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Retinal-Based Anion Pump from the Cyanobacterium *Tolypothrix campylonemoides*

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Abstract—In this work, TcaR rhodopsin from the cyanobacterium *Tolypothrix campylonemoides* was characterized. Analysis of the amino acid sequence of TcaR revealed that this protein possesses a TSD motif that differs by only one amino acid from the TSA motif of the known halorhodopsin chloride pump. The TcaR protein was expressed in *E. coli*, purified, and incorporated into proteoliposomes and nanodiscs. Functional activity was measured by electric current generation through the planar bilayer lipid membranes (BLMs) with proteoliposomes adsorbed on one side of the membrane surface, as well as by fluorescence using the voltage-dependent dye oxonol VI. We have shown that TcaR rhodopsin functions as a powerful anion pump. Our results show that the novel microbial anion transporter, TcaR, deserves deeper investigation and may be of interest both for fundamental studies of membrane proteins and as a tool for optogenetics.

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Keywords: microbial rhodopsin, photosensitive ion pump, optogenetics, proteoliposomes, bilayer lipid membrane

INTRODUCTION

Microbial rhodopsins comprise a large family of light-sensitive α-helical proteins with covalently bound retinal as a cofactor. These proteins have been found in microorganisms from all domains of life including viruses [1]. One of the main functions of these proteins is their ability to perform light-driven transport of protons, cations, and anions. The retinal-containing proteins participate in energy storage by the marine microorganisms, moreover, according to rough estimates, contribution of the rhodopsin-based photosynthesis to the total bioenergetics of biosphere is comparable or even exceeds contribution of the chlorophyll-based photosynthesis [2]. However, other functions of the proteins from this family are known: sensory functions, ability to form light-gated ion channels in the membrane [3], and even regulation of enzymatic activity [4]. Structural-functional studies of new microbial rhodopsins are very important for the development of new optogenetic tools [5]. Metagenomic analysis is often used for searching new rhodopsins; and during the search for new objects a lot of attention is paid to unusual pattern of the conserved amino acid residues. The most studied light-driven proton pump, bacteriorhodopsin of *Halobacterium salinarum*, has DTD motif (D85, T89, and D96 – key amino acid residues for proton transport) [6].

The gene encoding TcaR opsin have been found in the course of bioinformatics search of the open metagenomic databases [7]. It has been located in the genome of *Tolypothrix campylonemoides* VB511288 cyanobacteria isolated from the green biofilm found on the façade

Abbreviations: BLM, planar bilayer lipid membrane; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; PLs, proteoliposomes.

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of the building in the East-Indian city Santiniketan. Analysis of the TcaR amino acid sequence reveals the presence of the TSD motif. Rhodopsins from cyanobacteria with such motif have been characterized previously [8-10], they have the ability to pump anions in bacterial cells in response to illumination. The TSD motif differs by only one amino acid from the motif of the known chloride pump of the halorhodopsin, TSA [11].

In this study we present the data of functional studies of the TcaR rhodopsin using electrophysiological [12, 13] and optical methods, including experiments on determination of its ion selectivity in lipid vesicles and nanodiscs. Electrophysiological approach used in our study was developed by L. A. Drachev and A. D. Kaulen, and later was widely applied for the investigation of rhodopsins in the modification suggested by Bamberg et al. [14-16]. The method is based on measuring of electric photoresponses from the macroscopic planar bilayer lipid membrane (BLM) with bound proteoliposomes containing retinal proteins. This measuring approach has been widely accepted due to the fact that it has high sensitivity and, same as optical techniques, allows monitoring fast transient processes.

MATERIALS AND METHODS

Materials. Reagents from Sigma-Aldrich (USA) were used in this work (unless indicated otherwise).

Proteoliposomes with TcaR rhodopsin. Nucleotide sequence encoding TcaR (GenBank accession no. JXC B00 000 000) was optimized for expression in *Escherichia coli* cells using GeneArt software (Thermo Fischer Scientific, USA). Nucleotide sequence at the 5'-end was optimized with the help of the RNA Web-Server (Institute of Theoretical Chemistry, Vienna University) with the goal to reduce the possibility of hairpin formation at the RNA regions including ribosome binding sites. The gene was commercially synthesized (Eurofins, Luxemburg) and cloned into an expression vector pET15b (Novagen, USA) at XbaI and XhoI restriction sites. Hence, the gene was inserted in front of the LEHHHHHH sequence (polyhistidine sequence used later for nickel-chelate affinity chromatography). Expression in the cells of the *E. coli* C41(DE3) strain and purification of the TcaR protein was carried out according to the protocol described previously [17].

For preparation of proteoliposomes, a 1% (*w*/*v*) solution of a soybean azolectin in chloroform (Sigma, USA) was added to a glass flask. Chloroform was removed with the help of a rotary evaporator and vacuum pump. The obtained thin lipid film formed on the glass flask walls was resuspended in a solution containing 0.1 M NaCl (Applichem, Germany) and 2% (*w*/*v*) sodium cholate to produce final concentration of azolectin 1% (*w*/*v*). Lipid suspension was subjected to ultrasound treatment for 5 min at 4°C, followed by fast addition of a solubilized rhodopsin to final concentration 0.7 mg/ml and detergent-absorbing Bio-Beads SM-2 (Bio-Rad, USA). The obtained mixture was mixed in an orbital shaker for 2 h with minimal illumination, followed by replacement of the beads (this procedure was repeated for at least four times).

Nanodiscs with TcaR. Assembly of nanodiscs and incorporation of TcaR was carried out using standard techniques described previously in the literature [18]. Dimyristoyl phosphatidylcholine (DMPC) (Avanti Polar Lipids, USA) was used as a lipid. An elongated variant of the apolipoprotein-1 protein named MSP1E3D1 was used at a molar ratio DMPC/MSP1E3D1/TcaR = 100/2/3. Dry lipid film obtained by dissolving of DMPC powder in chloroform followed by evaporation on a rotary evaporator (Heidolph, Germany) was rehydrated by adding a detergent solution of 100 mM CHAPS to obtain a lipid/detergent ratio of 1 : 2. Next, a nanodisc-forming protein MSP1E3D1 was added to the solution of the target protein TcaR solubilized in micelles. The obtained mixture was incubated for 1 h at room temperature, followed by addition of a BioBeads™ sorbent (Bio-Rad) in order to remove detergent at a ratio of 1 g of the sorbent per 40 mg of detergent. Detergent removal was carried out for 3 h.

Planar bilayer lipid membrane was formed from a decane solution, which contained 2% diphytanoyl phosphatidylcholine and 0.04% (*w*/*v*) dimyristoyl ethylphosphatidylcholine (Avanti Polar Lipids), on an aperture in a partition separating a Teflon cell with a buffer solution into two parts [19]. Diameter of the aperture was 0.8 mm. Composition of a buffer solution varied and is presented in figure captions. All experiments were performed at room temperature (23-25°C).

Electric current was recorded under voltag-clamp conditions. Potential difference was applied to silver chloride electrodes connected through agar bridges into the Teflon cell to both sides of the membrane. Current was measured with the help of a patch-clamp amplifier OES-2 (OPUS, Russia), digitized with a NI-DAQmx (National Instruments, USA), and analyzed using a commercial WinWCP Strathclyde Electrophysiology Software, developed by J. Dempster (Strathclyde University, United Kingdom).

A halogen lamp Novaflex (World Precision Instruments, USA) with power density 0.77 W/cm^2 was used for BLM illumination. The lamp illuminated the cell from the front (*cis*) side, proteoliposomes were added to BLM from the *cis*-side.

Proteoliposomes (PLs) with incorporated retinal protein TcaR were added to the *cis*-side of the cell, which was closer to illuminator. A high-ohmic electrode was in the opposite part of the cell on the *trans*-side. Following addition of 25-50 μl of TcaR-proteoliposomes to BLM

and 60-min incubation in a buffer solution containing 10 mM Mes and 10 mM Tris (pH ≈ 7), lipid membrane was illuminated with a light in visible range of the spectrum. The BLM formed from the mixture of diphytanoyl phosphatidylcholine and cationic lipid dimyristoyl ethyl-phosphatidylcholine has been previously shown to facilitate adsorption of the negatively charged liposomes.

Significance of differences in data sets was evaluated with the Student's *t*-test.

Measurement of potential generation in suspension of proteoliposome containing TcaR was carried out by measuring fluorescence of the potential-sensitive dye oxonol VI (final concentration 2 μM**)** in a buffer containing 100 mM NaH_2PO_4 and 1 M NaCl (pH 7.2) as described previously [19]. Fluorescence intensity was recorded with a Panorama Fluorat-02 spectrofluorimeter (Lumex, Russia), excitation wavelength 590 nm and emission wavelength 620 nm (slits – 5 nm) at 15°C. After reaching the prescribed temperature 10-20 μl of proteoliposomes was added to the cuvette, which was further illuminated with a 1-mW green laser. To monitor generation of pH gradient in the liposomes, fluorescence of pH-sensitive dye, 9-aminoacridine (excitation – 425 nm, emission – 455 nm) was used.

Absorption spectra of TcaR were recorded with a Specord 50 spectrophotometer (Analytik, Germany).

RESULTS AND DISCUSSION

According to the literature data, pump activity of the retinal proteins is usually monitored using a simple technique based on measuring changes of pH in a liposome suspension with the help of a pH electrode, however, this method has low sensitivity and requires large amounts of the protein. A more complicated method exhibits a significantly higher sensitivity, in this method proteoliposomes are attached to the surface of a planar bilayer lipid membrane, but are not fused with it and remain intact on the BLM surface. It was shown that in such system illumination induces generation of transient electric potentials [12, 13] and transient currents across the BLM [14-16], which could be recorded with regular macroscopic electrodes. Addition of protonophores to such system usually results in the appearance of stationary photocurrents through the BLM, because the electrical accessibility of the inner aqueous phase of the proteoliposomes increases [16, 20].

After the start of illumination, a small and fast change of the BLM current towards negative values was observed (black curve, Fig. 1, a and b), which was followed by a return to the initial current values within a second time scale. Further addition of a choline chloride (Fig. 1a) or potassium fluoride (KF) (Fig. 1b) resulted in the immediate increase of the transient current as a response to switching illumination (light-gray curves), as well as in continuous increase of the transient current with time (dark-gray curves, recorded after 30 min). The sign of the observed transient current for PL with TcaR corresponds to the one for the PL with bacteriorhodopsin and indicates several possible ways of the protein functioning: pumping of protons or monovalent cations inside liposomes, pumping of monovalent anions outside of liposomes, or some other possibilities. Following the addition of tributyltin, which is a transporter of monovalent anions [21, 22], to both sides of the membrane resulted in the significant increase of not only initial, but also of the stationary current as a response to illumination (dotted curves). Addition of another known anion transporter, prodigiosin [23], instead of tributyltin, also resulted in the increase of initial transient and stationary BLM photocurrents (Fig. 1c). The results of experiments with the adsorbed PL on the effects of addition of 50 mM chloride ions followed by addition of 1 μg/ml of tributyltin on the amplitude of the initial photoresponse of BLM (4 experiments) are presented in Fig. 2. Initial photocurrent in the presence of 50 mM of chloride anion was taken as one unit. It can be seen that the addition of chloride ions causes increase of the initial current ($p < 0.0003$), and the following addition of anion transporters leads to the current increase ($p < 0.006$).

When an experiment was conducted in the medium with sulfate anion as a main anion in the electrolyte [medium composition: 10 mM Mes, 10 mM Tris, and 100 mM Na_2SO_4 (pH 7.0)], prolonged incubation of liposomes with the BLM also resulted in their adsorption on the surface of the planar lipid membrane, which was manifested by the appearance of a transient current on illumination (Fig. 3, black curve). However, in this case the amplitude of the transient current was lower in comparison to the one observed in the presence of chloride anion. Addition of protonophore (carbonyl cyanide m-chlorophenyl hydrazone, CCCP) increased the stationary current upon illumination (light-gray curve), and following addition of 60 mM sodium chloride (dark-gray curve) resulted in significant increase in the initial current induced in response to the illumination. Prodigiosin added at the end of the experiment significantly increased the stationary BLM photocurrent (Fig. 3, dotted curve).

In all experiments with adsorption of proteoliposomes on BLM, addition of potassium chloride, sodium chloride, choline chloride, or potassium fluoride, as well as addition of the transporters of monovalent anions (tributyltin and prodigiosin) resulted in the significant increase of the transient photocurrent. However, small transient photocurrents were observed after attachment of liposomes on the planar lipid membranes even in the absence of monovalent anions. It is likely related to the presence of chloride anions inside liposomes and their leakage from the electrodes' agar bridges to the solution.

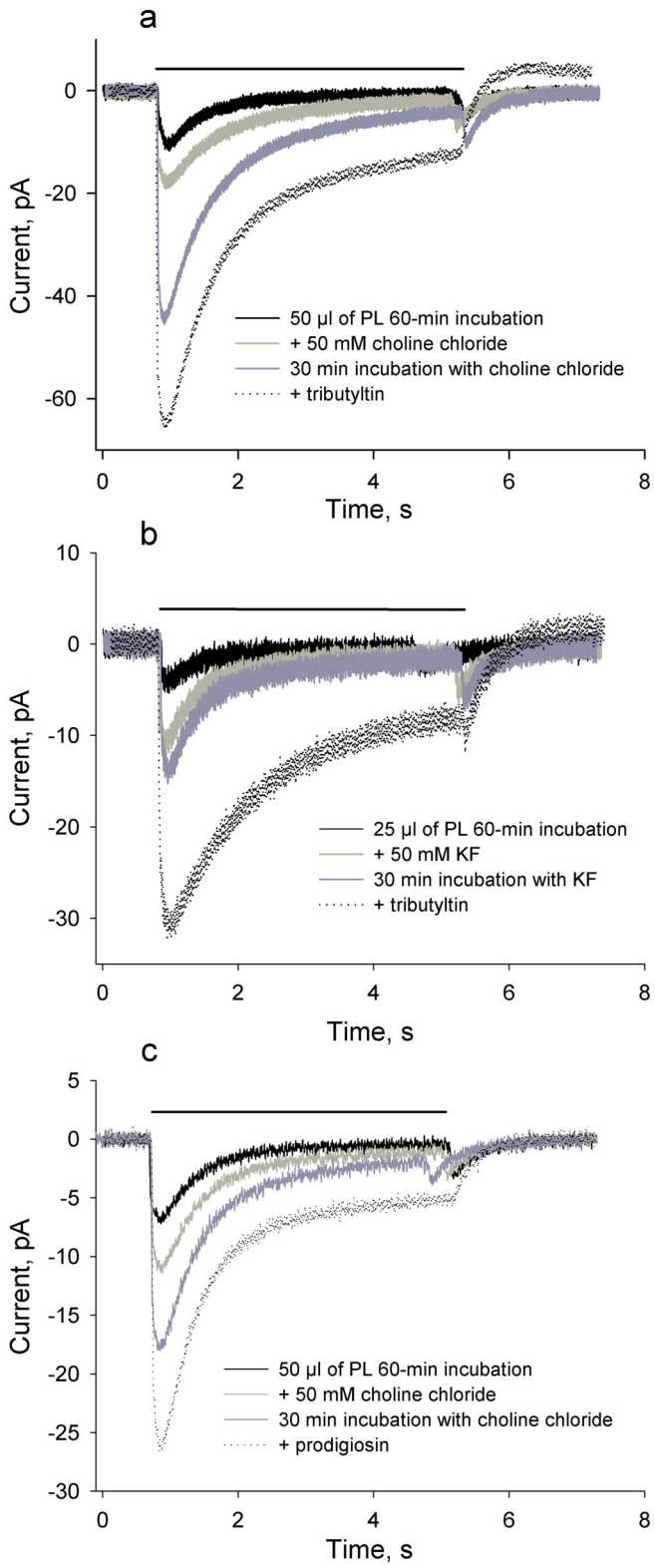

Fig. 1. Measurements of electrical current through BLM with adsorbed proteoliposomes containing TcaR at BLM potential 0 mV. Period of membrane illumination is shown with solid black line. a) BLM current during incubation with liposomes (50 µl) for 60 min (black curve); light-gray curve – BLM current immediately after addition of 50 mM choline chloride; dark-gray curve – after 30-min incubation with choline chloride; dotted curve – after addition of 1 µg/ml of tributyltin. b) BLM current during incubation with liposomes (25 µl) for 60 min (black curve); light-gray curve – BLM current immediately after addition of 50 mM KF; dark-gray curve – after 30-min incubation with KF; dotted curve – after addition of 1 µg/ml of tributyltin. c) BLM current during incubation with liposomes (25 µl) for 60 min (black curve); BLM current immediately after addition of 50 mM choline chloride; dark-gray curve – after 30-min incubation with choline chloride; dotted curve – after addition of 150 nM of prodigiosin. Buffer solution contained 10 mM Mes and 10 mM Tris (pH 7.0).

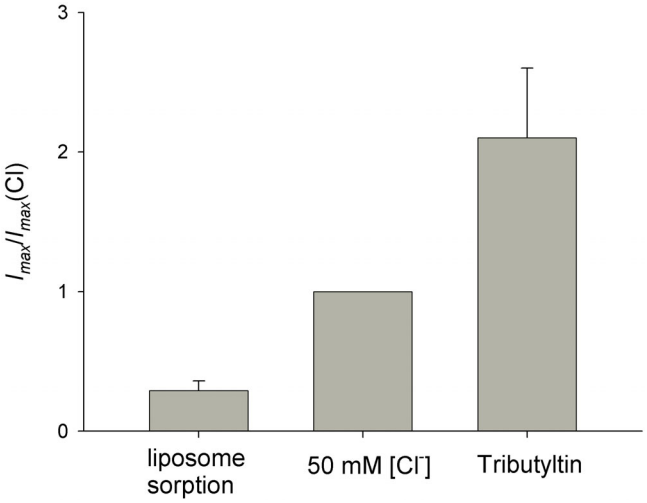

Fig. 2. Summarized results of four experiments with TcaR-proteoliposomes investigating effect of addition of 50 mM chloride ions and following addition of 1 μg/ml of tributyltin on amplitude of the initial BLM photoresponse. Initial photocurrent in the presence of 50 mM of chloride anion was taken as one unit.

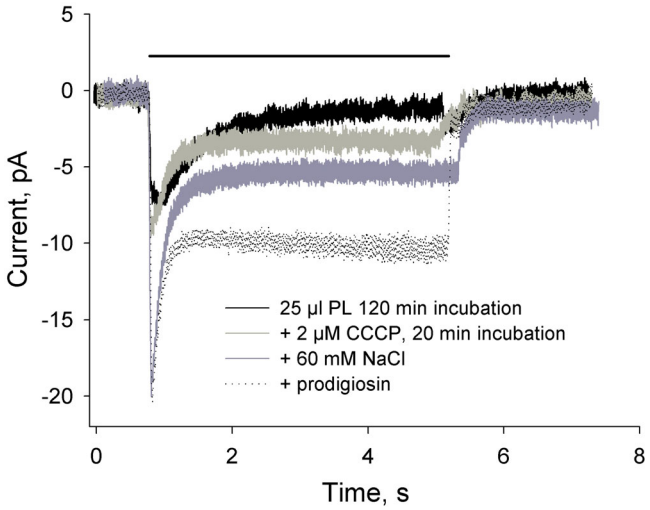

Fig. 3. Electric current in BLM with adsorbed proteoliposome containing TcaR in the medium containing 100 mM Na_2SO_4. Period of membrane illumination with white light is shown by solid black line. BLM current during incubation with liposomes (25 μl) for 120 min (black curve); light-gray curve – BLM current after addition of 2 μM CCCP; dark-gray curve – after addition of 60 mM NaCl; dotted curve – after addition of 150 nM prodigiosin. Buffer contained 10 mM Mes, 10 mM Tris, and 100 mM Na_2SO_4 (pH 7.0).

We prepared membrane nanodiscs with incorporated TcaR protein and measured photoresponses of BLM in their presence in different media. Nanodiscs does not have inner space filled with water, hence, the equivalent electric scheme in this case could differ from the experiments with liposomes. The results of measuring BLM photocurrent in the buffer with 100 mM NaCl (Fig. 4a), 100 mM Na_2SO_4 (Fig. 4b), and 100 mM potassium gluconate (Fig. 4c) after addition of 10 μl of nanodiscs at the *cis*-side of BLM are presented in Fig. 4. Nanodiscs rapidly adsorbed on the BLM, and already after 20 min BLM currents appeared in response to illumination. It is clearly seen that the significant transient currents were observed only in the case of the medium with sodium chloride (Fig. 4a; light-gray curve). In the presence of sodium sulfate or potassium gluconate the observed photocurrents were significantly lower (light-gray curves in Figs. 4, b and c). Moreover, in the case of 100 mM Na_2SO_4 (Fig. 4b) the first rapid response has even opposite sign. Addition of sodium chloride at the end of each experiment resulted in appearance of a large immediate current change in response to illumination (Fig. 4, b and c; dark-gray curves), or further increase of the initial current (Fig. 4a). This result allows us suggesting that sodium, potassium, sulfate, and gluconate ions are not transported by the pump. The stationary BLM photocurrent increased significantly in the presence of CCCP protonophore or anion transporter tributyltin (medium-gray and dotted curves in Fig. 5). In conclusion these experiments indicate the ability of the retinal-containing protein TcaR to transport chloride anions across the membrane in response to illumination.

The data presented in Fig. 1b allow us suggesting the ability of the TcaR protein to transport fluoride anions. Transport of hydroxyl anion by this protein also cannot be excluded. It was shown previously that the membrane fragments with halorhodopsin that has similar sequence of important amino acids (the TSA motif) could generate photocurrent in the presence Cl^-, Br^-, and I^-, but not in the presence of SO_4^{2-}, F^-, and NO_3^- [24]. However, depending on the illumination conditions halorhodopsin could function as a light-driven chloride pump or proton pump [25]. According to our results, transport of anions by the TcaR exposed to light is realized from inside of the liposomes to outside (which is equivalent to the transport into the cell cytoplasm). Hence, a direction of the active transport of anions by the TcaR and halorhodopsin is the same.

It was shown previously that the fluorescent dyes such as potential-sensitive oxonol VI and ΔpH-sensitive 9-acridine amine (9-AA) could be used for measuring activity of the rhodopsins in suspension [19, 26]. Typical response of oxonol VI fluorescence on addition to the suspension of proteoliposomes containing TcaR is presented in Fig. 5a. Significant decrease of the fluorescence signal is observed during illumination with green light, which suggests generation of membrane potential on the liposomes. Oxonol VI is an anionic dye and decrease of its fluorescence indicates generation of potential with plus sign inside the liposomes, which corresponds to the same polarity as in the similar experiments with bacteriorhodopsin [26]. It should be mentioned that the sensitivity of this method is significantly lower that of the method based on the measurement of photocurrent on BLM [19], hence, generation of large fluorescence responses

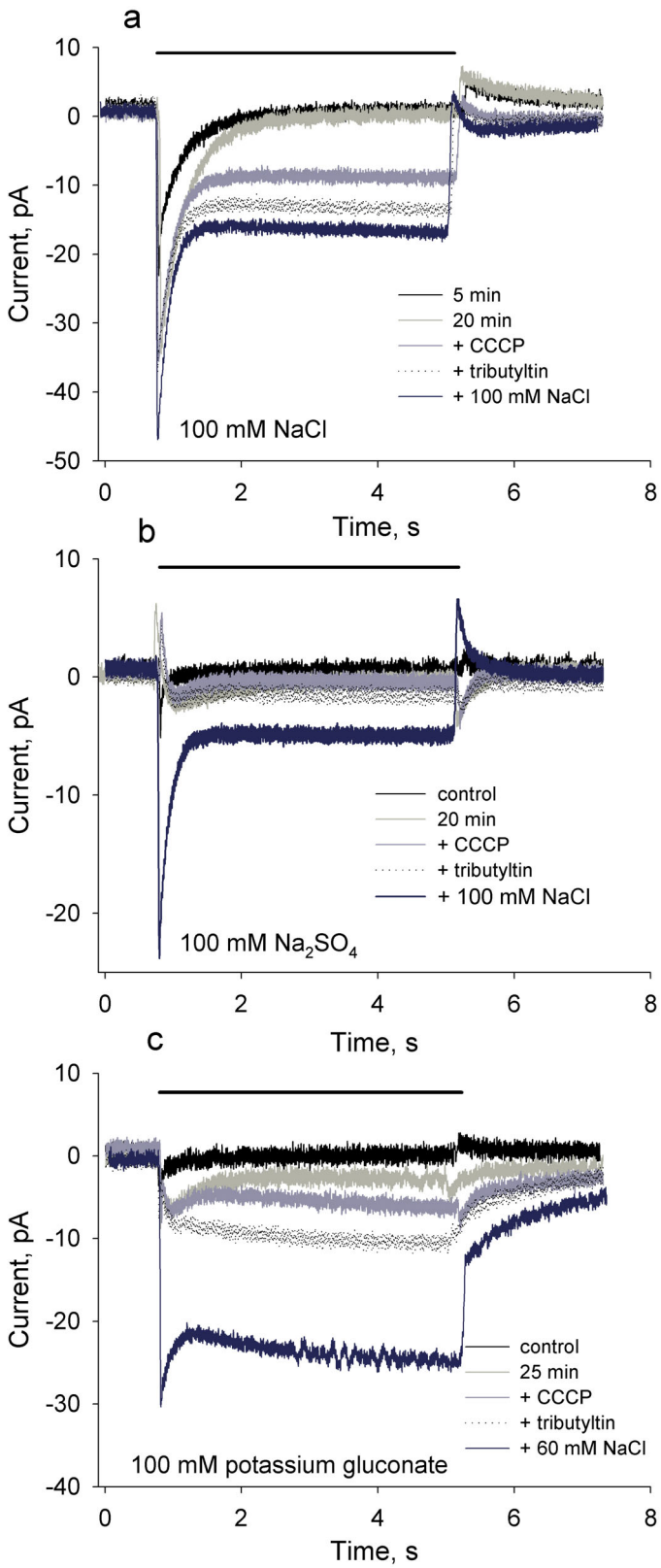

Fig. 4. Time-dependencies of electric current in BLM with adsorbed nanodiscs containing TcaR at BLM potential 0 mV. Period of the membrane illumination with white light is shown with solid black line. a) BLM current during incubation with nanodiscs (10 µl) for 5 min (black curve); light-gray curve – after 20-min incubation with nanodiscs; medium-gray curve – after addition of 2 µM of CCCP; dotted line – after addition of 1 µg/ml tributyltin; dark-gray curve – after addition of NaCl (to concentration 200 mM). Buffer solution contained 10 mM Mes, 10 mM Tris, and 100 mM NaCl (pH 7.0). b) Similar current time-dependencies in buffer solution containing10 mM Mes, 10 mM Tris, 100 mM Na_2SO_4 (pH 7.0). c) Similar current time-dependencies in buffer solution 10 mM Mes, 10 mM Tris, 100 mM potassium gluconate (pH 7.0).

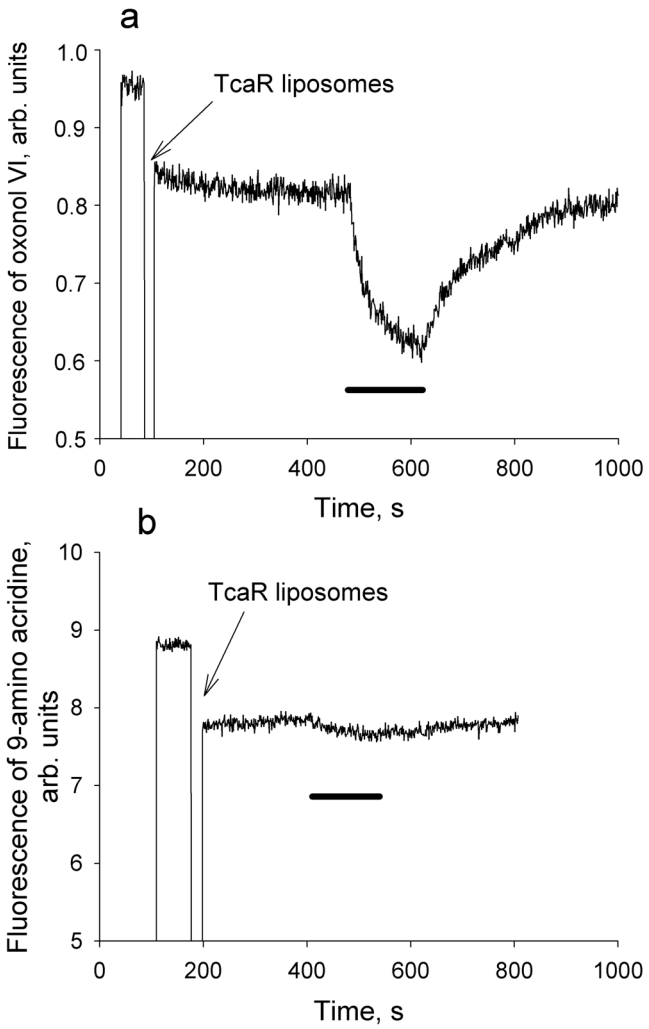

Fig. 5. Ion-transporting properties of TcaR in proteoliposome suspension measured by changes of fluorescence of 2 μM oxonol VI (a) and 4 μM 9-amino acridine (b). 10 μl of proteoliposomes were suspended in 1 ml of medium containing 1 M NaCl and 100 mM NaH_2PO_4 (pH 7.2). Period of membrane illumination with the light of green LED is shown with solid black line.

in the suspension of proteoliposomes with TcaR (Fig. 5a) indicates high efficiency of the TcaR pump. Similar experiments using the 9-AA dye demonstrated that no pH gradient is formed in this system (Fig. 5b).

A series of absorption spectra of TcaR in the absence of chloride anions (black curve) and in the presence of increasing concentrations of chloride anions (gray and dotted curves) is presented in Fig. 6. Position of absorption maximum of the TcaR protein in nanodiscs is at 531 nm, and a 5-nm shift of the absorption maximum towards short wavelength occurs after addition of 50-150 mM of chloride ions. Furthermore, optical density at the absorption maximum increased. These data are in good agreement with the effect of chloride anions on the absorption spectra of halorhodopsin investigated previously [8, 9, 27] and provide support to the notion that TcaR is an effective chloride pump.

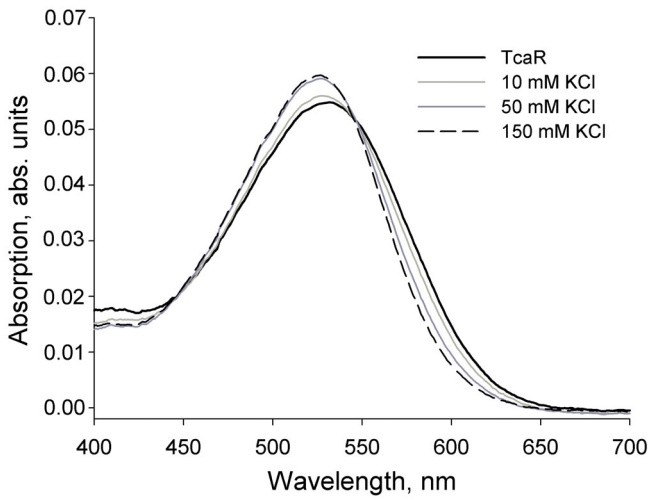

Fig. 6. Absorption spectra of TcaR in nanodiscs in the presence of varying concentration of potassium chloride. Measurement medium contained 10 mM Mes and 10 mM Tris (pH 6.5). Potassium chloride concentrations: 0 mM (black line), 10 mM (light-gray line), 50 mM (dark-gray line), and 150 mM (dashed line).

Analysis of amino acid sequence of TcaR and results obtained in this study allow us suggesting that TcaR exhibits functional similarity with halorhodopsin. Halorhodopsin participates in maintenance of salt balance in bacteria, and, it could be assumed, that physiological role of TcaR in cyanobacteria is similar. Considering that halorhodopsin is used in optogenetics, TcaR could also find its application niche among the used optogenetic tools. It seems that the TcaR protein deserves more detailed and comprehensive investigation including investigation of peculiarities of its photocycle, as well as its structure.

Contributions. V.I.G. concept of the study; A.A.A., T.I.R., Y.N.A., S.M.B., F.M.Ts. conducting experiments; Y.N.A., V.I.G., T.I.R. writing text of the paper. All authors participated in discussion of the obtained results and editing of the final text of the paper.

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Ethics declarations. The authors declare no conflict of interests in financial or any other sphere. This article does not contain any studies with human participants or animals performed by any of the authors.

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