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A New Anion-Sensitive Biosensor Using an Ion-Sensitive Field Effect Transistor and a Light-Driven Chloride Pump, Halorhodopsin

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ABSTRACT

A new biosensor sensitive to chloride anion using a light-driven chloride pump protein, halorhodopsin (hR), and an ion-sensitive field effect transistor (ISFET) has been developed. Membrane vesicles of halophilic bacteria containing hR were immobilized in the matrix of polyvinylbutyral resin on the surface of the ISFET. The gate voltage of this device changed in the min time scale under yellow light illumination. The response for chloride anion increased according to the increase of chloride anion concentration in the bulk aqueous phase. In the dark, the gate potential did not change even in the presence of chloride anion. These chloride-dependent gate potential changes of the hR-ISFET indicate that the chloride pumping by hR is active on the ISFET and that ISFET detects the light-dependent chloride transport by hR.

Index Entries: Ion-sensitive field effect transistor (ISFET); halorhodopsin; halophilic bacteria; chloride anion; ion-pump protein.

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INTRODUCTION

A biosensor that is an enzyme-coupled ion-sensitive field effect transistor (ISFET) was first proposed by Caras and Janata in 1980 (*1*). ISFET has been drawing attention as a microdevice since then. Much research on the ISFET biosensor has been reported. In earlier papers, water-soluble proteins that catalyze a reaction causing a pH change were coupled with ISFET, and these biosensors detected penicillin (*1*), urea (*2*), and glucose (*3*). Furthermore, microorganisms have also been utilized in constructing ISFET biosensors (*4*). It has been recently reported that membrane proteins, such as channels or pumps, were immobilized on the surface of an ISFET gate for the detection of cations. The acetylcholine receptor was used for recognition of Na^+ (*5*) and bacteriorhodopsin for that of H^+ (*6*). These studies clearly show that the membrane proteins sensitive to cations are active on the solid surface and can be applied to biosensing. To date, there have been no reports of any anion-sensitive ISFET biosensor. The knowledge about the importance of anions has been broadened, especially in the medical field. The determination of the concentration of chloride in blood is important for diagnosis of renal diseases. Therefore, we noted halorhodopsin as a chloride anion recognition molecule.

Halorhodopsin (hR) is a light-driven chloride anion pump in the cytoplasmic membrane of halophilic bacteria, *Halobacterium halobium* (*7,8*). The chloride transport properties and the photochemical properties of hR have been characterized with membrane vesicles prepared from the bacteriorhodopsin-deficient mutant strains (*9*). These studies revealed that hR has a light-induced cyclic reaction that is called a photocycle, and that hR transports chloride anions from outside to the inside of the cell (*9*). The photocycle and the chloride pumping of hR are strongly dependent on chloride anion (*10*). This chloride pumping is closely coupled with the photocycle, which is detected with flash spectrophotometry. A flash converts the original pigment absorbing maximally at 580 nm into an intermediate whose absorbance maximum is at 500 nm. Then the intermediate returns to the original pigment within 10 ms (*11,12*).

In this article, we report a new anion-sensitive biosensor in which hR is immobilized on the surface of the ISFET. The hR-ISFET shows the light-induced response dependent on chloride anion.

MATERIALS AND METHOD

Instruments

ISFETs and an electric circuit for measurement (*13*) (ISFET mV/pH meter) were purchased from Shindengen Electric Mfg., Saitama (Japan).

Preparation of Cell Membrane Vesicles

Halobacterium halobium strain $R_1S_4pm^-$, a mutant lacking bacteriorhodopsin, was grown at 40°C under aeration. The growth medium contained the following chemicals in 20 L: 5000 g of NaCl, 400 g of $MgSO_4 \cdot 7H_2O$, 40 g of KCl, 2 g of $CaCl_2 \cdot 2H_2O$, 60 g of trisodium citrate dihydrate, 200 g of Oxoid peptone (L37), 10 mL of $FeSO_4$ solution (22.8 g/500 mL), and 10 mL of $MnSO_4$ solution (0.18 g/500 mL). The pH of the medium was adjusted to 7.0 with NaOH. After the culture reached the stationary phase, the cells were collected with centrifugation at 9600*g* for 15 min and suspended in 4.28*M* NaCl. The suspension was frozen in liquid nitrogen and stored at −80°C.

hR-containing vesicles were prepared as follows: The harvested cells were disrupted by freeze–thaw method. The membrane fraction was collected with centrifugation at 100,000*g* at 4°C for 60 min, and washed with 4.28*M* NaCl until the supernatant became clear. To remove chloride anion, the vesicles were washed with 1*M* citrate buffer (pH 7.0) eight times.

Immobilization of hR on ISFET

The hR-containing vesicles were immobilized on an ISFET as follows: An ISFET was dipped into a 0.2% (w/v) of polyvinylbutyral (PVB) resin in dichloromethane and dried in air (*14*). The PVB-coated ISFET was immersed in the hR-containing vesicle suspension at room temperature for 1 h to immobilize the vesicles in the PVB matrix on the ISFET. Total protein concentration of the vesicle suspension, which was determined by the Lowry method with bovine serum albumin as a standard, was about 30 mg/mL.

Detection of Chloride Anion

The hR-ISFET was immersed in 1*M* citrate buffer (pH 7.0). In the dark, KCl solution was added, and the solution was stirred gently. The hR-ISFET was illuminated with halogen lamp (Shimazu AT-100HG) passed through a yellow glass filter (Irie Y-50) and an infrared absorbing filter (Irie IRA-25S). The gate voltage of the hR-ISFET was measured with a Ag/AgCl reference electrode and a salt bridge of potassium nitrate, which was used to prevent contamination of chloride ion from the reference electrode to the buffer solution. All experiments were carried out at room temperature.

Flash-Induced Absorbance Change

The spectroscopic activity of hR has been measured with flash-photolysis (*15*). Chloride anion was added as KCl solution to the membrane suspension, and the absorbance change induced by red flash ($\lambda > 620$ nm) was measured.

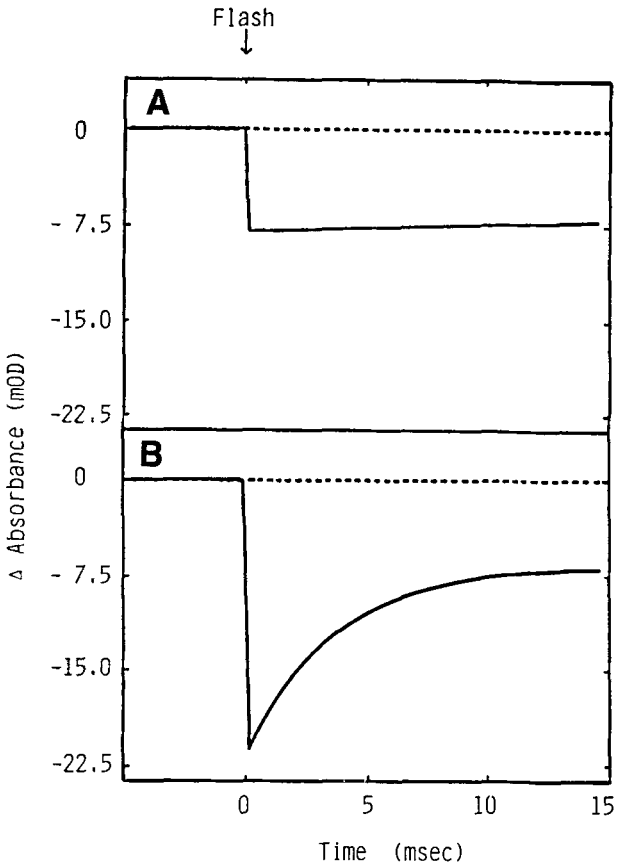

Fig. 1. Flash-induced absorbance change of membrane vesicles suspended in 1*M* citrate buffer (pH 7.0) at 580 nm (**A**) in the absence of chloride anion, and (**B**) in the presence of chloride anion (9.6 m*M*).

RESULTS AND DISCUSSION

Preparation of Chloride-Free hR and Chloride Dependence of hR

To prepare chloride-free hR, we washed the membrane vesicles with citrate buffer. We used citrate buffer because citrate anion is too large to interact with the anion-binding site in hR (*16*). Figure 1A shows a red flash-induced absorbance change at 580 nm in the hR-containing vesicles washed intensively with citrate buffer. A very slow recovery after a rapid decrease was observed. This slow component is attributed to sensory rhodopsin (sR), which does not have ion-pump activity (*11*). Chloride addition to the membrane vesicles causes an increase in the initial rapid change (Fig. 1B). This chloride-dependent component is attributable to hR (*10,11*). The amplitude of the flash-induced absorbance change of hR increased according to the increase of chloride ion concen-

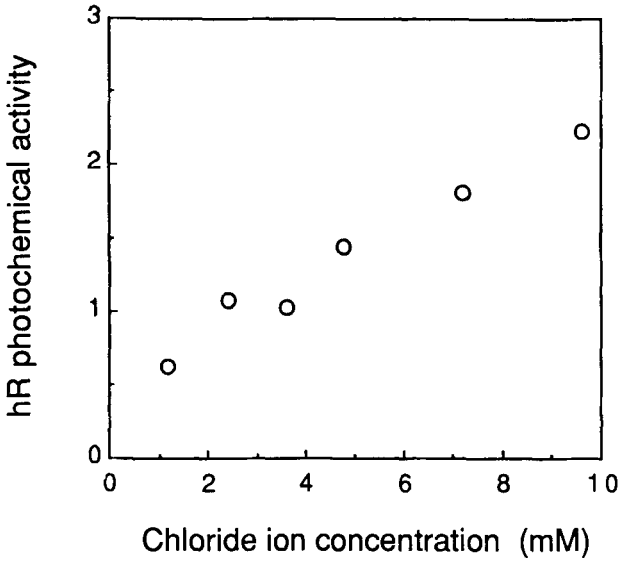

Fig. 2. Chloride anion concentration dependence of hR photocycling activity. Photocycling hR was determined by monitoring flash-induced absorbance changes at 580 nm. Membrane vesicles were suspended with 1*M* citrate buffer. KCl solution was added to the suspension.

tration, but the amplitude of hR signal was affected by not only chloride ion concentration, but also hR concentration. To correct the signal amplitude of hR from the dilution caused by the addition of chloride solution, sR signal is used as an internal standard because the amplitude of sR signal is not affected by chloride (*10*). The amplitude of the absorbance change of hR was divided by that of sR absorbance. Figure 2 shows the chloride concentration dependency of the corrected hR absorbance change. The chloride dependence agrees well with that of hR reported already by several groups (*10,16*). These results show that the intensive washing with citrate buffer makes chloride-free hR and that, even in the chloride-free condition, hR retains the ability to pump chloride anions.

Response of hR-ISFET Sensor to Chloride Anion

The chloride-free membrane vesicles suspended in the citrate buffer were used for immobilizing hR in the polyvinylbutyral (PVB) resin layer on ISFET. We measured the gate output voltage of hR-ISFET. The response curve of the hR-ISFET is shown in Fig. 3. In the chloride-free solution, yellow light caused a sharp fall of the gate voltage within 10 s, and took the photosteady state level. Yellow light then turned off, and the voltage returned to the dark level after a sharp rise. This response is owing to the light-sensitive properties of the ISFET itself as a silicon semiconductor. The output voltage under the steady illumination was dependent on the light intensity (data not shown). After addition of chloride anion, we

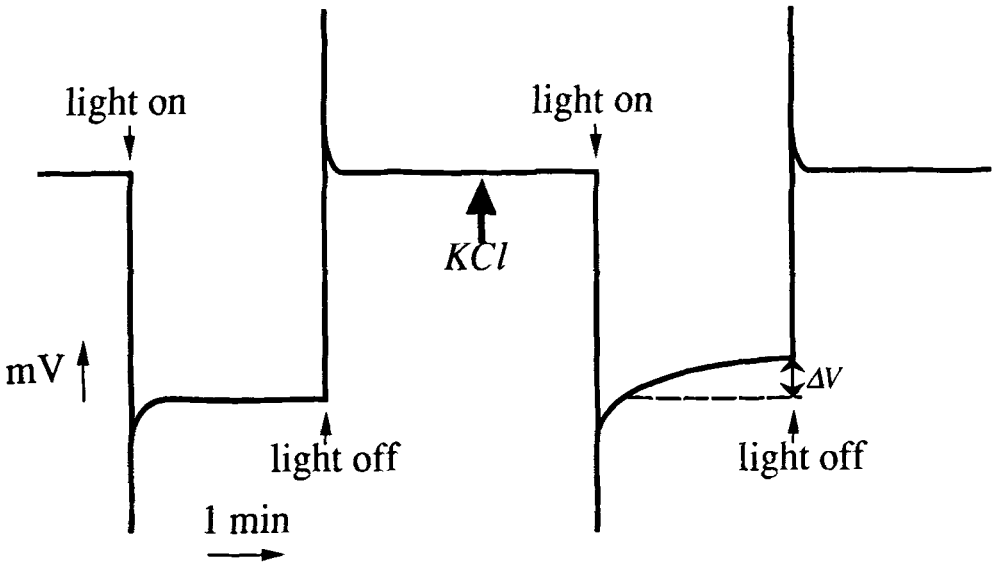

Fig. 3. Photoresponse curve of hR-ISFET before and after addition of Cl^-.

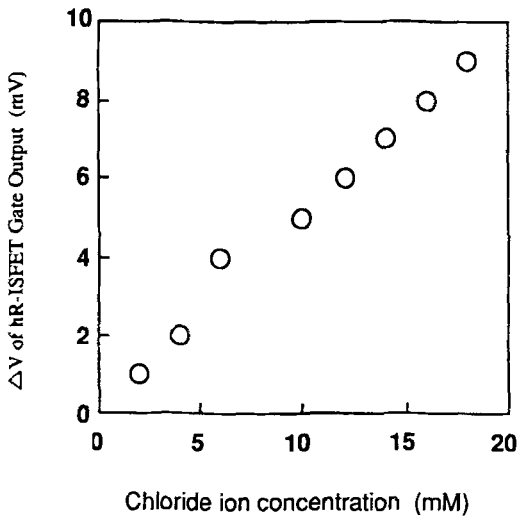

Fig. 4. Output change of hR-ISFET to chloride anion under light illumination. hR-ISFET was immersed in 1*M* citrate buffer (pH 7.0), and KCl was added to the buffer.

observed a chloride-dependent signal, which was a gradual voltage change from the photosteady-state level in the min time scale. Chloride anion concentration dependence of the voltage change was measured. The gate voltage difference (ΔV) between the output in the presence of Cl^- and that in the absence of Cl^- after 3 min on illumination was regarded as the response of the hR-ISFET to Cl^-. The magnitudes of the responses were plotted against chloride anion concentration (Fig. 4). The gate voltage difference is linearly related to the chloride ion concentration. The gate voltage was not changed by Cl^- addition in the dark, indicating that this chloride-

dependent response of the hR-ISFET requires light energy. Because this chloride-dependent signal was not observed for ISFET alone or the PVB-coated ISFET, hR-vesicle on the PVB-coated ISFET must be the cause for this light-induced chloride-dependent response. These results suggest that hR in the resin matrix on the ISFET has light-driven chloride pumping activity and that ISFET detects the electrochemical changes generated by hR.

CONCLUSIONS

We showed here a new chloride-sensitive biosensor consisting of an ISFET and a recognition element, hR, which is a light-driven chloride pump. hR kept its activity immobilized even in the resin matrix on the solid surface of the ISFET. This biosensor is superior in its simplicity and its greatly reduced size. Anion-pump protein is a useful element of an ISFET-based biosensor.

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