# Investigation of the light-induced proton pumping function of Bacteriorhodopsin

Hiroshi MATSUOKA*, Kazuhito KAJIWARA, Motohiko HIKUMA

Department of Bioscience and Bioengineering, The Nishi-Tokyo University,
2525 Uenohara, Yamanashi 409-01

## SUMMARY

The light-induced proton pumping function of bacteriorhodopsin (BR) was investigated by two methods. One was based on the simultaneous measurements of the pH values in the inner and outer liquids of BR-liposomes during the transient state after a shift from the light to the dark and the reverse. Some mathematical equations characterizing the mechanism of the proton transport through the membrane for the BR-liposomes were developed and could simulate well the dynamic aspect of the pH changes within the internal and external liquids of the BR-liposomes. The other was that BR was located on the surface of the ISFET with an acetylcellulose membrane containing phosphatidylcholine and cholesterol. A potential around the gate of this device rose rapidly and increased gradually during illumination. It showed the photoresponse, especially a gradual increase, for which the proton pump of BR was responsible. The effects of the light intensity on the BR-immobilized ISFET were also examined.

Keywords; Bacteriorhodopsin, liposome, light-induced proton pump, ISFET

## INTRODUCTION

Biomembranes are responsible not only for the isolation of the inner phase of a cell envelope from the outer but also for many biological functions such as energy conversion, information transmission, endocytosis, and so on. According to the model proposed by Singer et al. [23], biomembranes are supposed to consist of a fluid bilayer phospholipid in which various proteins float. These proteins are called membrane proteins and play major roles in the fu¹nctions high-lighted above.

Bacteriorhodopsin (BR) is one of the membrane proteins contained in the purple membrane (PM) of halophilic bacteria, such as *Halobacterium halobium*, which are found in natural brines of high concentration at or near saturation. It has the function of light-induced proton pumping across the membrane [19, 14, 21]. The observations of this function have been examined by using various reconstituted systems such as vesicles [14, 21, 3], black membranes [11, 4], and acrylamide gels [7]. Among them, the vesicle reconstitution method has been most widely used as a model for the cell membranes. Hellingwerf and coworkers [10] observed the light-induced proton pumping function with the artificial membrane incorporating BR and estimated the amount of protons transported across the membrane, by measuring the pH value of the illuminated suspension buffer of the vesicles. Beside this, many models of the proton transport in the BR-liposome have been proposed [9, 15, 20, 25, 26]. In most cases, however, the parameters necessary for featuring the transport phenomena were given without quantitative estimation. The reason why the physically significant parameters could not be determined in these studies might be ascribed to the fact that an accurate proton balance between the inner and outer liquids of the BR-liposomes was difficult to be established. Although the pH change in the internal liquid of the BR-liposomes has been measured in one study, the experiment was carried out only at the so-called steady state II [9] or the pH value was calculated on an assumption about the liposome volume which was difficult to be measured accurately [2, 9].

In this paper, we investigated the characteristics of the light-induced proton transport of

* to whom all correpondence should be addressed.

BR by two kinds of method. One was the simultaneous measurements of the pH values inside and outside of the BR-liposome. The inside pH was monitored by a fluorescence intensity change of a water-soluble pH-sensitive dye, pyranine, and the outside pH was done by a combined glass electrode. The correlation between a proton motive force and a proton flux was made clear by the method. The other was that BR was located on the surface of the ion selective field effect transistor (ISFET) with an acetylcellulose membrane containing phosphatidylcholine and cholesterol. A potential around the gate of this device rose rapidly and increased gradually during illumination. It showed the photoresponse, especially a gradual increase, for which the proton pump of BR was responsible.

## MODEL

Transport of ions through the membrane of BR-liposome in the 150 mM KCl solution are considered after Westerhoff [26]. Since the proton and the potassium permeation rate are much larger than that of chloride in this condition, it is sufficient that only the proton and the potassium transports are considerable [26]. The membrane potential and the pH difference are mainly caused by the proton pump activity of BR and only secondarily influenced by the passive process also described in Fig. 1 and by the possible feedback of $\Delta\tilde{\mu}_H$ on the proton pump. When the electrogenic proton pump of the BR is set into action by switching on the light, a membrane potential will be built up with a $t_{1/2}$ characterized by

$$t_{1/2}=0.303C\left(\frac{1}{R_{BR}}+\frac{1}{R_m}\right)^{-1} \qquad (1)$$

The value of $t_{1/2}$ is estimated as 1.1 s by Westerhoff and coworkers [26]. Therefore after 3 s of illumination the electroneutral flow condition (steady state I) approximately holds. In the steady state I, the proton pumping rate of the BR is nearly equal to the rate of facilitated transport of the proton and the potassium ion. It follows that after some 10 min the approximation of zero net proton flow (steady state II) will be operative. In the steady state II, the proton pumping rate is nearly equal to the facilitated transport of proton [26].

The model is constructed on the conditions that the electropotential difference across the membrane of the BR-liposomes is negligible in comparison with the chemical potential difference, as an appropriate amount of valinomycin is incorporated in the membrane to transport the potassium ions in the internal liquid countercurrently with the proton transport [18]. The inward proton flux is;

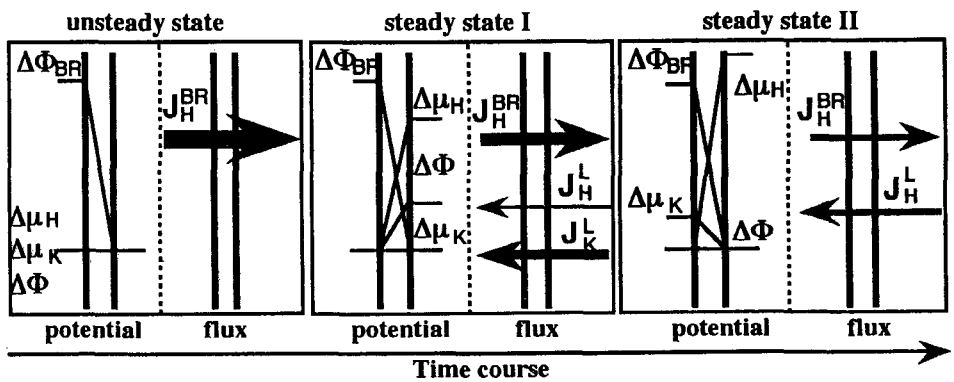


Fig. 1. Predicted potential difference and ion transport across the membrane in photo-activated BR-liposome. $\Delta\Phi_{BR}$:potential difference of photo-activated BR; $\Delta\mu_H$, $\Delta\mu_K$, $\Delta\Phi$ : proton, potassium, and electro potential differences, respectively ; $J_H^{BR}$:photo-activated proton flux, $J_H^L$, $J_K^L$: flux of proton and potassium ions, respectively.

$$J_H=S\{P-k_L([H^+]_i-[H^+]_e)\} \quad \text{(the light condition)} \tag{2}$$

$$J_H=-Sk_D([H^+]_i-[H^+]_e) \quad \text{(the dark condition)} \tag{3}$$

In general, $k_L$, is not equal to $k_D$. The inhibition coefficient, $k_I$, is defined as the coefficient of the proton leak associated with the proton pumping. After Ramirez [22],

$$k_I=k_L-k_D \tag{4}$$

The proton flux is zero at steady state II, ie.,

$$J_H=0 \tag{5}$$

By introducing Eq. (5) in Eq. (2),

$$P=k_L([\hat{H}^+]_i-[\hat{H}^+]_e) \tag{6}$$

Eqs. (2) and (3) are rewritten, respectively, as follows,

$$\frac{d[H^+]_i}{dt}=Q_L\{-[H^+]_i^2+u[H^+]_i+v[H^+]_i^{1-A}\} \quad \text{(the light condition)} \tag{7}$$

$$\frac{d[H^+]_i}{dt}=Q_D\{-[H^+]_i^2+v[H^+]_i^{1-A}\} \quad \text{(the dark condition)} \tag{8}$$

where,

$$A=\frac{pH_e-p\tilde{H}}{p\tilde{H}-pH_i}=\frac{B_iV_i}{B_eV_e}$$

$$u=[\hat{H}^+]_i[\tilde{H}^+]^{1+A}[\hat{H}^+]_i^{-A}[\hat{H}^+]_e$$

$$v=[\tilde{H}^+]^{1+A}$$

$$Q_L=\frac{2.303Sk_L}{B_iV_i}$$

$$Q_D=\frac{2.303Sk_D}{B_iV_i}$$

$Q_L$ and $Q_D$ are modified proton leak coefficients in the light and the dark, respectively.

We can estimate the proton pump activity, $P^*$, is

$$P^*=PS=\frac{Q_L([\hat{H}^+]_i-[\hat{H}^+]_e)AB_eV_e}{2.303} \tag{9}$$

The six parameters included in the right-hand side of Eq. (9) were determined experimentally from the pH and the buffering capacity of the external liquid.

## MATERIALS AND METHODS

### Materials

PM containing BR was prepared from the culture of *Halobacterium halobium* according to the method of Oesterhelt and Stoeckenius [19]. Soybean phosphatidylcholine (PC) type IV-S was purchased from SIGMA and purified by acetone-ether extractions [12]. Pyranine was purchased from Tokyo-kasei and purified as described previously [13]. Other reagents were of analytical grade.

**Preparation of the BR-liposomes**

BR was incorporated in liposomes under sonication by the method of Racker [21] with slight modification. Briefly, in the typical experiment, 10 mg-PM, 30 mg-soybean PC, and 5 mg-cholesterol were suspended in the buffer solution (150 mM KCl) and then treated by using an ultasonic homogenizer at 0 °C. A suspension of liposomes was prepared according to the procedure described previously [17]. The intravesicular pH was estimated from the fluorescence data as reported previously [17]. Determinations of the concentrations of lipid and protein were carried out as described by Ames [1] and Lowry [16], respectively.

**Simultaneous measurement of pH inside and outside of the BR-liposomes**

The simultaneous measurement method is described in the previous report [18] was slightly modificated. Briefly, the experimental setup for the fluorescence intensity of the pyranine solution comprised in the BR-liposomes is shown in Fig. 2. The neutral density filter cut off the light over 550 nm. The experiment was carried out with a 2.5 ml suspension at 25 °C. A fluorescence intensity of the suspension at the excitation wave lengths of 400 nm and 450 nm with the bandwidths of 10 nm was measured at an emission wave length of 510 nm with the bandwidth of 4 nm. Difference fluorescence intensity was measured with 2 ml of BR-liposome suspensions put in the reference and the sample cells. The intravesicular pH was estimated from the different fluorescence intensity as reported previously [17]. The external pH of the liposome suspension was measured with a micro combined glass pH electrode (2.5 mm D; Fuji-Kagakukeisoku), which was fitted to a port on a side of the glass vessel and connected to an Orion 701A ion analyzer. The data of the fluorescence intensity and the potential difference of the electrode were recorded and analyzed by personal computer (NEC-8801) which was interfaced with an amplifier and a high speed multichannel A/D converter.

**Preparation of the BR-liposomes immobilized on the surface of the ISFET**

Preparation of the BR-liposomes immobilized on the surface of an ISFET is shown in the previous report [24]. Briefly, the ISFET, as shown in Fig. 3, was coated with a thin layer of silver,using the silver mirror method and then the Ag-covered ISFET was coated with porous

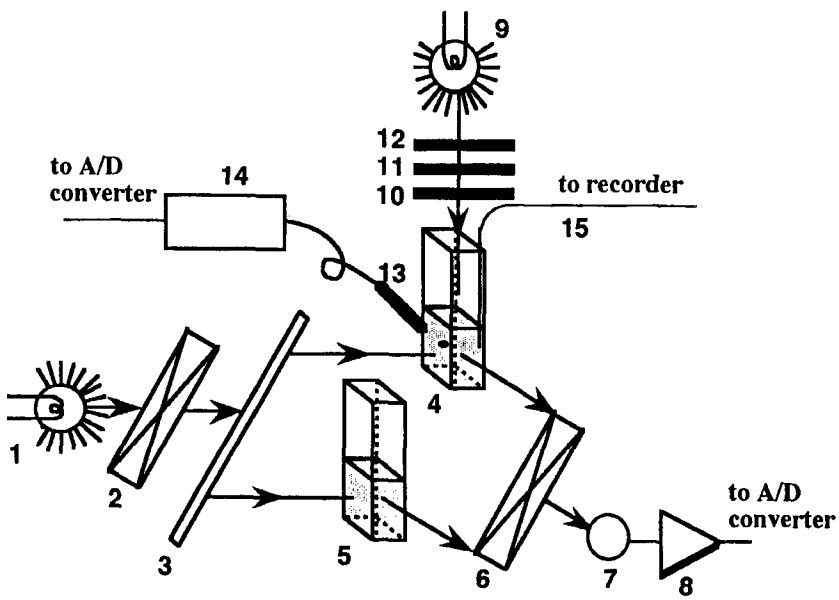


Fig. 2. Experimental setup: 1. 300 W xenon lamp; 2. monochrometer; 3. chopper; 4. sample cell; 5. reference cell; 6. monochrometer; 7. photomultiplier; 8. amplifier; 9. 150 W halogen tungsten lamp; 10. water filter; 11. neutral density filter; 12. interference filter; 13. pH electrode; 14. ion analyser; 15. themcouple

acetylcellulose (AC). The coated AC was 50 μm thick. Thereafter, the ISFET was placed in 1 ml of n-decane solution containing 200 mg of soy bean PC and 33 mg of cholesterol, so that a PC layer was formed around the porous AC membrane of the ISFET. After adding 40 mM $CaCl_2$ to the sonicated vesicle suspension, the PC-soaked ISFET was placed in it for 60 min at room temperature, so that the PM-containing vesicles would become associated with the PC layer [5]. The ISFET was then transferred into another 5 ml of 50 mM Tris-HCl buffer containing 150 mM KCl at pH of 7.0. A source follower circuit was used to operate the ISFET with an Ag/AgCl reference electrode (TOA Co., type HS-907). The potential difference of the membrane of PM and PC on the surface of the modified ISFET was equal to the change of the source gate potential difference. A slide projector (Elmo, model S-300) with a 300 W tungsten lamp was used for the illumination (Fig. 4)

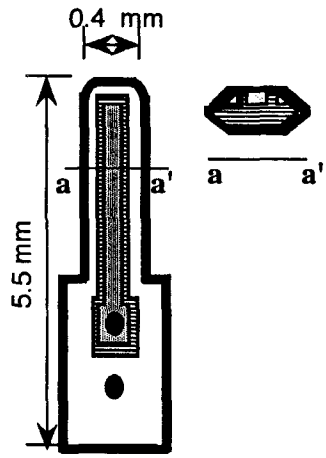


Fig. 3. Diagram of ISFET.
□ source (positively doped)
▤ gate (negatively doped)
▩ drain (positively doped)

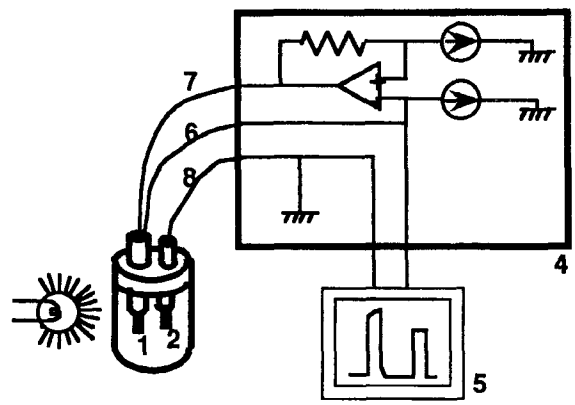


Fig. 4. Experimental setup for the photo-sensitive device model: 1. ISFET; 2. reference electrode; 3. 300 W halogen tungsten lamp; 4. source follower circuit; 5. recorder; 6. source; 7. drain; 8. ground

## RESULTS AND DISCUSSION

### Simultaneous measurement of pH inside and outside of the BR-liposomes

Figure 5 shows that an experimental result concerning simultaneous changes of both the external pH and the internal fluorescence intensity of the BR-liposomes. The results indicates that the inner liquid became acidic in the light. The pH difference between the inner and outer liquids at the steady state II was within 1 pH unit, although it varied according to the light intensity, the BR concentration, and the temperature (data not shown). The reproducibility of the experimental results was good when the intensity of the actinic light was changed within the range adopted in this experiment. However, when the light intensity exceeded a critical value (about 30 mW $m^{-2}$), the pH difference at the steady state II sometimes decreased in successive experiments, even though the experimental conditions were the same. It was thought that the electropotential difference across the membrane of the BR-liposomes was negligible in comparison with the chemical potential difference, because the ratio of valinomycin to PC was $1.5 \times 10^{-3}$ g/g [18]. On addition of an excess amount of gramicidin as proton channels to the suspension of the BR-liposomes, the fluorescence difference generated in the light disappeared (data not shown), as reported by Bell and coworkers [3].

### Rates of proton pump and leak

Two examples of the time dependent pH changes of the inner and outer liquids of the

BR-liposome system on the shifts from the dark to the light and the reverse are shown in Fig. 6. The simulation curves, which were calculated from Eqs. (7) and (8), represent the experimental tendency well although the parameters of $Q_L$ and $Q_D$ used were obtained by fitting to the experimental result with the method of least square. The value of $P^*$ obtained from Eq. (9) using the estimated values of $Q_L$, $A$, $B_e$, and $V_e$ increased with the intensity of light, as shown in Fig. 7. Values of $k_L$ and $k_D$ in Fig. 8 were obtained from Eqs. (7) and (8) using the estimated values of $Q_L$, $Q_D$, $S$, $V_i$, and $B_i$. The values of all parameters used in the simulation were obtained from the literatures of [17]. The values of $k_D$ were constant irrespective of the light intensity in the previous light condition. The values of $k_I$ increased to a certain extent with the light intensity.

We made the simulation model as regarding to be the proton pump activity constant. The inhibition coefficient $k_I$ might be composed of two parts: the back pressure, of which the effect was independent of the light intensity, and the actinic light-induced open channels of the BR. The existence of the back pressure effect of the BR was demonstrated from the result that the interpolated value of $k_I$ to zero light intensity had a definite value (Fig. 8).

**Typical photoresponse of the BR-ISFET**

A typical photoresponse of the modified ISFET is shown in Fig. 9. The increase of 'Δ' in Fig. 9*(A)*) was observed as soon as illumination started. The continuous increase (so called at the steady state II in Fig. 1 and indicated as 'α' in Fig. 9*(A)*), which leveled off in 25 min (data not shown), was followed during the illumination. A rapid decrease of potential was observed when the illumination was stopped. The potential difference of the decrease was almost the same as that of the initial increase. Following the rapid decrease after stopping the illumination, the potential decreased gradually to the initial value. This photo-induced response pattern was obtained repeatedly for more than 5 h after the preparation of the ISFET. To estimate the contribution of BR to the potential response, 200 mM hydroxylamine hydrochloride (HH), which bleaches BR [8], was added to the buffer solution of the experiment cell. In this modification, the extent of the initial rapid increase was reduced as shown in Fig. 9*(B)* and the gradual increase could no longer be seen. It was estimated that the inherent photo effect of the ISFET was about 80 % of the observed initial response.

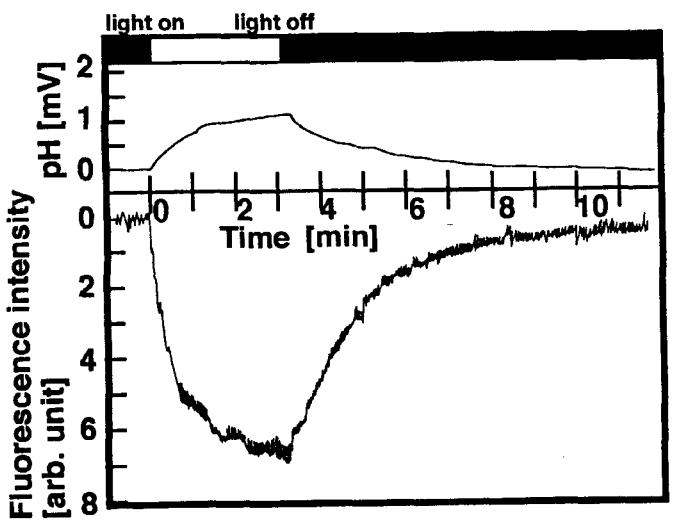


Fig. 5. Experimental recording of transient changes of both fluorescence intensity of inner liquid and pH of outer liquid of BR-liposomes. BR conc. =0.032 g/l; PC conc. 10.0 g/l; valinomycin conc. =15 mg/l.

Figure 10 shows the effect of the breaking time (less than 1 s of illumination), which was much shorter than the time constant of the gradual decrease. Therefore, a temporary decrease of the potential was observed. The extent of this potential decrease was the same as the value of the rapid initial increase and which recovered completely after illumination was restarted. Figures 9*(A)* and 10 suggest that at least two mechanisms exist in this system; a rapid one and a gradual one. Kagawa et al. [12] suggested that both a membrane potential and a gradient of proton across the membrane were formed during illumination in the BR-containing vesicle system. According to the model proposed by Drachev et al. [5], the BR-carrying vesicles are

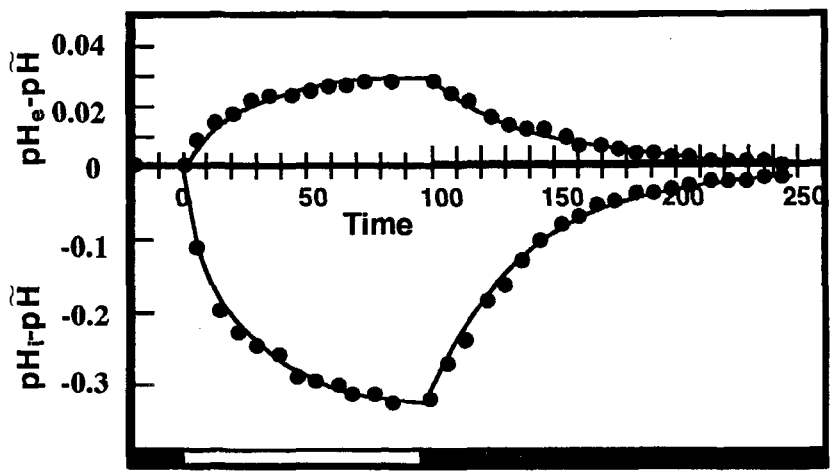


Fig. 6. Simulation curves of internal and external pH changes of BR-liposomes in the light condition and the dark condition.
$p\tilde{H}$=6.80, A=0.079, $Q_L$=92, $Q_D$=52

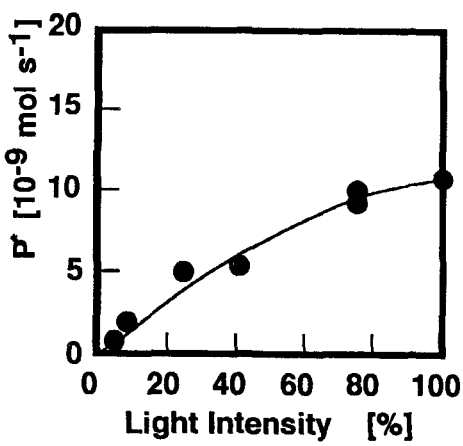


Fig. 7. Effect of light intensity on modified proton pumping activety. 100% light intensity corresponds to 30 mW $m^{-2}$.

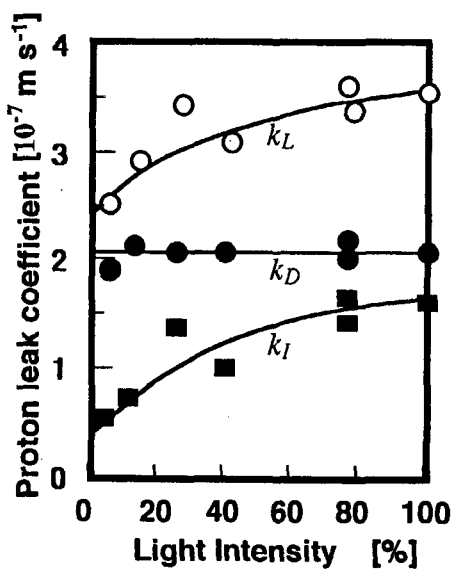


Fig. 8 Effect of light intensity of proton leak rate coefficients.

associated with the surface of the prepared ISFET which retained spherical vesicles holding the buffer solution inside. They also discussed the permeability of the protons across the intactvesicle membrane [6]. According to this, proton uptake/leakage took place between the inner phase of the associated PM-containing vesicles and the outer buffer solution, and simultaneously protons diffused across the phospholipid layer on which vesicles were associated. The membrane potential was created by the light-driven proton uptake, which was detected as the rapid response of this system. The continuous light-driven proton uptake might have caused the gradient of the proton concentration across the vesicle membrane. Accumulated protons might have diffused towards the surface of the ISFET, which were then detected as a gradual increase in the response.

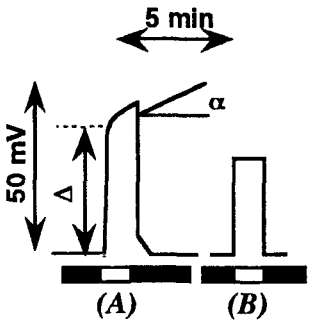


Fig. 9. Typical photoresponce of the modified ISFET *(A)* and after BR bleaching by 200 mM of HH *(B)*. ▭: light on, ▬: light off

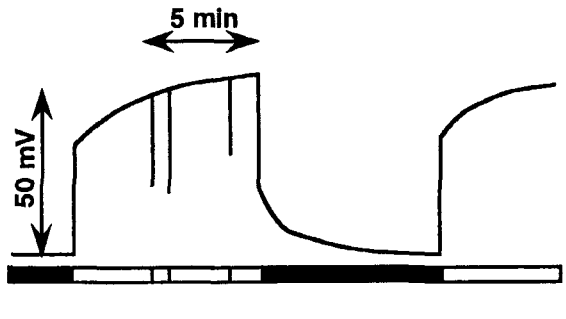


Fig. 10. Short cut of illumination. Arrows indicates that the illumination was temporarily removed for less than 1 s. ▭: light on, ▬: light off

**Effect of Illumination Intensity**

The relationship between the photoresponse of the modified ISFET and the intensity of the light was examined by changing the voltage applied the halogen tungsten lamp. Figure 11 shows that both the initial rapid increase (Δ) and the rate of gradual increase ( α) were facilitated by the light becoming stronger, to the extent of a 20 % increase between 3 x $10^4$ lux and 7 x $10^4$ lux. A trend of saturation was observed when the intensity exceeded 1 x $10^5$ lux. It might be suggested that there was a limit to the number of photons which were absorbed by the BR molecules.

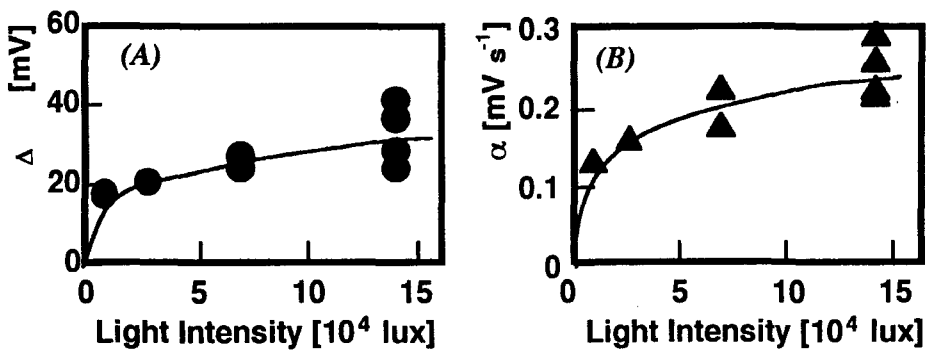


Fig. 11. Relationships of the initial rapid increase*(A)* and the gradual increase rate*(B)* of the response against the light intensity.

## CONCLUSIONS AND SCOPE

The model equations could simulate the experimental observations well, as shown in Fig. 6. In the model the four kinds of proton fluxes were suggested; the proton pump of the BR in the light, the reverse flow of proton with its back pressure, the proton leak through the light-activated defected BR, and the proton leak through the inert BR and PC.

On the other hand, The light-induced effect of the reconstituted membrane containing BR was measured by ISFET. As the system has the great advantage of an ISFET photoeffect, this method provides new prospects for using and studying the interface between biological substances including other types of membrane proteins and conventional semiconductor devices. It is hoped that this work also shows the potential for biochip development, where biological substances will be used as electronic components.

### Nomenclature

| | | |
|---|---|---|
| $A$ | buffering intensity ratio | [-] |
| $B$ | buffering capacity | [mol $m^{-3}$] |
| $C$ | electrical capacity across the liposomal membrane | [F] |
| $[H^+]$ | proton ion concentration | [mol $m^{-3}$] |
| $J_H$ | proton flux rate | [mol $s^{-1}$] |
| $k$ | rate coefficient of proton leak | [m $s^{-1}$] |
| $P$ | proton pump activity | [mol $m^{-2}$ $s^{-1}$] |
| $P^*$ | modified proton pump activity | [mol $s^{-1}$] |
| $Q$ | modified proton leak coefficient | [$m^3$ $mol^{-1}$ $s^{-1}$] |
| $R_{BR}$ | electrical response of the BR | [Ω] |
| $R_m$ | electrical response of membrane | [Ω] |
| $S$ | mean interfacial area of total liposomes | [$m^3$] |
| $V$ | volume | [$m^3$] |

<Subscripts>

| | |
|---|---|
| $D$ | in the dark |
| e | external liquid phase of liposomes |
| $I$ | inhibition of proton pumping |
| i | internal liquid phase of liposomes |
| $L$ | in the light |

<Superscripts>

| | |
|---|---|
| ^ | steady state II |
| ~ | initial state |

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