcitation (Fig. 4a). Compared to the previously characterized ACRs from *G. theta*, channel closing in *Psu*ACR1 at pH 7.4 was ~8 times faster (Fig. 4b). The current decay was fit with 3 exponentials. The third, slowest component made the least contribution to the overall amplitude. This minor component was also detected in current traces recorded from the earlier characterized *Gt*ACR1, but was not included in the previous analysis (12). The voltage dependencies (IE curves) of the amplitudes of the three decay components of *Psu*ACR1 photocurrents are shown in Fig. 4c. The reversal potentials of all components were the same within experimental error. The time constants (τ) of all components were only weakly voltage-dependent (Fig. S2), in contrast to previous observations in *Gt*ACR1 (12). The rate of the peak current recovery was measured in double-flash experiments. For *Psu*ACR1 it was ~5 times faster than that for *Gt*ACR2, and ~8 times faster than that for *Gt*ACR1 (Fig. 4d).

At the reversal potential for channel currents a fast negative signal was recorded, the time course of which was limited by the time resolution of the recording system (Fig. 5a). Such fast currents from rhodopsins could reflect either initial stages of the charge transfer associated with the formation of K and L intermediates of the photocycle, or intramolecular proton transfer (13–14). The inward direction of the fast current recorded from *Psu*ACR1 corresponded to that of the initial charge transfer associated with retinal isomerization (15). The amplitude of this current in *Psu*ACR1 did not depend on the V_h or pH of the bath in the tested range (Fig. 5b and c), which further indicated the origin of this current as retinal isomerization. Similar fast negative signals have previously been measured earlier in several CCRs and rhodopsin proton pumps expressed in HEK293 cells (16).

DISCUSSION

We demonstrate that *Psu*ACR1, a rhodopsin homologous to *G. theta* ACRs, acts as a light-gated anion channel. The finding of an ACR in a second cryptophyte genus among only a handful of cryptophyte algae sequenced indicates that ACRs are likely to be widely distributed among cryptophyte species. The kinetics of *Psu*ACR1 currents is ~8 times faster than that of *G. theta* ACRs, which is an advantage for some optogenetic applications, i.e., when inhibition of individual spikes in neurons firing at a high frequency is needed. Also, the maximal spectral sensitivity of *Psu*ACR1 is 5 nm and 50 nm red-shifted relative to that of *Gt*ACR1 and *Gt*ACR2, respectively, which allows using the light of longer wavelengths that better penetrates biological tissue. Although the amplitude of *Psu*ACR1 photocurrents in cultured mammalian cells is ~10-fold smaller than those of the currents generated by *G.*

theta ACRs, with non-ACR hyperpolarizing rhodopsins amplitudes comparable to those of *PsuACR1* could be achieved only at the expense of dramatically (2 orders of magnitude) slower kinetics (17).

ACRs are distinguished from CCRs by their strict anion selectivity and >20-fold greater unitary conductance. Gating of *GtACR1* comprises two separate mechanisms with opposite dependencies on the membrane voltage and pH involving different amino acid residues (12). The action spectra of photocurrents under conditions that dramatically alter relative contribution of the two gating mechanisms were identical within the accuracy of our measurements, which indicated the absence of spectrally identifiable heterogeneity of the unphotolyzed state of *GtACR1* (12). Similar kinetic components were resolved in currents generated by *PsuACR1* in response to laser flashes, but their voltage dependencies were much weaker than those of the corresponding components of *GtACR1* currents, which suggests that the strong voltage dependence is rather a characteristic of a particular ACR species than that of the entire group. Quantitative comparison of the kinetics of currents generated by ACRs with those by CCRs is difficult because the latter have mostly been studied upon stimulation with pulses of continuous light when several intermediates of the photocycle can contribute to the response, rather than under single-turnover conditions. Two components of the laser flash-induced current decay have been reported for a hybrid CCR from *Volvox* (18), but their voltage and pH dependencies were not tested.

Cryptophytes evolved by secondary endosymbiosis between a non-photosynthetic host and a red alga (19) and are phylogenetically very distant from green (chlorophyte) algae, such as the model organism *C. reinhardtii* (20). Nevertheless, cryptophytes, like green flagellates, are capable of phototactic orientation (21–23). The role of receptors for phototaxis in green flagellates is played by CCRs (24). Periodic photoexcitation of CCRs caused by periodic shading in the rotating alga signals the flagella to turn the cell in the direction that minimizes the membrane electrical potential oscillation. Periodic excitation of ACRs could in principle serve a similar role in the phototaxis orientation process, but there is insufficient data on cryptophyte phototaxis to assess this possibility.

An assembly of the only completely sequenced cryptophyte genome, that of *G. theta*, predicts 53 protein models showing similarity to type I (microbial) rhodopsins (1). The presence of such a large number of opsin genes in a single organism has also been found in the dinoflagellate *Oxyrrhis marina* (25), which probably reflects the evolutionary origin of these algal groups as secondary endosymbiotic chimeras. Among all predicted *G. theta* rhodopsins, the ACRs are the closest homologs of CCRs from green algae. A reasonable hypothesis is that ACRs act as receptors for phototaxis in cryptophyte flagellates. An extremely low abundance of ACR mRNA in algal cells (E.G. Govorunova and J.L. Spudich, unpublished data) is consistent with such a role.

The maximal spectral sensitivity of *G. theta* photoaccumulation is in the blue region of the spectrum (26), which roughly correlates to the absorption of ACRs from this organism. The maxima of the phototaxis action spectra measured in other cryptophyte species range from 420 to 560 nm and do not correspond to the absorption maxima of their phycobilin pigments

(23), earlier considered for a receptor role (26). These data are consistent with a role of ACRs as phototaxis receptors, but further study is needed to reach a firm conclusion.

The direction of ACR photocurrents depends on the electrochemical potential for Cl^- across the cell plasma membrane. Although the resting membrane potential of cryptophytes is currently not known, that of the giant unicellular marine alga *Acetabularia mediterranea* is -170 mV (27). Assuming that the cytoplasmic Cl^- concentration in cryptophytes is within the range found in other cells, at such highly negative membrane potentials ACRs would generate depolarizing photocurrents (such as those recorded from a freshwater *Cryptomonas* (28)) even in marine cryptophytes such as *G. theta* and *P. sulcata*. On the other hand, analysis of photoreceptor currents in *C. reinhardtii* and other green flagellates demonstrate a major contribution of yet unidentified Ca^{2+} channels (29). Phototactic orientation in *Cryptomonas* is inhibited by the removal of Ca^{2+} from the external medium (30–31). Therefore, it is possible that depolarizing photocurrents recorded in *Cryptomonas* (28) also reflect the contribution of secondary Ca^{2+} channels in addition to photocurrents generated by the channelrhodopsins. Direct probing of a possible role of ACRs in cryptophyte phototaxis by gene knockout would help resolve these issues but requires the development of means for molecular genetic manipulation of these microorganisms.

CONCLUSION

PsuACR1 from the marine cryptophyte alga *Proteomonas sulcata* is a light-gated anion channel with fast closing kinetics. Although its photocurrents are ~10-fold lower than those of *GtACRs*, when high temporal resolution is needed in very fast-firing neurons, its faster kinetics as well as its most red-shifted spectral sensitivity among so far known natural ACRs make it a candidate tool for inhibitory optogenetics. The finding of an ACR in a second cryptophyte genus among only a handful of cryptophyte algae sequenced indicates that ACRs are likely to be widely distributed among cryptophyte species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a–c) Photocurrents generated by *PsuACR1* in HEK293 cells at the membrane potentials changed in 20-mV steps from –60 mV at the amplifier output (bottom to top) in response to a 1-s light pulse (520 nm). The Cl[–] concentrations of the pipette and bath solutions were as indicated in the legend. (d) The voltage dependencies of the peak current (IE curves) measured at the Cl[–] gradients indicated in the legend. The data (mean values ± SEM, $n = 4–9$ cells) were corrected for liquid junction potentials.

Figure 2.

Shifts of the E_{rev} of *PsuACR1* photocurrents upon variation of the anion (left axis) or cation (right axis) composition of the bath obtained by subtraction of the value measured with non-permeable Asp^- (for anions) or non-permeable NMG^+ (for cations) from the value measured with the indicated anion or cation, respectively. The data are mean values $\pm$ SEM ($n = 3-9$ cells). The data for *CrChR2* are shown for comparison.

Figure 3.

The action spectra of *PsuACR1* photocurrents at pH 7.4 (black squares and black solid line) and 5.4 (red circles and line). The data are mean values $\pm$ SEM ($n = 3-5$ cells). The action spectra of *G. theta* ACRs are shown as dashed lines for comparison.

Figure 4.

(a) Typical photocurrents (black solid lines) generated by *PsuACR1* in response to laser photoexcitation (6 ns, 532 nm) at indicated voltages at the amplifier output. The current decay was fit with three exponentials (red dashed lines), from which the time constants shown on the plot were derived. (b) Channel closing rates of three known ACRs calculated as reciprocals of the time needed for the peak amplitude to decrease to 50%. (c) The voltage dependence of the amplitudes of current decay components. (d) The dark recovery of the peak current for *PsuACR1*, *GtACR1* and *GtACR2* measured in double-flash experiments with 5-ms light pulses of the saturating intensity at 520, 515 and 470 nm, respectively. The numbers on the plot show the dark interval required for 50% peak recovery for each protein. The data points in panels b-d are mean values $\pm$ SEM ($n = 3-8$ cells).

Figure 5.

(a) Fast negative current recorded from *PsuACR1* at the reversal potential for channel current (solid line, left axis) and exponential fit of its rise and decay (dotted line), and channel current recorded from the same cell at -60 mV at the amplifier output (dashed line, right axis). (b) Voltage dependencies of fast negative current (squares and solid line, left axis) and channel current (circles and dashed line, right axis) in a typical cell expressing

PsuACR1. (c) The dependence of fast negative current on the bath pH. The data points are mean values $\pm$ SEM ($n = 3-5$ cells).

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