BRIEF COMMUNICATION

MEMBRANE POTENTIAL INDUCED BY EXTERNAL ELECTRIC FIELD PULSES CAN BE FOLLOWED WITH A POTENTIOMETRIC DYE

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ABSTRACT A potential-sensitive dye was recently used to measure the spatial variation in the membrane potential induced by an externally applied electric field. In this work, we demonstrate that the time course of these induced potentials can also be followed. Two experimental systems were explored. Dye fluorescence from HeLa cells could be modulated by a train of field pulses; the relative fluorescence change measured with a lock-in amplifier was linear with the field and similar to the fluorescence responses obtained in the static measurements. A model membrane system consisting of a hemispherical bilayer allowed convenient measurement of the dye absorbance change as a function of the bathing solution conductivity. The charging time of the membrane was inversely related to the aqueous conductance as predicted by the theoretical solution to Laplace's equation.

INTRODUCTION

External electric fields can produce a variety of profound biochemical or physiological changes in cells, tissues, organs, or whole animals. Applications include direct gene transfer into animal (Neumann et al., 1982; Potter et al., 1984) and plant (Fromm et al., 1985; Fromm et al., 1986) cells, monoclonal antibody production (Lo et al., 1984), and drug delivery (Muehlig et al., 1984; Zimmermann et al., 1980). At the cellular level, simple electrostatic theory suggests that the interior of the cell is essentially fixed at a uniform potential, with the entire voltage drop across the cell experienced by the insulating shell surrounding it, the plasma membrane. Any consequences of the application of an electric field (e.g., dielectric breakdown, cell fusion, cell permeabilization) (Zimmermann et al., 1981) must, therefore, be traced back to the induced change in the membrane potential.

The spatial and temporal variation of the membrane potential induced by an external field can be calculated for ordinary cell geometries such as spheres or elipsoids by solving Laplace's equation with the appropriate boundary conditions. Eq. 1 is a solution of this problem for a spherical geometry:

$$V_m = \frac{9\lambda_o\lambda_i\,[1 - e^{-t/RC_m((2\lambda_o\lambda_i/2\lambda_o+\lambda_i)+\lambda_m(R/d))}]\,Ed\cos\theta}{2(\lambda_o + \lambda_m)(2\lambda_m + \lambda_i) - 2(\lambda_o - \lambda_m)(\lambda_i - \lambda_m)[(R - d)/R]^3}, \quad (1)$$

where V_m is the local transmembrane voltage drop, λ_m, λ_i, λ_o are the specific conductivities of the membrane and inner and outer media, respectively, R is the cell (particle) radius, d is the membrane (dielectric shell) thickness, C_m is the membrane capacitance, E is the externally applied electric field intensity, and θ is the angle between the applied field direction and the normal to the membrane plane at the point V_m is measured. A simpler form of Eq. 1, in which the membrane conductivity was assumed to be sufficiently low to permit simplification of the exponential, was employed by Farkas et al. (1984) in their analysis of the time course of electrophotoluminescence from a suspension of photosynthetic blebs. They were able to obtain values for the membrane capacitance that were in agreement with more traditional electrical measurements.

To verify that the cell membrane can be treated as a simple insulating shell and to map out membrane potentials induced on cells or cell clusters with more complex

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geometries, an experimental method based on a membrane-staining potentiometric dye and a digital video microscopy system was developed (Gross et al., 1986). The ability of the molecular probe to detect local electric fields together with the data manipulation capability of the imaging system, permits a detailed analysis of the spatial variation of the membrane potential at the resolution limit of an optical microscope.

In this paper, we show that the potentiometric dyes can follow the potential changes induced by electric field pulses that are too fast for the relatively long acquisition times (30 ms) necessitated by the video technology employed previously. Thus, a general method, applicable to any cell type, for following and mapping membrane potentials induced by external electric fields, is made available.

MATERIALS AND METHODS

The hemispherical bilayer system employed previously in this laboratory to characterize the potentiometric responses of dyes (Loew, 1982; Loew and Simpson, 1981) was modified so that a square wave applied to a pair of parallel Pt plates flanking the hemispherical bilayer served to supply an external field (Fig. 1). The optics and electronics were otherwise identical except that a waveform analyzer (model 6000; Data Precision, Danver, MA) was used to determine the time course of the optical signal triggered by the train of square voltage pulses. A Pt wire probe electrode suspended just above the bilayer provided a convenient reference for the second channel of the waveform analyzer. The KCl solutions bathing the bilayer were prepared from double distilled deionized water and their conductivity determined with an impedance bridge (model 1650-B; Gen Rad, Inc., Concord, MA). Di-4-ANEPPS (synthesized according to Hassner et al., 1984) was included in the outer bathing solution at the indicated concentrations; the membrane was stained within the time required for thinning to a bilayer (~15 min).

HeLa cells were grown to ~25% confluency in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) containing 10% fetal calf serum on 22 × 30 mm glass coverslips. The cells were stained with a 10-μM solution of di-4-ANEPPS in Earle's balanced salt solution (Gibco) at room temperature for 10 min. The excess dye was washed away and the coverslip mounted on the electric field chamber described by Gross et al. (1986). Electric field pulses of up to 100 V/cm were applied to the chamber with a power supply (model BOP-500M; Kepco, Inc., Flushing, NY) controlled by a 10-V square wave from a function generator (model 3010, B & K Precision, Chicago, IL). Fluorescence was excited at 546 nm and detected at >610 nm with a Universal microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with incident light fluorescence and with a photometer containing a variable aperture rectangular measuring diaphragm. The diaphragm was set to measure the fluorescence from a small patch of the cell membrane that was approximately perpendicular to the field direction (i.e., θ in Eq. 1 is 0° or 180°). The signal detected by a photomultiplier tube (model PF1011; RCA, Lancaster, PA) was amplified and sent to a lock-in amplifier (model 5203; EG & G PAR, Princeton, NJ) which was referenced to the aforementioned square wave.

RESULTS AND DISCUSSION

The potentiometric probe di-4-ANEPPS (Fluhler et al., 1985; Hassner et al., 1984) was used to measure the potential induced by a train of electric field pulses on both cells under a microscope and on a model membrane in a spectrophotometer cuvette. In the former, modulation of dye fluorescence was determined, and in the latter experiments, the transmitted light intensity was monitored.

The linearity of the dye response with applied electric field intensity was established for several cell types by Gross et al. (1986) by using the digitized fluorescence from video images. The fields were applied for periods of 3 s while incoming video frames were averaged. In this work, to demonstrate that an AC field can evoke an equivalent

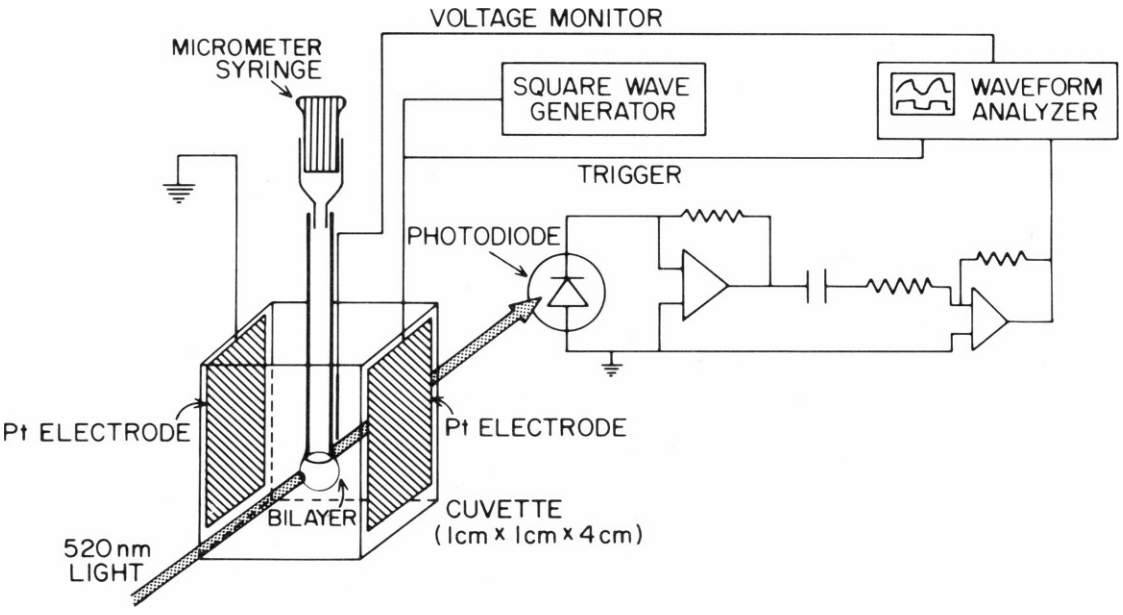

FIGURE 1 Experimental arrangement for measuring the membrane potential induced by a uniform electric field across a hemispherical lipid bilayer. Light from a monochromator is focused on one side of the bilayer so that ~0.1 of its surface area is illuminated. The transmitted light is detected with a photodiode and the AC-coupled signal sent to the waveform analyzer, which is triggered by the same square wave generator used to supply electric field pulses to the membrane.

optical response, we used a photomultiplier tube as a fluorescence detector on the microscope and employed a 250-Hz square wave symmetric about ground to apply the field. The output of the detector was sent to a lock-in amplifier, which was referenced to the field.

HeLa cells were stained with di-4-ANEPPS and the relative fluorescence response from a small patch of membrane perpendicular to the field direction was determined by comparing the total signal from the photomultiplier to the differential signal recorded by the lock-in amplifier. The amplitudes of the induced membrane potentials were calculated from the preexponential part of Eq. 1 and plotted against the relative fluorescence change in Fig. 2. The slope of the plot is 9.5%/100 mV, within experimental error of the sensitivity of the dye determined on several cells using the video system (Gross et al., 1986) and on a voltage clamped hemispherical bilayer (Fluhler et al., 1985). The lock-in amplifier thus provides a convenient and rapid readout of membrane potential changes. In addition, since the rate of membrane charging would be reflected in a shift in the phase of the measured signal relative to an externally applied electric field, the method can provide kinetic information interpretable via Eq. 1.

The "hemispherical" bilayer allowed a more direct determination of the time course for the development of the membrane potential. Since its geometry is closer to spherical than hemispherical (Fig. 1), the data can be treated safely with Eq. 1. Moreover, the bilayer can be bathed in any electrolyte and can include ionophores or other membrane modifiers; thus, well controlled biophysical studies are permitted. Either fluorescence or transmittance responses to membrane potential can be monitored (Loew, 1982). We chose to concentrate on the transmitted light response of di-4-ANEPPS because of the simpler optical arrangement in electric field experiments with opaque electrodes (Fig. 1).

The response to an oscillating electric field could be detected either with the lock-in amplifier or by signal averaging. The sign of the response depends on whether the beam is focused on the face of the bilayer closer to the positive or ground electrode; no potentiometric signal is obtained from the bottom of the bilayer as predicted from the cosine dependence in Eq. 1. The size of the response was typically 2×10^{-4} of the total transmitted light signal at 520 nm for a 100-mV membrane potential but is much less consistent than relative fluorescence changes (Loew, 1982). Once again, the dye response was linear in membrane potential (Fig. 2). This is a necessary condition if one

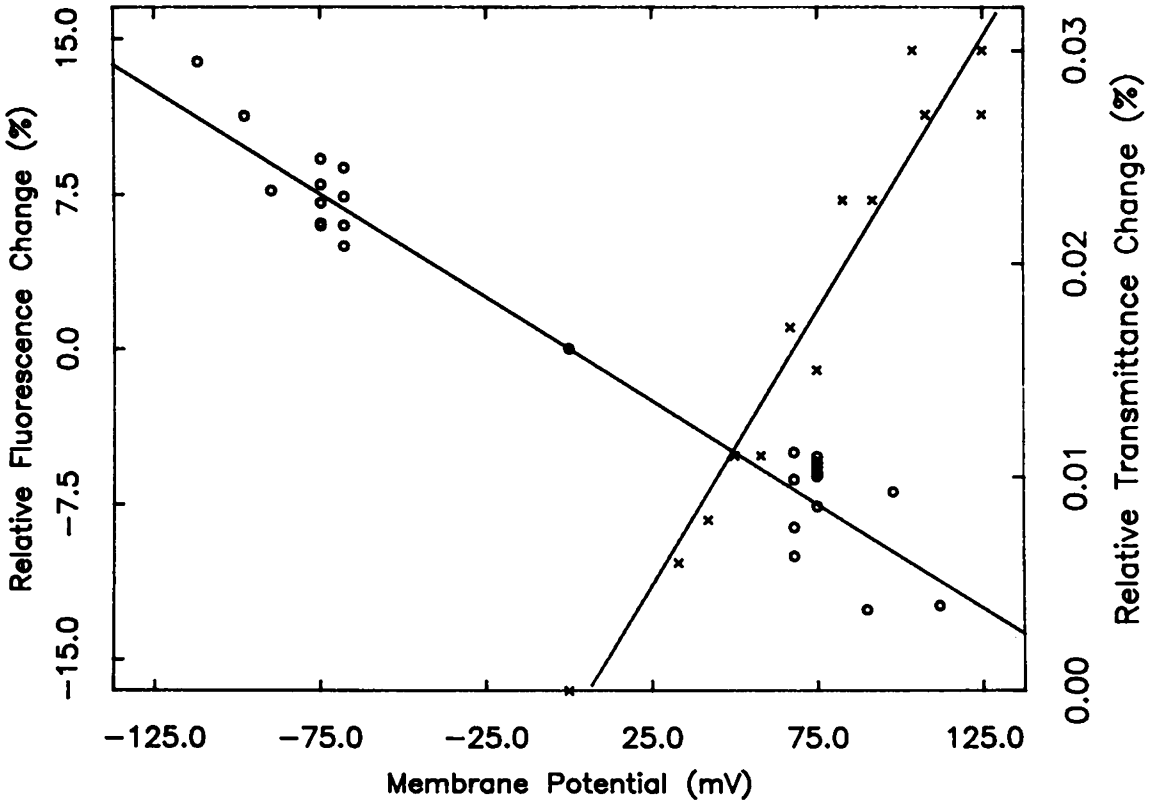

FIGURE 2 Linearity of the dye response on HeLa cells (O, fluorescence; data from five different cells) and hemispherical bilayer (×, transmittance). V_m was calculated from the steady-state form of Eq. 1 ($t = \infty$) at $\theta = 0°$ and 180°, assuming $\lambda_o = \lambda_i \gg \lambda_m$ (cf. Farkas et al., 1984). The value of R was determined with the aid of a stage micrometer for the HeLa cells and a stereomicroscope fitted with a calibrated eyepiece graticule for the hemispherical bilayer. The external field applied to the cells was measured via a pair of Pt electrodes flanking the coverslip as in Gross et al. (1986). The field on the bilayer was taken directly from the voltage on the Pt plates. *Solid lines*, least squares fits to the respective data.

is to use the response to measure the kinetics of the development of the potential. Fig. 3 shows the optically detected time course of the membrane potential when a 6-kHz bipolar square wave of 0.9 V/cm amplitude is applied to a bilayer of 3-mm diam. in 100 mM KCl. Since the hemispherical bilayer can last for up to 24 h, long averaging times required for this experiment are possible. A lower time resolution was sufficient for most experiments, permitting a smaller effective frequency bandwidth in the detection electronics; thus, data of quality comparable to Fig. 3 could usually be obtained with <1,000 sweeps.

The optical signal rises with a single exponential as predicted by Eq. 1. Since the system is adequately described by a *RC* equivalent circuit, the time constant is determined by the solution conductivity (11 μs for the experiment in Fig. 3). The kinetics are independent of dye concentration but do depend on [KCl] since this controls λ in Eq. 1. The slope of a plot of membrane charging rate vs. electrolyte conductivity (Fig. 4) allows us to obtain the membrane capacitance. From Eq. 1, $t^{-1} = 2\,\lambda/3\,RC_m$, assuming λ_m is insignificant and $\lambda_o = \lambda_i = \lambda$. R was adjusted to 1.5 mm for each determination of t^{-1} as determined with a stereo microscope mounted in front of the electric field cuvette. The value obtained, 0.71 μF/cm^2, is within the range reported for direct electrical measurements on this membrane (Tien, 1974).

We have demonstrated that the voltage drop produced by a train of external field pulses across the membrane of a cell or model system can be followed with a voltage-sensitive dye. Since the dye is sensitive to the electric field at a molecular spatial resolution, it can detect local potentials induced by the external fields; these integrate to zero over the entire membrane surface and are not, therefore, amenable to microelectrode measurements. Indeed, the

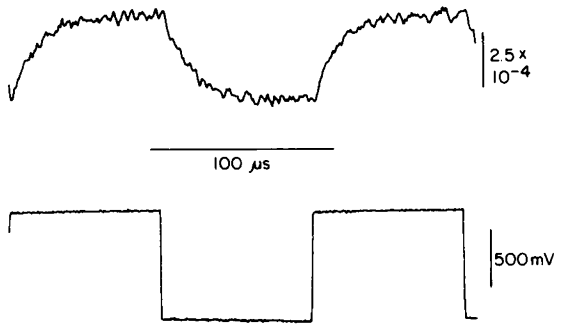

FIGURE 3 Membrane potential induced on a hemispherical bilayer at 500-ns data acquisition time. The time constant of the electronics/photodiode combination is <1 μs. A hemispherical bilayer in 100 mM KCl is stained with a saturated solution of di-4-ANEPPS (~0.3 μM). The AC-coupled transmitted light signal averaged over 70,000 sweeps is displayed (*top*) with a calibration bar indicating the relative change. *Bottom*, the voltage measured with a probe electrode near the bilayer. The applied field had a peak to peak amplitude of 1 V/cm symmetric about ground, corresponding to a membrane potential oscillating between −120 and +120 mV in the illuminated area.

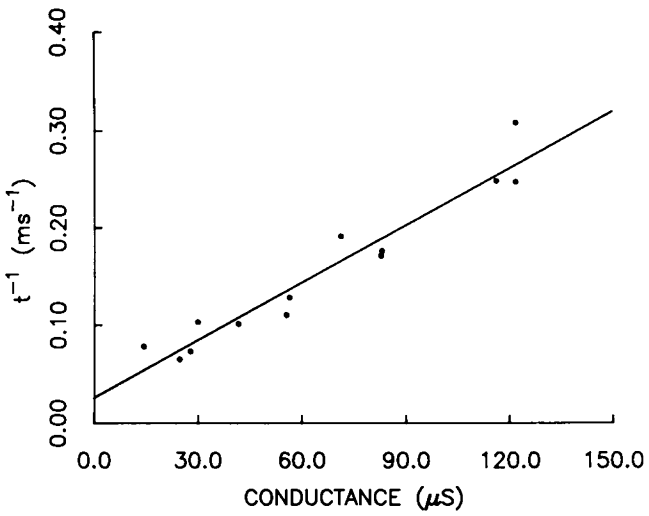

FIGURE 4 Linear dependence of the rate constant for charging of the bilayer membrane on the conductivity of the bathing solution.

simplicity and flexibility associated with the application of external fields should prompt the development of new electrophysiological applications now that the method for measuring the membrane potential has been established. For example, the risetime of the potential in cells can be extremely fast since it will take less time to charge up a membrane of smaller surface area (submicrosecond for cells <100-μm diam.); therefore, by allowing λ_m in Eq. 1 to be a function of V_m, one could model the behavior of voltage gated channels with a temporal resolution much greater than that permitted by patch-clamp techniques.

Perhaps the most important impact of this work will be in the direct study of external field effects on cells. The extraordinarily important new applications for external field techniques (e.g., cell fusion, electroporation) have been largely based on purely empirical studies of the effects of field strength, pulse duration or frequency, etc., on the desired biological transformation. In many applications, optimization of these parameters was not even considered. We believe that the availability of the optical techniques described herein and by Gross et al. (1986) make it possible to define these parameters and thus ultimately promote the development of new and better applications of the external field methods.

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