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# Glutamate residue 90 in the predicted transmembrane domain 2 is crucial for cation flux through channelrhodopsin 2

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ABSTRACT

Channelrhodopsin 2 (ChR2) is a microbial-type rhodopsin with a putative heptahelical structure that binds all-*trans*-retinal. Blue light illumination of ChR2 activates an intrinsic leak channel conductive for cations. Sequence comparison of ChR2 with the related ChR1 protein revealed a cluster of charged amino acids within the predicted transmembrane domain 2 (TM2), which includes glutamates E90, E97 and E101. Charge inversion substitutions of these residues significantly altered ChR2 function as revealed by two-electrode voltage-clamp recordings of light-induced currents from *Xenopus laevis* oocytes expressing the respective mutant proteins. Specifically, replacement of E90 by lysine or alanine resulted in differential effects on $H^+$- and $Na^+$-mediated currents. Our results are consistent with this glutamate side chain within the proposed TM2 contributing to ion flux through and the cation selectivity of ChR2.



## 1. Introduction

Channelrhodopsin 2 (ChR2) is a retinylidene protein of 737 amino acids that is expressed in the eye spot of the green alga *Chlamydomonas reinhardtii* and supposed to evoke photoresponses [1,2]. Illumination of *Xenopus laevis* oocytes expressing ChR2 with blue light ($\lambda_{max}$ = 480 nm) induces a transient peak current followed by a persisting stationary one, carried mainly by protons and other monovalent cations. Additionally, $Ca^{2+}$, but not $Mg^{2+}$, also permeates ChR2 [1,3]. The presumptive ion pore of ChR2 is located within the first 315 amino acids, as a correspondingly truncated ChR2 retains the electrophysiological properties of the full-length protein [1]. ChR2 shows 15–20% sequence homology to the microbial-type rhodopsins bacteriorhodopsin (BR), halorhodopsin (HR) and sensory rhodopsins (SRs). Their seven transmembrane domain structure [4–8] is consistent with hydropathy plots of ChR2 [2], and spectroscopic data as well as measurements on lipid bilayers suggest that ChR2 is a leaky proton pump [9]. The precise topology, the mechanism of its proton pump activity, as well as the location of its ion channel pore, however, remain elusive.

Here, we investigated whether a cluster of glutamates localized at positions 90, 97 and 101 of the predicted TM2 of ChR2 is implicated in ion permeation and/or cation selectivity. Neutral substitution of E97 has been previously shown to result in modest changes in current amplitude [10]. We report that charge inversions at these positions impair ChR2 currents, and that residue E90 appears to be particularly important for both $H^+$ and $Na^+$ conductance. Our results suggest that TM2 contributes to the lining and selectivity filter of ChR2's intrinsic ion channel.

## 2. Materials and methods

### 2.1. Plasmids and cRNA synthesis

cDNAs encoding a truncated version of ChR2 (AA1–315) or a C-terminal fusion of the truncated ChR2 sequence to YFP (ChR2-YFP; see [1]) were cloned into pGemHE [11] using its BamHI and XhoI sites. Mutations were introduced by site-directed mutagenesis using the QuickChange Mutagenesis Kit (Agilent Technologies, Santa Clara, USA) and confirmed by sequencing. After linearization at a unique NheI site, these ChR2-encoding plasmids were used as templates for cRNA synthesis with the mMessage T7 Ultra Kit (Ambion, Austin, USA).

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### 2.2. Oocyte preparation and cRNA injection

*Xenopus laevis* oocytes were isolated and maintained as described [12,13]. Oocytes were injected with approximately 20 ng of cRNA and incubated at 18 °C for 3 days in standard ND96 (in mM: NaCl 96, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1, Hepes/NaOH 5; pH 7.4; 50 μg/ml gentamycin) containing 1 μM *all-trans*-retinal. Non-injected oocytes from the same batch incubated in parallel were used as controls.

### 2.3. Electrophysiology

Two-electrode voltage-clamp (TEVC) recordings from oocytes were performed using a Turbo TEC-03X amplifier and CellWorks (V3.7) data acquisition software (all from npi, Göttingen, Germany) as described previously [14]. ChR2 currents were induced by illumination with an HBO 100 lamp (Zeiss, Göttingen, Germany) equipped with a band pass filter (450 ± 25 nm, AHF Analysentechnik, Göttingen, Germany). Light was focussed by a lens on a 2 mm light guiding fiber (PG-R-FB1500; Laser Components, Olching, Germany). Illumination was controlled by a computer-triggered shutter (Uniblitz VCM-D1; Vincent Associates, Rochester, USA).

Routinely, oocytes were held at a membrane potential ($V_m$) of −60 mV. Transmembrane voltage changes from −80 to +40 mV were applied in 20 mV steps. After holding the respective $V_m$ for 1.5 s, illumination was for 3 s; 1.5 s after termination of the light pulse, the membrane potential was changed to a new $V_m$. Oocytes were superfused successively with $Na^+$ or *N*-methyl-glucamine (NMG) containing buffers (in mM: NaCl 110 or NMG 110; $MgCl_2$ 2; Hepes 5, adjusted to pH 4.0 (Na4.0), 7.6 (Na7.6) or 9.0 (Na9.0) with HCl or NaOH, respectively).

### 2.4. Data evaluation

Current values were determined using CellWorks (V3.7) software. Mean values (±SEM) were calculated from 8 to 18 TEVC-measurements obtained with oocytes from at least three frogs. For *I*/*V*-relation curves, absolute values were normalized to Na7.6, if not indicated otherwise.

## 3. Results

### 3.1. Charged residues in TM2 might form an amphipathic helix

To identify residues of ChR2 that could be involved in the formation of the ion channel, we aligned ChR2 to ChR1, another light-gated channel protein from *Chlamydomonas reinhardtii* originally thought to be $H^+$-selective [15,16]. Assuming that membrane permeating cations should be stabilized by hydrophilic or negatively charged side chains, we identified three glutamate residues, E90, E97 and E101, within the predicted α-helical TM2 (Fig. 1A; see also Refs. [2,10]). These residues and a lysine residue at position 93 are conserved in both ChR2 and ChR1 but absent in BR, which acts exclusively as a proton pump [17]. The side chains of these charged residues are predicted to lie at one side of the TM2 α-helix and thus could create a negatively charged hydrophilic microdomain within the transmembrane core of ChR2 (Fig. 1B).

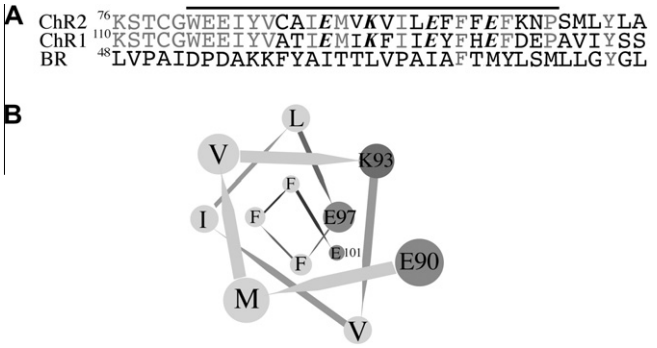


**Fig. 1.** Amphipathic nature of the putative TM2 region of ChR2. (A) Alignment of partial amino acid sequences including the putative TM2 regions (black bar) of ChR2, ChR1 and BR. Conserved amino acids are indicated in gray. Charged TM2 residues found in both ChR2 and ChR1, but not in BR, are shown in bold italics. (B) α-Helical wheel plot of the TM2 residues 90–101 of ChR2 viewed from the intra- to the extracellular side. Note that all charged side chains (E90, K93, E97 and E101, highlighted in dark gray) face one side of the putative α-helix.

### 3.2. Characteristics of light-induced ChR2 currents

A truncated version of ChR2 lacking the large intracellular C-terminal domain ($ChR2_{1-315}$) has been shown to generate photocurrents identical to that of the full-length protein [1], and a fluorescent fusion protein of $ChR2_{1-315}$ (ChR2-YFP) is widely used for light-induced manipulation of neuronal activity [18–20]. To facilitate the eventual usage of mutants with altered ion conductance for biological applications, we generated all substitutions in the ChR2-YFP construct. In all our recordings, the properties of ChR2-YFP were indistinguishable from those of the unfused $ChR2_{1-315}$ protein (compare Fig. 2A, B and Supplementary Fig. S1; see also Table 1).

Illumination of ChR2-YFP expressing oocytes held at a $V_m$ of −80 mV in NaCl solution at pH 7.6 (Na7.6) induced a biphasic, inwardly directed current consisting of a transient and a stationary component that persisted until the end of the light pulse (Fig. 2A; see also Ref. [1]). Non-injected control oocytes did not display light-induced currents (data not shown). At +40 mV, current flow was outwardly directed (Fig. 2A). The current–voltage (*I*/*V*) analysis of these light-induced currents disclosed a pronounced inward rectification with a reversal potential ($V_{rev}$) of −9 ± 2 mV (Fig. 2B and Table 1).

To determine the contribution of different cations to the total ChR2-mediated current, we used buffers containing different $H^+$ and $Na^+$ concentrations. When decreasing the external $H^+$ concentration to pH 9.0 (Na9.0), only a small reduction of the inward currents was observed at negative membrane potentials, and the $V_{rev}$ values were not significantly altered (Fig. 2B). This is consistent with ChR2-mediated inward currents at neutral and alkaline pH being predominantly carried by $Na^+$ ions. In contrast, at pH 4.0 light-induced currents were increased and only inwardly-directed at all membrane potentials analyzed (Fig. 2B). Apparently $H^+$ is the predominantly conducted ion species in Na4.0. This interpretation was confirmed by analyzing the *I*/*V*-relations derived from ChR2-YFP expressing oocytes superfused with solutions, in which $Na^+$ was replaced by NMG. The currents generated in NMG4.0 were not significantly different from those obtained upon Na4.0 superfusion (Fig. 2B and Table 1). However, when the external $H^+$-concentration was decreased to pH 9.0 in the absence of $Na^+$ (NMG9.0), only small light-induced currents were detected, and $V_{rev}$ was shifted to strongly negative membrane potentials (Table 1). The outwardly directed currents in NMG7.6 and NMG9.0 most likely resulted from ChR2-mediated cation efflux, probably $K^+$, as observed in recordings from Na7.6 or Na9.0 superfused oocytes held at positive $V_m$ (Fig. 2B). Since the absorption spectrum of ChR2 is insensitive to changes in pH [21], the different current amplitudes cannot be attributed to changes in excitation wavelength sensitivity.

### 3.3. Charge inversion of TM2 glutamates impairs ChR2 currents

To investigate whether the residues E90, E97 and E101 contribute to ChR2 conductance and/or ion selectivity, we generated the