Video Article

Measuring the Induced Membrane Voltage with Di-8-ANEPPS

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Abstract

Placement of a cell into an external electric field causes a local charge redistribution inside and outside of the cell in the vicinity of the cell membrane, resulting in a voltage across the membrane. This voltage, termed the induced membrane voltage (also induced transmembrane voltage, or induced transmembrane potential difference) and denoted by ΔΦ, exists only as long as the external field is present. If the resting voltage is present on the membrane, the induced voltage superimposes (adds) onto it. By using one of the potentiometric fluorescent dyes, such as di-8-ANEPPS, it is possible to observe the variations of ΔΦ on the cell membrane and to measure its value noninvasively. di-8-ANEPPS becomes strongly fluorescent when bound to the lipid bilayer of the cell membrane, with the change of the fluorescence intensity proportional to the change of ΔΦ. This video shows the protocol for measuring ΔΦ using di-8-ANEPPS and also demonstrates the influence of cell shape on the amplitude and spatial distribution of ΔΦ.

Video Link

The video component of this article can be found at http://www.jove.com/video/1659/

Protocol

Part I: Preliminary steps

1. In this experiment Chinese hamster ovary cell line (CHO-K1) is used. Cells are plated in Lab-Tek II chambers (2 wells, 4 cm^2 each) (Nalge Nunc, Germany) at ~$0.7x10^5$ cells/ml in HAM-F12 culture medium supplemented with 8% fetal calf serum, 0.15 mg/ml L-glutamine, 16 mg/ml gentamicin (all from Sigma-Aldrich, Steinheim, Germany), and 200 units/ml Crystacillin (Pliva, Zagreb, Croatia), and incubated in 5% CO_2 at 37°C. Alternatively, cells can also be plated on #1 glass cover slips (0.13 to 0.16 mm thick), coated with a cellular adhesive such as polylysine.
2. Incubate the cells in their culture medium. Incubation lasting 2 to 4 hours yields cells that are still roughly spherical, but firmly attached to the surface with a small part of their membrane. Alternatively, after 16 to 20 hours of incubation, cells are fully attached to the surface and have more complex shapes, but most of them are still not dividing.
3. Prepare a 10 mM stock solution of di-8-ANEPPS (Invitrogen, Eugene, Oregon, USA) by adding 843 µl of DMSO (Sigma-Aldrich, Steinheim, Germany) to 5 mg of the dye in the original Invitrogen vial. The stock solution can be stored in a refrigerator at 4°C for several months. Before starting the experiments, warm up the the solution until the crystals of DMSO dissolve.
4. Some cell lines may require the use of pluronic to ease the dye incorporation into the cell membrane. Pluronic can be purchased in 20% stock solution in DMSO (F-127, Invitrogen, Eugene, Oregon, USA), or a stock solution of the same concentration can be prepared by dissolving pluronic in DMSO. Stock solution of pluronic can be stored at room temperature.

Part II: Loading the cells with di-8-ANEPPS

1. Mix 3 µl of 10 mM di-8-ANEPPS and 2.5 µl of 20% pluronic in 1 ml of the Spinner modification (calcium-depleted version) of the Minimum Essential Medium SMEM (medium M8167 or M4767, Sigma-Aldrich, Steinheim, Germany) in a 1.5 ml Eppendorf tube. This yields a "loading solution" containing approximately 30 µM di-8-ANEPPS and 0.05% of pluronic. For other cell types, their native culture media can be used instead of SMEM.
2. Replace the culture medium in the Lab-Tek chamber with the loading solution. Transfer the chamber to the refrigerator for 10 min at 4°C. At this temperature, internalization of the dye through the plasma membrane is largely inhibited.
3. After staining, gently wash the excess dye two to three times with pure SMEM.
4. Leave 1.5 ml of SMEM in the chamber.

Part III: Experiment and image acquisition

1. The cells are observed using a fluorescence microscope (in our case Zeiss AxioVert 200, Zeiss, Germany) equipped with an oil immersion objective (x63, NA 1.4), a monochromator (Polychrome IV, Visitron, Germany) and a cooled CCD camera (VisiCam 1280, Visitron, Germany). The images are acquired with MetaFluor 7.1.1 and processed in MetaMorph 7.1.1 (both Molecular Devices, Downingtown, PA, USA), but other similar acquisition software can also be used. Set the excitation wavelength in the acquisition software to 490 nm and choose a suitable emission filter for ANEPPS, for example, a band-pass filter centered at 605 nm (605/55m, dichroic 565 DCXR, Chroma, Rockingham, VT, USA). If a monochromator is not available, an excitation filter centered at 490 nm can be used instead.
2. Place the chamber with cells onto the microscope stage, position the electrodes at the bottom of the chamber and connect them to the pulse generator. The medium should cover the electrodes.
3. To maintain cell viability and reduce the heating, the pulse should be of sufficiently low amplitude and short duration. In this experiment a square pulse with 35 V amplitude and 50 ms duration (low enough to avoid electroporation) is generated using a DC voltage supply and a custom-made microprocessor-controlled switcher device. Alternatively, a commercial pulse generator, such as 33210A (Agilent, Santa Clara, CA, USA) connected to an amplifier or a commercial stimulator, such as S88 (Grass, West Warwick, RI, USA), can be used. The pulse is delivered to two parallel Pt/Ir wire electrodes with 0.8 mm diameter and 4 mm distance between them, creating an electric field of approximately 88 V/cm between the electrodes and inducing ΔΦ. The exact field distribution between the electrodes used in this experiment is described elsewhere [1].
4. Find the cells of interest. Apply a single electric pulse or a sequence of pulses. For each pulse, acquire two fluorescence images: one immediately before the pulse (the control image), and one during the pulse (the pulse image). Due to the low response of di-8-ANEPPS, the changes in the fluorescence are difficult to discern by the naked eye and become apparent only after processing. The pulse delivery must be synchronized with the image acquisition, which is a feature of the acquisition software. To avoid photobleaching and possible heating, the illumination can be limited to the duration of the pulse.

Part IV: Image processing and analysis

1. Open the images in MetaMorph software. For each pulse, subtract the background in both the control and the pulse image.
2. Choose a cell and set the region of interest so that it corresponds to the membrane. Measure the fluorescence intensities along this region in the control and pulse image and transfer the values to a spreadsheet.
3. For each pulse, subtract the control data from the pulse data, and divide the result by control data to obtain the relative fluorescence changes. If a sequence of pulses is applied, the values of relative fluorescence changes determined for each pulse can be averaged to get a more reliable measurement.
4. Transform the relative fluorescence changes into ΔΦ using a calibration curve. A rough estimation of this curve can be obtained from the literature, but for higher accuracy, it has to be measured for each particular setup. For the cells and the setup used in this experiment a 6% decrease in fluorescence corresponds to a 100 mV increase in ΔΦ [2].
5. Finally, plot the voltage as a function of the relative arc length in a graphing software package such as Excel (Microsoft Corp., Redmond, WA, USA), Sigma Plot (Systat Software Inc., San Jose, CA, USA), or Origin (OriginLab Corp., Northampton, MA, USA). The curve can also be smoothed using a suitable filter, such as the moving average filter.

Discussion

Measurements of the induced membrane (transmembrane) voltage, ΔΦ, can be important in various experimental settings, such as studies of voltage-gated membrane channels, action potential propagation, cardiac cell stimulation, or cell membrane electroporation [3, 4, 5, 6, 7]. With simple cell shapes, ΔΦ can be calculated analytically. For example, for a spherical cell, ΔΦ is given by the Schwan s equation, which states that the voltage is proportional to the field strength and cell size and follows the cosine function along the membrane [8, 9]. For more complicated cell shapes, ΔΦ can deviate considerably from the cosine and must be determined either numerically, using a computer [2, 10, 11], or experimentally, using a potentiometric dye [12, 13, 14, 15].

One of the potentiometric dyes widely used for this purpose is di-8-ANEPPS (di-8-butyl-amino-naphthyl-ethylene-pyridinium-propyl-sulfonate), a fast dye with excitation and emission spectra dependent on the membrane voltage, which allows noninvasive observations of the variations of the ΔΦ on the cell membrane and to measure its value. In this video, we show an experimental approach for determination of ΔΦ by using di-8-ANEPPS.

The dye was developed by Professor Leslie Loew and colleagues [13, 14] at the University of Connecticut and belongs to the class of fast-response dyes. di-8-ANEPPS is nonfluorescent in water and becomes strongly fluorescent when it incorporates into the lipid bilayer of the cell membrane. A change in ΔΦ results in a change of the intramolecular charge distribution and corresponding changes in the spectral profile and intensity of the dye's fluorescence. The fluorescence intensity of di-8-ANEPPS varies proportionally to the change of ΔΦ; the response of the dye is linear for voltages ranging from −280 mV to +250 mV [4, 16]. Relatively small changes in fluorescence of the dye, uneven membrane staining, and dye internalization make di-8-ANEPPS less suitable for absolute measurements of membrane voltage, e.g. its resting component, although such efforts were also reported [17]. It is, however, suitable for measuring larger changes in membrane voltage, such as the onset of induced membrane voltage in nonexcitable cells exposed to external electric fields [12, 13], or action potentials in excitable cells [4, 5]. Although not applied here, di-8-ANEPPS also allows determination of ΔΦ by ratiometric measurements of fluorescence excitation [18] or emission [19], which increases the sensitivity of the response and reduces the abovementioned effects. As di-8-ANEPPS stains the membrane, it can also be used plainly as a membrane marker [2].

One of the drawbacks of the dye is that it is prone to photobleaching, so that prolonged exposures to the strong light should be avoided. Calibration of the dye is performed with either (i) potassium ionophore valinomycin and a set of different potassium concentrations in external medium [2,18], or (ii) patch-clamp in voltage clamp mode [17].

Finally, with the measurements of ΔΦ on spherical cells and cells of more complex shapes, the video demonstrates the influence of the cell shape on the amplitude and spatial distribution of ΔΦ. Thus for spherical cells ΔΦ is close to a cosine, in agreement with Schwan's equation, while for more complicated cell shapes the spatial distribution of ΔΦ is more intricate [20]...

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