Dual-Wavelength Ratiometric Fluorescence Measurements of Membrane Potential†

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ABSTRACT: This work shows that the voltage across membranes in two very different preparations, lipid vesicles in suspension and individual HeLa cells under a microscope, is linearly related to the ratio of fluorescence excited from the two wings of the absorption spectrum of a voltage-sensitive dye. The dye di-4-ANEPPS [1-(3-sulfonatopropyl)-4-[β-[2-(di-*n*-butylamino)-6-naphthyl]vinyl]pyridinium betaine] is well characterized from earlier investigations and responds via a rapid (less than millisecond) spectral shift to membrane potential changes. The resultant small change in fluorescence intensity monitored at a single wavelength is useful for measurements of temporally well-defined voltage transients such as action potentials. The dual-wavelength approach described in this work extends the usefulness of this fast potentiometric dye by filtering out complex or artifactual changes in fluorescence intensity and providing a voltage-dependent signal that is internally standardized. Thus, rapid measurements of membrane potential are made possible in nonexcitable cells.

Fast fluorescent indicators of membrane potential have been developed for applications involving excitable cell and tissue preparations (Cohen et al., 1974; Ross et al., 1977; Gupta et al., 1981). They are well suited to measuring transient voltage changes, such as action potentials, but have only rarely been applied to nonexcitable cells [Freedman and Novak (1983) and Gross et al. (1986) are noteworthy and possibly unique exceptions]. The primary reason for this is that the voltage changes of interest in nonexcitable cells are often small—typically 10 mV—and can only be measured with much more sensitive, but slowly responding, redistribution dyes (Freedman & Laris, 1981; Waggoner, 1979, 1985; Freedman, 1988). Fast dyes generally have relative fluorescence changes in the range of 1–20%/100 mV—much too low to give reliable readings of small changes. Also, variable staining and the interference of artifactual changes in fluorescence intensity have made it difficult to quantitate even large fast potential changes; the dyes are primarily used to indicate the passage of electrical transients rather than to measure their amplitudes.

We have developed a series of fast potentiometric dyes that undergo a small spectral shift in response to voltage changes across a lipid bilayer membrane (Fluhler et al., 1985) via a putative electrochromic mechanism (Loew et al., 1978, 1979). For the best of these dyes, di-4-ANEPPS,[1] this shift is enough to produce a 10%/100 mV fluorescence change at the optimal combination of excitation and emission wavelengths. Since the dye fluorescence is extremely sensitive to environment (e.g., binding to membrane causes a 100-fold increase in fluorescence quantum yield), the emission intensity can never directly measure membrane potential and has been used primarily to monitor potential transients; i.e., the scope of its usefulness is similar to that of other fast dyes. But the spectral shift has never been fully exploited.

The success of dual-wavelength ratiometric methods for measurements with fluorescent cation indicators (Grynkiewicz et al., 1985; Tsien & Poenie, 1986; Rink et al., 1982) is largely due to the ability of this approach to eliminate artifactual variations in total dye fluorescence from the assay. Thus cells in suspension can be assayed without concern for small variations in the level of dye loading from sample to sample. More importantly, optically heterogeneous specimens, such as single cells under the fluorescence microscope, can have their cation distributions mapped via digital ratio imaging (Tsien & Poenie, 1986). The avalability of several commercial dual-wavelength fluorescence spectrometers and imaging systems has made this technology accessible to many laboratories. While dual-wavelength differential absorbance techniques have been occasionally applied to potentiometric indicators (Freedman & Hoffman, 1979; Bashford et al., 1979, and references cited therein), dual-wavelength ratiometric fluorescence measurements have not been previously implemented for membrane potential. Clearly, this approach should be applicable to a dye that undergoes a potential-dependent spectral shift, especially given the need to extract a small response from intensity fluctuations that are likely to be large. In this work, we present results with di-4-ANEPPS showing that the ratio of fluorescence excited at two wavelengths is a reliable measure of membrane potential.

Experimental Procedures

Lipid Vesicles. Solutions were buffered with 20 mM HEPES/Tris, pH 7.0 (22 °C), and contained either 100 mM K_2SO_4 (high K buffer) or 300 mM sucrose and 1 mM K_2SO_4 (low K buffer). Lipid vesicles were prepared by sonicating 20 mg of egg phosphatidylcholine (Sigma Chemical Co., St. Louis, MO; type XI-E) with 1 mL of the appropriate buffer under argon to clarity by using a cylindrical bath sonicator (Laboratory Supplies, Hicksville, NY). For fluorescence measurements, 30 μL of the vesicle suspension was diluted into 3 mL of buffer containing 0.5 μM di-4-ANEPPS (Hassner et al., 1984). The ratio of the high and low K buffers was varied to set different concentration gradients across the vesicle membrane. Upon addition of 1.8 μM valinomycin (Aldrich,

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[1] Abbreviations: di-4-ANEPPS, 1-(3-sulfonatopropyl)-4-[β-[2-(di-*n*-butylamino)-6-naphthyl]vinyl]pyridinium betaine; EBSS, Earle's balanced salt solution.

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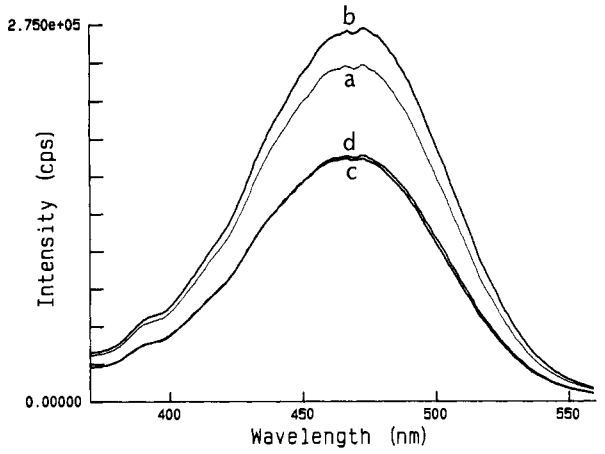

FIGURE 1: Excitation spectra of lipid vesicles containing 0.5 μM di-4-ANEPPS. (a) A 20 mg/mL stock solution of lipid vesicles containing 100 mM K_2SO_4 was diluted 100-fold into a cuvette containing a solution of the dye, 1 mM K_2SO_4, and 297 mM sucrose. The emission wavelength was 610 nm. (b) As in (a) but after addition of 1.8 μM valinomycin. (c) The buffers used in (a) are reversed so that $[K^+]$ is 2 mM inside the vesicles and 200 mM outside. (d) As in (c) but after addition of 1.8 μM valinomycin.

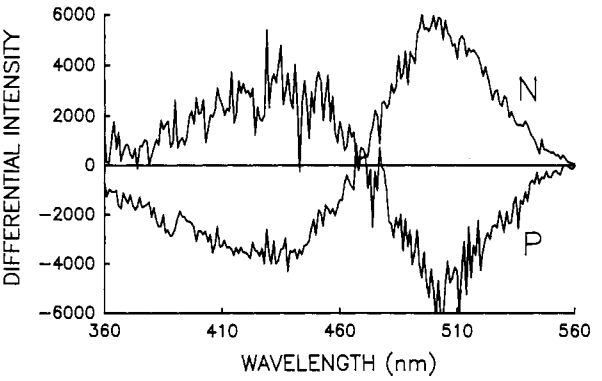

FIGURE 2: Potential-dependent wavelength shifts revealed by normalized difference spectra. The spectra in Figure 1 were normalized to the same integrated intensity (determined from the area under each curve). The difference between these spectra obtained after and before valinomycin addition is displayed. The curves labeled N and P correspond to postvalinomycin potentials of −128 and +128 mV, respectively.

Milwaukee, WI), a K^+ diffusion potential V is generated as given by the Nernst equation: $V = -(RT/F) \ln ([K^+]_{in}/[K^+]_{out})$. Because no other alkali cations are present, the Nernst equation is sufficient for the description of the membrane potential in this system. The fluorescence of the vesicle suspension was measured with a Spex CM dual-wavelength fluorescence spectrometer (Spex Industries, Edison, NJ). Conventional corrected emission or excitation spectra can be obtained with this instrument, which also has the ability to monitor emission excited from two excitation monochromators; excitation is rapidly alternated between the two excitation wavelengths via a 400-Hz chopper.

Cells in an Electric Field. HeLa cells were cultured in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) and plated onto 22 × 30 mm glass cover slips. Before use, cover slips were washed several times with Earle's balanced salt solution (EBSS) (Gibco), cooled to 4 °C, and stained for 7 min with a 2.5 μM solution of di-4-ANEPPS in EBSS, which also contained 0.05% Pluronic F127 (a kind gift of BASF, Inc., Wyandotte, MI). The cover slip was then washed again with fresh EBSS and mounted on the electric field chamber described by Gross et al. (1986). Fluorescence was imaged from the cells with a 100× NA 1.30 objective on a Leitz Ortholux II fluorescence microscope (Leitz, Rockleigh, NJ) upon which was mounted a silicon-intensified target camera (Model SIT-66; Dage MTI, Michigan City, IN). The video output was digitized and analyzed with an image processor (Recognition Technology, Westborough, MA). Field pulses, applied via a bipolar power amplifier (Model BOP 500M; Kepco, Flushing, NY), were synchronized to the data acquisition and opening of fluorescence excitation shutters via an 80286-based microcomputer (Standard 286; Compuadd, Austin, TX). Typically, a sequence of three images was collected with field positive, no field, and field negative; each image was the average of 16 video frames (0.5 s), and cells were exposed to both field and exciting light for the minimum amount of time to obtain a stable image (0.6 s/image).

RESULTS

Lipid Vesicles. Figure 1 displays four excitation spectra of lipid vesicle suspensions containing 0.5 μM di-4-ANEPPS. The top pair is before and after addition of valinomycin to a suspension in which the ratio of K^+ inside to outside the vesicles is 100:1, and the lower pair is for a suspension in which the ratio is reversed; thus, valinomycin induces K^+ diffusion potentials of −118 and 118 mV, respectively. Clearly, the effect of valinomycin is not simply a spectral shift, and the effect is different in the two suspensions. This is partly because of a potential-dependent shift in the emission spectrum [data not shown but see Fluhler et al. (1985)], which manifests itself as a change in intensity at the fixed emission wavelength used for the excitation scan. Another, in this case artifactual, contributor to the overall change in intensity is direct interaction of valinomycin with the dye, which is evident when the potassium gradient across the vesicle membrane is set to 0 (data not shown). This effect appears to be larger for the vesicles prepared in high K^+ buffer.

The wavelengths with the maximum positive and negative changes resulting purely from the shift in excitation spectrum were 440 and 505 nm, respectively. This was determined by normalizing the areas of the excitation spectra obtained at 0 and −118 mV to the same integrated intensity and then finding the maximum and minimum in the difference between the (Figure 2). The ratio of fluorescence excited by these two wavelengths was recorded for a series of vesicle suspensions containing varying potassium gradients as described under Experimental Procedures. The fluorescence ratios were all similar prior to addition of valinomycin (despite the differences in intensity evident in Figure 1) but separated in proportion to the membrane potential after the addition (Figure 3). The stabilities of the postvalinomycin ratios attest to the stability of the potential generated by the valinomycin-mediated K^+ diffusion. The slope of the correlation shown in the inset to Figure 3 is 0.09/100 mV. Significantly, the ratios determined for the two null potential cases—no concentration gradient across the vesicle membrane, but with 2 or 200 mM K^+, respectively—were identical (both points are plotted in the inset to Figure 3 but appear superimposed); clearly the dye spectrum is not shifted by either valinomycin or potassium but only by the membrane potential. The reproducibility of these measurements was better than 0.01 ratio unit, so that potentials can be determined with a precision of 10 mV. Figure 4 shows a record obtained with wider slits and a shorter data interval to try to determine the time course of the change; the rise time of the change is within the limits of the mixing time (determined by injecting an ethanol solution of a fluorescent dye into a stirred cuvette containing the vesicle buffer).

Cells. It has been shown that di-4-ANEPPS can be used to map out the membrane potential induced by an external

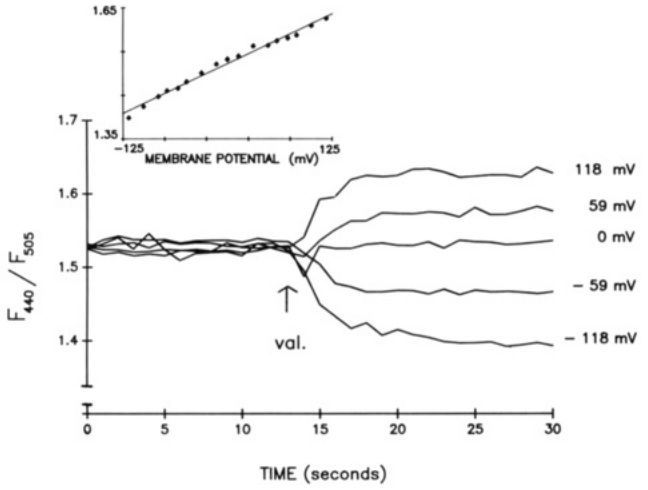

FIGURE 3: Ratio of fluorescence excited at 440 and 505 nm for a series of vesicle suspensions containing various K^+ concentration gradients. Valinomycin is added at 15 s, and the resultant membrane potentials are indicated to the right. The inset shows a plot of the fluorescence ratios averaged over the last 10 s for a series of 18 such experiments as a function of the membrane potential. The line is the result of a linear regression fit (r = 0.995). [Di-4-ANEPPS] = 0.5 μM; emission wavelength = 610 nm; excitation slits set at 1.0 mm; emission slits set at 3.0 mm; 0.3-s integration time, 1 s/point.

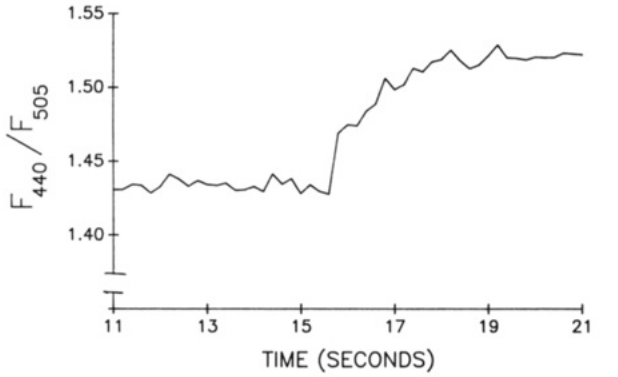

FIGURE 4: Kinetics of the dye response within the mixing time for addition of valinomycin in an experiment similar to those displayed in Figure 2. The time resolution was increased to 0.2 s/point (0.06-s integration time) and the noise reduced by opening the excitation slits to 2 mm (other settings as in Figure 2). [K^+] is 200 mM inside and 2 mM outside the vesicles.

electric field along the surface of a cell (Gross et al., 1986). For a round cell the potential varies as the cosine of the angle between the membrane normal and the field direction and can be precisely related to the amplitude of the applied field via a solution to Laplace's equation [see Gross et al. (1986) for details]. This is a somewhat complex experimental protocol but has the advantage of permitting the precise control over the induced membrane potential required for the evaluation or calibration of a potentiometric dye. The work by Gross et al. used the 546-nm line of a mercury arc lamp for excitation and a 590-nm barrier filter for emission in a fluorescence microscope connected to a digital video imaging system. In Figure 5, this experiment was repeated along with one employing a 450-nm excitation filter. To accentuate the response of the dye, these images are differences between digital images obtained with and without an applied field. The dye responds with the predicted cosine dependence, indicating hyperpolarization on the pole facing the anode and depolarization on the cell surface facing the cathode. Also clear are the opposite changes in fluorescence intensity at the two wavelengths.

A series of images was obtained at different applied fields and with both blue and green excitation for four round or

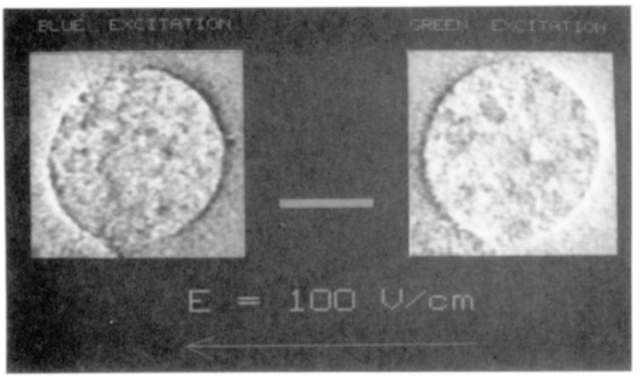

FIGURE 5: Di-4-ANEPPS mapping the membrane potential induced by an electric field applied to a HeLa cell. Digital images obtained before application of the field were subtracted from the fluorescence excited with the field on; a null difference was set to the center gray level of 128. The image on the left results from 450-nm excitation and the image on the right from 530-nm excitation.

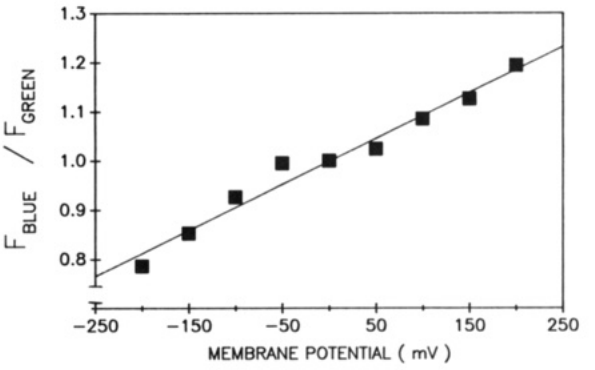

FIGURE 6: Ratios of intensities from excitation at 450 and 530 nm vs membrane potential calculated from a solution of Laplace's equation for a spherical dielectric shell in a conducting medium. The points were derived from four sets of measurements obtained with varying applied fields and cell sizes.

nearly round cells (simplifying calculation of the potential). The intensity was integrated over a ca. 10° arc of membrane at each pole for each of the images, the same patches being used for each member of a blue/green pair. The ratio of the integrated intensities is plotted against the calculated membrane potential in Figure 6. The slope of this line is 0.093/100 mV (r = 0.999). Since the wavelength and band-pass combinations in this experiment are not the same as those used for the vesicle suspension, the identity of the slopes in Figures 3 and 6 is coincidental.

Discussion

This work shows that the voltage across membranes in two very different preparations, lipid vesicles in suspension and individual HeLa cells under a microscope, is linearly related to the ratio of fluorescence excited from the two wings of the absorption spectrum of a voltage-sensitive dye.

The total fluorescence from lipid vesicles stained with di-4-ANEPPS has a complex dependence on membrane potential and, in our experimental protocol, includes some artifactual contribution from interaction of the dye with valinomycin (Figure 1). This complexity is filtered out when the ratiometric treatment is applied, leading to a simple linear correlation (Figure 3). It is noteworthy that the correlation in the inset to Figure 3 was derived from only the ratios obtained after the valinomycin was added to establish a membrane potential and is not relative to the null potential state; i.e., the ratio is a direct measure of the potential and need not be restricted to measuring changes. The spectral shift, which underlies the

sensitivity of the dual-wavelength ratio to potential, is fast [less than milliseconds (Ehrenberg et al., 1987)]. The instrumentation which has become available for dual-wavelength measurements permits the rapid acquisition of data to take full advantage of this speed for the study of rapid potential changes in vesicle (or cell) suspensions (Figure 4).

That the approach does work for cells is indicated by the results presented in Figures 5 and 6. Furthermore, these results show that measurements can be made via quantitative imaging of single cell fluorescence—similar to the analysis of Ca^{2+} in individual cells (Tsien & Poenie, 1986). It should be emphasized, however, that calibration will be required for each specific application. At the least, this is because different filter and lamp combinations will provide different light intensities at the two excitation wavelengths. Also important in this regard is the possibility that surface potential or more specific interactions of the dye with membrane constituents may offset the null potential spectrum relative to that of the dye on a model membrane. Procedures for calibrating voltage-sensitive dyes in cell suspensions have been well established (Freedman & Laris, 1981) and can now be readily extended to measurements on individual cells via the ratiometric approach described in this paper. Finally, it should be noted that many potentiometric dyes undergo spectral shifts as part of their response to potential changes, so that the approach need not be confined to the particular dye chosen in this work.

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