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A novel method for measuring membrane conductance changes by a voltage-sensitive optical probe

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This study presents a method whose principles enable using a voltage-sensitive optical probe, to quantitatively measure conductivity changes elicited in membrane vesicles and cells. The procedure is based on the fact that the amplitude of the transmembrane potential difference, established across a membrane by an external electric field, is decreased when membrane conductivity is increased upon incorporation of ionophores into the membrane. The method was applied to osmotically swollen thylakoid membranes whose membrane conductivity was changed by the addition of gramicidin or ionomycin. The electric field induced stimulated luminescence from photosystem I (electrophotoluminescence-EPL) was used as a voltage-sensitive optical probe. We calculated the induced conductance changes by using a calibrated EPL vs external electric field response curve and measuring the ionophore-mediated attenuation of the EPL signal. The calculated ionophore-unmodified conductance of the thylakoid membrane yields a value of 171 ± 56 nS/cm. The value of the membrane conductance, modified by 10 nM gramicidin was found to be 190 ± 56 nS/cm. The modified membrane conductance and the membrane conductance changes induced by 1 μM ionomycin in the presence of $CaCl_2$ were found to be 186 ± 3 nS/cm and 15 ± 3 nS/cm, respectively.

Conductance change; Voltage-sensitive optical probe; Electrophotoluminescence; Ionophore; Photosynthetic membrane

1. INTRODUCTION

Over the last two decades, voltage-sensitive optical probes have been used to monitor changes in the membrane potential of cells and lipid vesicles [1,2]. These probes can be divided into fast and slow responding probes, according to the speed of their response to the potential change. Photosystems I and II (PS I and PS II, respectively) of the photosynthetic membrane, which carry the light-driven electron transport process, were found to be fast and highly sensitive voltage-responding optical probes. Delayed light luminescence from these membrane complexes was found to be stimulated, by up to 3 orders of magnitude, when exposed to high external electrical fields [3–6]. The electrical field-induced stimulated luminescence (electrophotoluminescence-EPL) is elicited by precursors originating in PS I and PS II [7], reflecting enhanced rate of charge recombination. This high sensitivity of the EPL to the electric field-induced transmembrane potential difference led to the use of the light-induced charge-separation in PS I as a voltage-sensitive optical probe for monitoring ionophore-mediated ion-transfer [8]. This study made use of the fact that the amplitude of the transmembrane potential difference established across a membrane by an external electrical field was decreased when membrane conductivity was increased upon incorporation of ionophores into the membrane. An extension of this approach has recently been applied when analyzing the response of an extrinsic optical probe in a spherical lipid bilayer containing gramicidin molecules [9].

The present study presents a methodology whose principles allows one, by using voltage-sensitive optical probes, to measure quantitatively conductivity changes elicited in membrane vesicles and cells either by incorporating ionophores into the membrane or by electrically inducing conductivity changes in it by electroporation.

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2. MATERIALS AND METHODS

Broken chloroplasts were prepared from spinach, pea or tobacco according to Avron [10]. The broken chloroplasts were stored at −180°C in order to preserve their photosynthetic activity for a long period [11]. In every set of experiments the concentrated thylakoids were thawed and incubated at 50°C for 3 min (heat inactivation) in order to deplete the thylakoids from their photosystem II-related electrophotoluminescence activity [7] which is less sensitive to the electrical field [13]. An optimum time of 3 min was found to deplete most of the photosystem II-related activity while preserving the photosystem I activity. After heat inactivation the thylakoids were resuspended under hypotonic conditions. The concentration of the stored stock of broken thylakoids was 6–10 mg/ml, and they were diluted 1000-fold in 5 mM Mes-NaOH buffer, pH 5.5, or in 1 mM Tris-HCl, pH 7.4. After 15 min at room temperature, they were kept on ice. This swelling process yields spherical vesicles known as swollen thylakoids or 'blebs'. These vesicles, composed of a single membrane with occasional patches on it have a size distribution of radii of 1–10 μm, with an average radius of 4 μm. The analysis of size distribution was done by microscopic visualization.

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Gramicidin D (Dubos, Sigma Chemical Co., St. Louis, MO) was used for calibration at concentrations of 10 nM and ionomycin (Calbiochem AG, Lucerne) was used as a carrier model. The ionophores were added from concentrated ethanolic solutions (1 mM) while continuously stirring the vesicle suspension. The experimental set-up for electrophotoluminescence measurements was described elsewhere [6]. The experiment was initiated by a pre-illumination, for 120 ms with a light projector. The light was filtered by a 4-96 glass filter (Corning Glass Works, Corning, NY) limiting the exciting wavelength to approximately 400–600 nm. After a dark time of 230 ms, an external electrical field pulse was applied. The electrical field-induced luminescence (EPL) was filtered by a model RG 665 cut-off filter (Schott Glass Technologies Inc., Duryea, PA) and was monitored on a fast oscilloscope (model 2430A, Tektronix Inc., Beaverton, OR) interfaced to a compatible IBM PC computer. In all cases, the amplitude of a particular EPL signal was taken at its maximum. The electric field pulse was delivered by a high voltage pulse generator (Velonex model 360), capable of delivering voltage pulses of 200–2500 V.

3. RESULTS AND DISCUSSION

It has previously been shown [6,8] that the EPL amplitude is sensitive to changes in membrane conductance. The attenuation of the EPL due to the increase in membrane conductivity results from the decrease in the electrical field induced in the membrane. The stationary local electric field (E_m) induced in a vesicular membrane upon exposure to externally applied electric field (E_{ex}) was previously shown [6] to be:

$$E_m = \frac{3(R/d)\cos\theta}{2+3(R/d)(\sigma_m/\sigma_o)} \cdot E_{ex} \quad (1)$$

where E_m is the electric field induced in the membrane, R is the radius of the vesicle, d is the thickness of the membrane, E_{ex} is the external applied electric field, θ is the angle formed by the vector of the electric field and the radius vector of the vesicle to a certain point on the membrane where the local electric field is induced, σ_m is the specific membrane conductance, σ_o is the specific conductance of the medium, where the specific conductivities of the inner (σ_i) and the outer (σ_o) media are equal ($\sigma_i = \sigma_o$) and $R \gg d$. As it can be seen from eqn 1 the E_m induced by an externally applied electrical field is dependent on the specific membrane conductance, so that changes in it will influence the E_m, and consecutively the EPL signal which depends on it. This phenomenon was observed upon addition of gramicidin molecules to a suspension of thylakoids [6,8]. This fact can be used to make indirect measurements of changes in the membrane conductance by measuring the change in the EPL signal, provided we give a quantitative measure to the attenuation of the signal. The empirical dependence of EPL signal on E_{ex} is shown in fig.1. It shows a non-linear relation with the E_{ex}. However, between 1000 V/cm and 2000 V/cm, it could be taken as linear function of E_{ex} with a linear correlation coefficient larger than 0.95. The EPL signal is an explicit function of E_m rather than

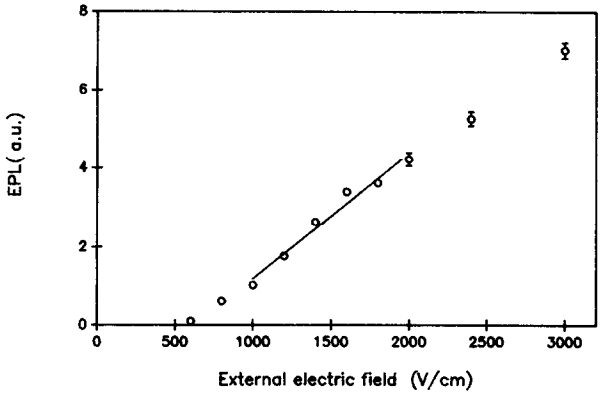

Fig.1. EPL signal as a function of the external applied electric field. Swollen thylakoids were incubated in 5 mM Mes-NaOH buffer, pH 5.5, at a temperature of 5°C. (The absence of the error bars implies that the open circle is bigger than the standard deviation.)

of E_{ex} itself. Eqn 1 can be rewritten in terms of the ratio between E_m and E_{ex}. Thus:

$$E_m = K_1 \cdot E_{ex} \quad (2)$$

where

$$K_1 = \frac{3(R/d)/\cos\theta}{2+3(R/d)(\sigma_{m1}/\sigma_o)}$$

and σ_{m1} is the specific non-modified membrane conductance (before alteration by ionophore addition). Thus the EPL signal depends on a multiplication of the E_{ex} times a factor K_1, which is related to σ_m and where its absolute value is unknown. We introduce a new parameter of an effective electric field (E_{ef}) which is defined by the following relationship:

$$E_{ef_2} \cdot K_1 = E_{ex} \cdot K_2 = E_m \quad (3)$$

where E_{ef_2} is the external electric field which would give such an EPL signal if K_2 was equal to K_1 and K_2 is defined by:

$$K_2 = \frac{3(R/d)\cos\theta}{2+3(R/d)(\sigma_{m2}/\sigma_o)}$$

and σ_{m2} is the modified specific membrane conductance altered, for example, by electroporation or by the addition of ionophore. Eqn 2 can be expressed in terms of E_{ef} as $E_m = E_{ef_1} \cdot K_1$ where $E_{ef_1} = E_{ex} \cdot K_1/K_1$. Thus E_{ef_1}, which is the effective electric field before any change in σ_{m1} has taken place, is actually equal to E_{ex}. If all parameters are constant including E_{ex}, and we can use the EPL signal dependence on E_{ex} as a calibration curve (in the linear range) we can elucidate E_{ef} directly, by matching the attenuated EPL signal with the corresponding external field. The division of E_{ef_1} (before membrane conductance alteration) by E_{ef_2} (after membrane conductance alteration) gives the following relationships:

$$E_{ef_1} = E_{ex}K_1/K_1$$

$$E_{ef_2} = E_{ex}K_2/K_1$$

Thus,

$$E_{ef_1}/E_{ef_2} = K_1/K_2$$

or

$$E_{ef_1}/E_{ef_2} = \frac{2+3(R/d)(\sigma_{m2}/\sigma_o)}{2+3(R/d)\cdot(\sigma_{m1}/\sigma_o)}$$

After rearranging, we obtain:

$$[2d\sigma_o/(3R)][(E_{ef_1}/E_{ef_2}) - 1] = \sigma_{m2} - (\sigma_{m1}E_{ef_1}/E_{ef_2}) \quad (4)$$

By measuring the attenuation of the EPL signal induced by the addition of gramicidin we can obtain an additional equation for the induced change in membrane conductance (delta σ_m)

$$\Delta\sigma_m = \sigma_{m2} - \sigma_{m1} \quad (5)$$

$\Delta\sigma_m$ can be estimated from the contribution of gramicidin to the membrane conductance change (see below). We can elucidate the values of σ_{m1} and σ_{m2} by the simultaneous solution of eqn 4 and eqn 5 yielding:

$$\sigma_{m1} = [(2d\sigma_o/3R)[(E_{ef_1}/E_{ef_2}) - 1] - \Delta\sigma_m]/[1 - (E_{ef_1}/E_{ef_2})]$$

$$\sigma_{m2} = \sigma_{m1} + \Delta\sigma_m \quad (6)$$

Once we calculate the value for σ_{m1} we can further calculate any ionophore-induced conductivity change by inserting the experimental E_{ef_1} and E_{ef_2} values into eqn 4.

The estimation of the average change in membrane conductance of a suspension of swollen thylakoids upon addition of gramicidin was carried out by calculating the average number of gramicidin channels per vesicle. Due to the highly hydrophobic character of gramicidin, one can expect that all the added ionophore ends up in the thylakoid membrane. In this case we assume an equal concentration distribution of gramicidin among all vesicles. Addition of gramicidin (at concentration of 10 nM) yields number of active channels equal to the half of the added gramicidin molecules [14]. Thus the specific conductance change induced by the addition of gramicidin is given by the following relationship:

$$\Delta\sigma_m \text{ (gramicidin)} = n\cdot\gamma\cdot d/A$$

where n is the total number of gramicidin channels, γ is the single channel conductance, A is the total area of the vesicles and d is the thickness of the membrane. The study was carried in a medium of low ionic strength containing ~3 mM sodium. The single channel ion conductance at this concentration was found to be 0.28 pS by extrapolation based on the experimental data of Neher et al. [15]. Furthermore since the attenuation of membrane conductivity by gramicidin was studied under high transmembrane potential we have taken into account the voltage dependence of gramicidin [16] resulting in a single channel conductance of ~0.085 pS (for 500 mV transmembrane voltage difference). It should be stressed that though there may exist some error in the calculated absolute values of membrane conductances, due to the various approximations used, the influence of such an error does not affect much the calculated conductance change.

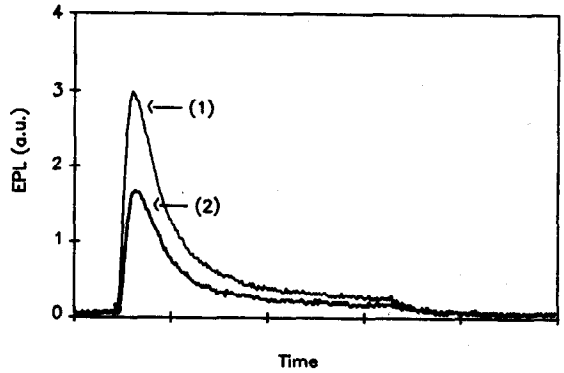

Fig.2. Gramicidin induced attenuation of the EPL signal. The EPL was induced by an external electrical field of 1600 V/cm which was applied during 200 μs. Conditions are the same as in fig.1. The time base is 70 μs/div. (Curve 1) Control (in the absence of ionophore). (Curve 2) In the presence of 10 nM gramicidin.

The calculated, ionophore-unmodified, membrane

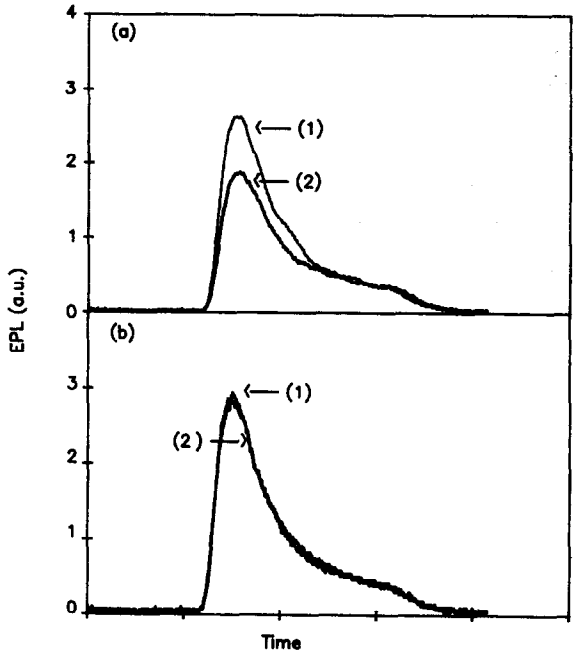

Fig.3. Ionomycin induced attenuation of the EPL signal. Swollen thylakoids were incubated in 1 mM buffer Tris-HCl, pH 7.4, at a temperature of 10°C; the EPL was induced by an external electric field of 1000 V/cm which was applied during 100 μs. The time base is 50 μs/div. (Curve 1) Control (in the absence of ionophore). (Curve 2) In the presence of 1 μM ionomycin. (a) In the presence of 0.5 mM $CaCl_2$ in the medium. (b) In the presence of 0.5 mM $MgCl_2$ in the medium.

conductance yields a value of 171 ± 56 nS/cm. This value of σ_{m1} enables us by using eqn 4 to calculate the ionophore-modified membrane conductance (σ_{m2}) for any ionophore. We applied this approach to study the conductance changes induced by ionomycin, a calcium-specific ion carrier. The attenuation of the EPL signal by gramicidin is shown in fig.2. The attenuation of the EPL signal mediated by ionomycin in the presence of $CaCl_2$ and $MgCl_2$ is shown in fig.3a and b. It can be seen that addition of ionomycin in the presence of $CaCl_2$ caused a significant attenuation in the EPL signal; however, no significant attenuation of the EPL was observed in the presence of $MgCl_2$. These results are in accordance with the high specificity of ionomycin towards calcium [17]. The calculated value of the membrane conductance, modified by 10 nM gramicidin, as calculated from the attenuation in the EPL signal, was found[1] to be 190 ± 56 nS/cm. This value is of the same order of magnitude when compared to the value of 80 nS/cm found by measuring the rotation of a single swollen thylakoid vesicle in a rotating electric field under similar gramicidin concentration [18]. The modified membrane conductance and the membrane conductance changes induced by ionomycin in the presence of $CaCl_2$ were calculated to be 186 ± 3 nS/cm and 15 ± 3 nS/cm, respectively.

In summary, we describe a quantitative method for measuring by optical methods membrane conductance changes mediated either by ionophores or by electroporation. The method, based on the use of a voltage-sensitive optical-probe, can be extended to the measurement of conductance changes in cells and vesicles. This can be carried out by employing one of the available external voltage-sensitive optical probes [1,2].

[1] The standard deviation around the average of the σ_{m1} and σ_{m2} values results from the high variation among the different experiments. However, the variation of $\Delta\sigma_m$ is much smaller (17.7 ± 0.5 nS/cm for 10 nM gramicidin.

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