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Functional expression of bacteriorhodopsin in oocytes allows direct measurement of voltage dependence of light induced H^+ pumping

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Abstract We report on the first successful expression of the light driven H^+ pump, bacteriorhodopsin, into the plasma membrane of oocytes from *Xenopus laevis*. The light induced photocurrents which reflect the pumping of H^+ by BR were analysed under voltage clamp conditions. At least 100 active BR molecules per μm^2 were expressed in the plasma membrane so that both the voltage clamp and giant patch clamp method could be applied. We show that H^+ pumping by BR is modulated by the membrane potential, i.e. the pump current shows strong voltage dependence in the range measured between −165 mV to +60 mV.

Key words: Bacteriorhodopsin; H^+-pump; Voltage dependence; *Xenopus* oocyte

1. Introduction

The light driven H^+ pump, bacteriorhodopsin (BR), serves as a model system for ion pumping [1]. Although an overwhelming body of biochemical and biophysical studies on the structure and function of bacteriorhodopsin is available [2–4], the vectoriality of pumping and the voltage dependence of transport are not well understood. The study of the voltage dependence of BR is of great interest since in the halobacterium the H^+ pumping by BR can create an electrochemical gradient corresponding to a proton motive force of up to −280 mV [5]. The direct measurement of voltage dependence is difficult to study because of the small size of the bacteria or reconstituted proteoliposomes. Spectroscopic and flux measurements under different imposed voltages were done with vesicles or bacteria but not under well defined voltage clamp conditions [6–8]. Direct electrical measurements have been untertaken with BR membrane sheets capacitively coupled to black lipid membranes (BLM) or to thin films or in oriented membrane suspensions, yielding valuable insight into the function of BR (for review see [9]). Since these methods did not allow the investigation of the voltage dependence of H^+ pumping, some attempts have been made to incorporate BR into planar lipid membranes [10,11]. However, the pumps must be fused into the artificial membrane with a single orientation, to avoid a superposition of two oppositely directed pump currents. Up to now, no procedure to achieve this requirement reproducibly has been reported. To circumvent difficulties of mixed orientation and to measure the photocurrent elicited by the pump with electrophysiological methods, we expressed BR into the plasma membrane of oocytes of *Xenopus laevis*. We report here two methods to study the electrical properties of the pump: (a) the usual voltage clamp and (b) the so-called giant patch clamp method [12,13]. Under voltage clamp conditions the pump current induced by BR in the whole cell was studied. The giant patch clamp method allows to analyse the pump under cell-free conditions which give optimal control of the electrolyte on both sides of the oocyte plasma membrane.

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Abbreviations: BLM, black lipid membrane; BR, bacteriorhodopsin; bop, bacterio-opsin; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; HEPES, *N*-[2-hydroxyethyl]piperazine-*N′*-[2-ethanesulfo nic acid]; MOPS, 3-[*N*-morpholino]propanesulfonic acid; NMG, *N*-methyl-D-glucamine; TEA, tetraethylammonium; V_h, holding potential.

2. Materials and methods

2.1. Enzymes and reagents

Restriction enzymes were obtained from New England Biolabs. Calf intestine phosphatase and T4 DNA ligase were obtained from Boehringer Mannheim. Fragments used for ligation were separated on agarose gels and purified by use of the QIAEX kit from QIAGEN (Hilden). The T7-Cap scribe kit was obtained from Boehringer Mannheim. Salts were p.a. grade and obtained from Merck (Darmstadt), EGTA, HEPES and MOPS were from Sigma.

2.2. Preparation of plasmid DNA and mRNA synthesis

Plasmid pGEMHE, a derivative of pGEM3z (Promega, Madison, WI), was used for in vitro generation of mRNA transcripts. Plasmid pGEMHE contains the 3′ and 5′ untranslated regions of the b globin gene from *Xenopus* [14]. A promoter-free *bop* gene, encoding bacterio-opsin including its signal sequence, was isolated from pBSbop [15] by cleavage with *Bam*HI and *Hin*dIII and ligated into pGEMHE, behind the T7 RNA-polymerase promoter. *E. coli* was transformed by the RbCl method [16]. Plasmids from *E. coli* were isolated by the method of Birnboim and Doly [17]. *Nhe*I linearized plasmid DNA was used for the in vitro generation of mRNA with the T7-cap scribe kit.

2.3. Oocyte microinjection and incubation

Oocytes where prepared as described elsewhere [18], in short: 50 nl mRNA (100 to 400 ng/μl) were injected per oocyte. Expression seemed to saturate above 10 ng. The oocytes were incubated for 3 to 5 days at 18°C with 1 μM retinal in the medium in order to reconstitute the BR from bacterio-opsin. After about 4 days at 18°C the level of expression became constant and the measured pump currents did not show a further increase. As a control injected oocytes were also incubated without retinal. As a further control oocytes were injected with water and incubated in retinal containing medium. Typically 20 oocytes were injected per batch.

2.4. Voltage clamp and patch clamp

To determine the light induced pump currents a two electrode voltage clamp (Turbo TEC-05, npi, Germany) was used. Voltage pulses between −165 mV to +60 mV were applied. Alternatively the pump currents were recorded in the cell-free mode by using an excised 'giant' patch configuration as described by Hilgemann [13]. The typical aperture of the patch pipette tip was 20 μm diameter.

All presented current recordings are filtered at 20 Hz. As a light source a mercury (Osram HBO, 100 W) or a xenon (Osram XBO,

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150 W) lamp or a continuous He-Ne laser were used. In order to activate the pump, the light was focused on the oocytes for one second. When using the lamps, always a heat filter and either a cutoff filter $\lambda > 515$ nm (Schott, OG 515) or an interference filter (580 nm with 10 nm bandwidth) were used.

For the determination of the action spectrum, the xenon lamp in combination with narrow bandwidth interference filters was used. Light from a helium neon laser with 4 different wavelengths was applied for complementary experiments on the action spectrum. The energy output was measured for each wavelength and photocurrents were corrected to a constant photon flux.

In order to minimize the offset current due to the variety of channels and transporters in the oocyte membrane, the bath solution contained for whole cell voltage clamp experiments 90 mM NaCl, 20 mM TEA-Cl, 5 mM $BaCl_2$, 2mM $CaCl_2$, 10 mM MOPS, pH 7.6. The pipette filling solution was 3 M CsCl, 0.6 M TEACl.

In the giant patch experiment the pipette was filled with (mM) 145 NMG-Cl, 5 KCl, 2 $MgCl_2$, 2 $BaCl_2$, 0.5 $CdCl_2$ and 10 HEPES, pH 7.4. The bath solution consisted of (mM): 100 NMG, 40 NaCl, 20 TEA-Cl, 2 $MgCl_2$, 10 EGTA and 10 HEPES, adjusted to pH 7.4 with HCl. The temperature was 21–24°C.

3. Results

3 to 5 days after the microinjection of mRNA the light induced currents were measured under voltage clamp conditions. Fig. 1 shows an experiment demonstrating photocurrents after irradiation with yellow light by using a cutoff filter ($\lambda > 515$ nm). Such a light response was obtained in every batch of injected oocytes. Usually more than 5 cells per batch were measured and a variable photoresponse, typically 25 nA (ranging from 15 to 50 nA) at −40 mV holding potential, was obtained from 10 batches. The sign of the current indicated that positive charges are outwardly transported. This demonstrates that BR pumps the protons to the extracellular medium. In order to prove that the light induced current is due to the proton pumping of the expressed BR, a series of control experiments were carried out.

Uninjected oocytes and oocytes injected with water did not show any photoeffect (irrespective of retinal incubation) other than a small negligible change of the background conductance (data not shown). This is probably due to a light induced temperature effect.

Oocytes injected with bop mRNA, but not treated with retinal, showed a marked, but small, photocurrent, usually 10% of the amplitude found in cells incubated with retinal. This implies that some retinal is already present in the oocytes. Overnight incubation in retinal containing solution resulted in a strongly enhanced photocurrent when tested 12 hours later.

As a further control experiment for functional expression of BR we determined the action spectrum of the photocurrent under voltage clamp conditions at −40 mV. Fig. 2A shows that

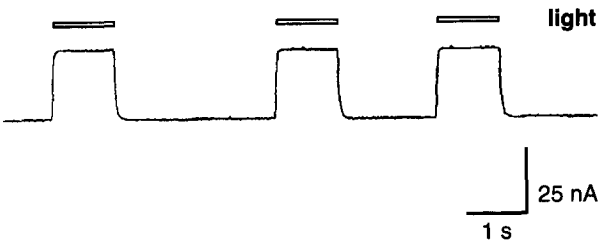

Fig. 1. Current recording from a voltage clamped oocyte with responses to light pulses of 1 sec. duration. V_h = −40 mV. Light source: HBO 100, OG 515.

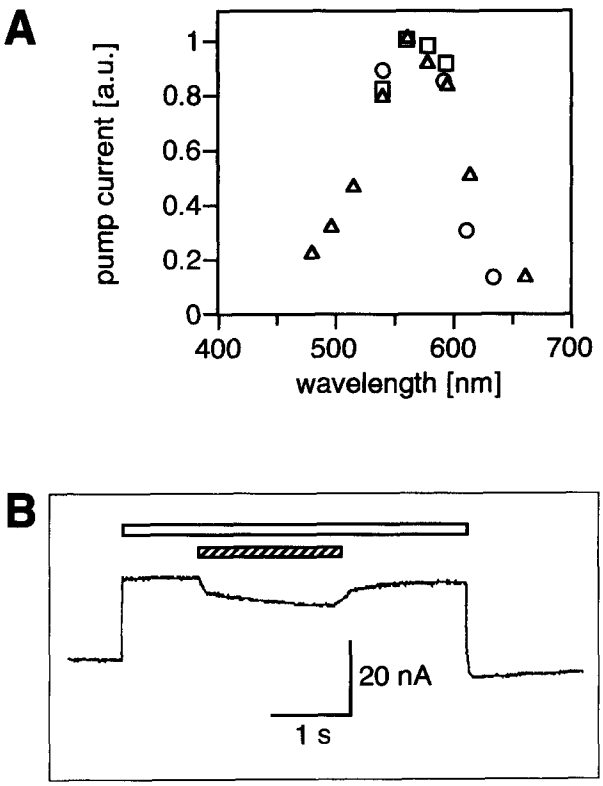

Fig. 2. (A) Action spectrum of the light induced current at V_h=−40 mV, obtained with Xenon gas arc lamp and different narrow bandwidth interference filters (△,□) or a Helium Neon laser(○). (B) Inhibition of light induced signal by blue light. At a holding potential of −40 mV light above 515 nm (open bar) was applied, then the cut-off filter was removed (hatched bar) for about 2 s.

the maximal stationary photocurrent occurs between 550 and 600 nm, the spectral range which coincides with the maximal absorption of BR at 568 nm.

As shown in Fig. 2B, the stationary photocurrent can be quenched by blue light. This is in agreement to the observation that the photocycle of BR is shortened in a two photon process. The M intermediate decays to BR_{568} after the absorption of a blue photon under reprotonation of the Schiff base from the extracellular side, i.e. the proton is taken up from the same side, where it was released during the light induced formation of M. This well known phenomenon is due to the light induced cis trans isomerization of the chromophore retinal [19,20].

Finally we demonstrate that a light activated current can also be measured under cell-free conditions (Fig. 3). A large piece of membrane was excised from the oocyte. The opening of the pipette tip had a diameter of 20 μm. This modified patch clamp method is necessary in order to get enough BR molecules activated in an excised patch. Indeed 1–2 pA photocurrents were measured in this configuration. The sign of the current indicated the same direction of pumping as under voltage clamp conditions of the whole oocyte. Also in this case the quenching of the stationary photocurrent by addition of blue light could be demonstrated (trace b in Fig. 3).

3.1. Current-voltage relationship of BR

The light induced pump currents were recorded at different holding potentials under voltage clamp conditions (Fig. 4A).

These data indicate that the photocurrent depends strongly on the applied potential in the range of −165 to +60 mV (Fig. 4B). Over a wide range the current-voltage curve is linear. As expected for a single orientation of the protein in the plasma membrane the current could not be inverted. All results were obtained under non-saturating light conditions. With conventional light sources only about 2 W/cm^2 light intensity (515 nm < λ < 800 nm) can be achieved, which is not sufficient to reach light saturation.

4. Discussion

We have shown that direct electrophysiological methods can be applied, when bacteriorhodopsin is expressed functionally in the plasma membrane from oocytes. From the size of the current in Fig. 1, 30 nA, measured at 0 mV, 2×10^{11} charges per second are transported. Assuming a turnover of 100 s^{-1} for the photocycle of BR [21], and 1 H$^+$ transported per photocycle, at least 2×10^9 BR molecules are expressed in the plasma membrane. (Since light saturation was not achieved, this estimation gives only the lower limit.) The capacitance of this cell was 150 nF and assuming 1 μF/cm^2, the density is at least 1.3×10^8 BR molecules per mm^2 or 130 per μm^2. This estimation is also confirmed by the giant patch experiment (Fig. 3). Compared with the density of BR in purple membranes of halobacteria with 10^5 μm^{-2} [2], the density in the oocyte plasma membrane is about 0.1% of that in the purple membrane. If however a turnover of 5 s^{-1} for proton pumping [21] is assumed the ratio increases to more than 2%.

Although functional expression in *S. pombe* was already demonstrated a few years ago [15,22] it was still surprising that the archaebacterial protein bacteriorhodopsin could be expressed in an amphibian cell. It was additionally satisfying from the electrophysiological investigations that the direction of light induced current flow was always positive, i.e. from the cytoplasm to the extracellular space. Incorporation of BR into artificial black lipid membranes (BLM) was reported before [9–11], but the main difficulty with this approach was control of the orientation of the BR molecules. For membrane insertion of a protein in a living cell it should be expected that a single orientation is achieved to a much higher degree than by insertion with reconstitution methods, but independent proof would be favourable. One indication of highly oriented membrane insertion is that the light induced current did not invert in the investigated voltage range, up to −165 mV, even though this current is clearly voltage dependent, as can be seen from Fig. 4.

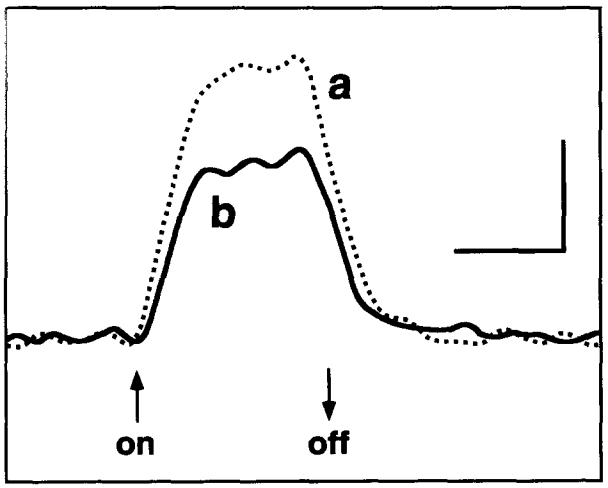

Fig. 3. Superimposed patch current recordings from a giant excised patch at 0 mV. Traces are averages of 10 successive recordings. a, 515 nm < λ < 800 nm, b, 320 nm < λ < 800 nm. Scale bars = 0 ms and 0.5 pA.

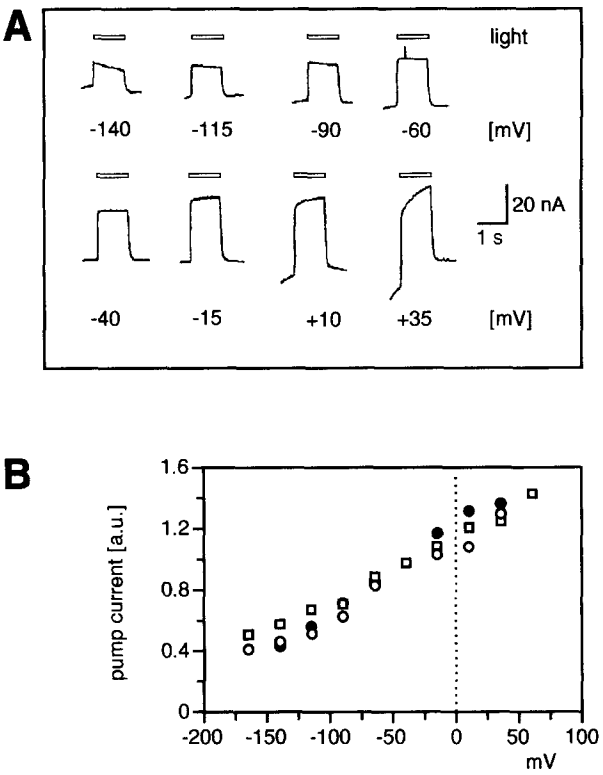

Fig. 4. (A) Light induced outward currents in a voltage clamped oocyte at different holding potentials: from −140mV to +35 mV in 25 mV steps. 1 sec light pulses, 515 nm < λ < 800 nm. (B) Current-voltage relationship of normalized (to −40 mV) light induced currents from A (●), from an experiment using 580 nm narrow bandwidth interference filters (□) and from an experiment using a helium neon laser emitting at 594 nm (○).

With the above mentioned limitations concerning saturation of BR molecules with light, it can be predicted by extrapolation of the *I/V* curve that complete inhibition or even reversal of pump current has to occur at potentials beyond −200 mV under our conditions of illumination. This is in good agreement with earlier estimations of membrane polarization reached in a living bacterium [5] and with measurements of BR incorporated in BLM's [10]. In the voltage range below −100 mV the shape seems non-linear and due to this nonlinearity no reversal could be extrapolated [11], but this should be put to experimental test. The scatter of the data does not allow a conclusion on the exact shape of the current-voltage curve but further attempts to suppress endogenous oocyte conductances might improve the signal to noise ratio. For energetic reasons no complete reversal of the pumping process is expected. However, reversal of current by reversal of pump direction is a possibility if an alternative transport mechanism could be induced by the applied potential, as it was shown for BR mutants [23] at a potential of 0 mV. The shape of the current-voltage relationship is currently under investigation with respect to illumination conditions,

bath solutions and especially pH. Unfortunately, in the case of the oocyte the internal pH is not under control of the investigator, but application of the giant patch method would be ideal to address this question.

To understand the linear current-voltage behaviour the reaction scheme of the photocycle can be written down as follows:

$$BR \rightarrow K \leftrightarrow L \leftrightarrow M \leftrightarrow N \leftrightarrow O \rightarrow BR$$

From earlier work we know, that the main electrogenic event is the M decay [24,25]. Braun et al. [11] concluded from their observed non-linear voltage dependence of the pump current that the electrogenic M decay is rate limiting. The approximately linear relation which we observed could be explained by the assumption that the voltage dependent M to N transition step is fast and reversible, whereas a following voltage independent step is rate limiting. In other words, depending on the sign of the applied voltage, the N or the M intermediate is enriched, because the potential favours the forward or the backward reaction, respectively. A similar relationship was found, for example, for the current-voltage curve of the Na^+,K^+-ATPase in heart cells where the electrogenic Na translocation is not rate limiting in the reaction cycle. Over a wide range of the applied voltage a linear dependency was obtained [26], as long as the Na^+ translocation step was not made irreversible by the absence of external Na^+.

The present results demonstrate that for studying the voltage dependence of BR the oocyte system is advantageous over the system where BR is incorporated into BLM's, which is burdened with uncertain sidedness. On the other hand BLM measurements with adsorbed BR containing membrane fragments ('BLM sandwich') are a powerful method to investigate substrate dependence, kinetics and vectoriality of proton pumping at a voltage of 0 mV.

The combination of measurements with the BLM sandwich technique and the presented new oocyte system will allow us to get more insight into the pumping mechanism regarding vectoriality and voltage dependence of electrogenic steps in the reaction cycle.

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