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Rapid evaluation of a protein-based voltage probe using a field-induced membrane potential change

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ABSTRACT

The development of a high performance protein probe for the measurement of membrane potential will allow elucidation of spatiotemporal regulation of electrical signals within a network of excitable cells. Engineering such a probe requires a functional screen of many candidates. Although the glass-microelectrode technique generally provides an accurate measure of a given test probe, throughputs are limited. In this study, we focused on an approach that uses the membrane potential changes induced by an external electric field in a geometrically simple mammalian cell. For quantitative evaluation of membrane voltage probes that rely on the structural transition of the S1–S4 voltage sensor domain and hence have non-linear voltage dependencies, it was crucial to introduce exogenous inwardly rectifying potassium conductance to reduce cell-to-cell variability in resting membrane potentials. Importantly, the addition of the exogenous conductance drastically altered the profile of the field-induced potential. Following a site-directed random mutagenesis and the rapid screen, we identified a mutant of a voltage probe Mermaid, exhibiting positively shifted voltage sensitivity. Due to its simplicity, the current approach will be applicable under a microfluidic configuration to carry out an efficient screen. Additionally, we demonstrate another interesting aspect of the field-induced optical signals, ability to visualize electrical couplings between cells.

1. Introduction

Elucidating spatiotemporal flows of electrical signals within a network of excitable cells is arguably one of the most significant challenges in modern physiology. While the conventional glass-microelectrode technique permits sensitive and reliable measurements of electrical activities in a single cell or a few cells, it is difficult to address these activities spatiotemporally. Spatially resolved measurements with excellent time resolutions have been achieved with voltage-sensitive organic dyes [1–3]. However, when applied to a complex network of heterogeneous cells, it is normally difficult to discriminate the activities in specific type of cells. The use of a protein-based optical probe, which can be genetically encoded under the control of cell-type specific promoters, may enable such discrimination in measurements. Since the first report of a genetically-encoded voltage probe [4], a number of protein-based voltage probes have been engineered [5–19]. Especially, the probes based on a voltage-sensing phosphatase (e.g. VSFP2.1 [5], Mermaid [6], ElectricPk [14], ArcLight [16], VSFP-Butterfly [17]) or a microbial rhodopsin (e.g. Arch [12]) exhibit improved capabilities in reporting electrical activities than the earlier channel based probes. Thus far, individual action potentials of single neurons in vitro, voltage dynamics of a beating heart in zebrafish, spontaneous as well as sensory-evoked cortical activities in living mice, and activities at neurite branches in fly brain have been successfully detected [5–19]. In spite of the recent progresses, however, the existing protein-based voltage probes do not yet reveal the sufficiently precise spatiotemporal regulation of electrical activities in a complex excitable system. Evidently, development of a better performing probe is still essential.

In engineering a protein-based voltage probe, totally rational design has been impractical. Even a small modification, which appears minor, could lead to unexpected improvements. A representative example is found in the recent report of an engineered voltage probe, ArcLight [16]. In their report, a point mutation on the surface of a fluorescence reporter coupled to a voltage-sensor domain remarkably enhanced the responsiveness by an unknown mechanism. Thus, functional screening of many candidates is crucial for the development of a better performing probe. While simultaneous single cell photometry and voltage-clamp recordings provide accurate measurements, throughputs in hand-

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operated patch-clamp recordings are limited. Automated electrophysiology might increase efficiency. However, the technique is still not very feasible because of its high cost and lack of flexibility in freely customizing to experimental configurations.

One approach could be a use of the membrane potential changes induced by an external electric field ($\Delta\Psi$). $\Delta\Psi$ has been also termed as "induced transmembrane voltage". Theoretical and experimental studies have quantitatively addressed the spatial distributions and temporal development of $\Delta\Psi$. The key result from these studies is that $\Delta\Psi$ can be calculated for simple cell geometries under some assumptions, and the solution is basically consistent with the experimental observations [20–29]. $\Delta\Psi$ has been actually used to observe response from a rhodopsin-based voltage probe [11].

However, as we show in this report, this approach is not directly applicable to evaluations of voltage probes that rely on the structural transition of the S1–S4 voltage sensor domain, for example, from a voltage sensing phosphatase [30], because such probes generally exhibit non-linear responses to transmembrane voltage which is the sum of $\Delta\Psi$ and the resting membrane potential. The uncertainty in resting potentials brought unavoidable ambiguity to the probe responses. In contrast, organic dyes and rhodopsin-based probes normally exhibit linear optical responses to physiologically relevant voltage changes, which do not bring such uncertainties. In this report, to overcome this issue, we stabilized resting membrane potential levels by stably introducing exogenous potassium conductance. We then show that $\Delta\Psi$ is drastically altered by the added conductance. The knowledge on $\Delta\Psi$ allowed us to evaluate the performance of a non-linear voltage probe under a conventional epi-fluorescence microscope without using the currently prevalent microelectrode techniques. Finally, we also reveal another interesting aspect of the field induced optical responses, which enable visualization of electrical couplings between cells.

2. Materials and methods

2.1. Cell preparations

N2a cells (a mouse neuroblastoma cell line) and HEK293 cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum in a standard incubator (5% CO_2, 37 °C). Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Recordings were made ~24 h post-transfection. Single N2a cells were prepared as follows. Three to five hours before recording, the medium was replaced by phosphate-buffered saline with 1 mM EDTA and nominally without calcium ion, and cells were collected by gentle mechanical dissociation. N2a cells were re-suspended in the culture medium and re-plated on a 35-mm glass-bottomed dish to isolate single cells. HEK293 cells were used for visualization of electrical couplings. Di-4-ANEPPS was loaded immediately before measurement by incubating non-transfected cells with 1 mL of loading solution that contained 5 μM Di-4-ANEPPS in Hank's Balanced Salt Solution (HBSS) for 7 to 10 min at room temperature. Cells were then washed 3 times with 1.8 mL of HBSS containing 15 mM HEPES (pH 7.4).

2.2. Optical imaging

Recordings were made using an inverted microscope (IX71; Olympus, Tokyo, Japan) equipped with a 40× objective (N.A. 1.3), a stable 75-W xenon lamp (UXL-S75XB; USHIO, Tokyo, Japan), and a C-MOS camera (Orca-Flash2.8; Hamamatsu Photonics, Hamamatsu, Japan). The camera was operated using HC-image software (Hamamatsu Photonics, Hamamatsu, Japan). In the experiment shown in Fig. 1S, another C-MOS camera (Neo, Andor Technology, Belfast, UK) which was kindly lent by a local distributor was used. A hand-made electrode unit consisting of a pair of parallel platinum wires ($\phi = 0.3$ mm) with a 7.0-mm gap was immersed into the glass-bottomed dish containing single cells. A square voltage pulse was applied by using a high-speed bipolar amplifier (BA4825; NF Corporation, Yokohama, Japan). To evaluate steady-state optical responses of protein sensors which can have slow activation kinetics at low voltage, we used a pulse of relatively long duration (600 ms), unless otherwise noted. The field strength was set at 5–6 V/mm. When higher strength was used, we sometimes recognized detrimental effects to the cells. The acquisition of images during the pulse was initiated at 400 ms after its onset. The camera exposure time was 40 ms/frame. To acquire fluorescence enough to quantify the spatial distributions of the optical responses, four consecutive images of the cells were acquired before and during the pulse, which were then time-averaged. After subtraction of dark signals, a region confined by 2 concentric circles was manually fit to the image of the membrane, which was then divided into 24 regions of interest (ROIs). Signals were integrated over the ROIs to measure fluorescence intensity (F) as a function of polar angle. A change in the fluorescence intensity by the pulse (ΔF) was normalized to the initial intensity (F) to calculate the fractional fluorescence change ($\Delta F/F$ [%]). In the experiment described in Fig. 1S, pulse duration and camera exposure time were 100 ms and 5 ms/frame, respectively. The image analyses were carried out using a homebuilt program that operates in IDL (Research Systems). The excitation filter, dichroic mirror, and emission filters used were ex535/50, dm565, and em610/75 for Di-4-ANEPPS; ex450/20, dm465, and em507/65 for mUKG; ex450/20, dm465, and em595/64 for mKOκ; and ex438/24, dm458, em483/32 for CFP, respectively.

2.3. Electrophysiology and molecular biology

Whole-cell patch clamp recordings were performed using an Axopatch 200B patch clamp amplifier (Axon Instruments). The pipette solution contained (in mM): NaCl 5, KCl 10, HEPES 10, KOH 130, MgATP 2.5, Na_2GTP 0.3, and EGTA 1 (pH adjusted to 7.3 with methanesulfonic acid). The bath solution was HBSS containing 15 mM HEPES (pH 7.4). The pipette resistance ranged between 4 and 6 MΩ. Simultaneous photometry was performed as described previously [6]. Plasmids were constructed using conventional molecular biology techniques. Site-directed random mutagenesis was performed as described previously [31].

2.4. Generation of a stable N2a-kir cell line

DNA encoding the mouse Kir2.1 channel was subcloned into pEF6 vector (Invitrogen). After transfecting N2a cells with the plasmid, stable transformants were screened by culturing cells in the presence of Blasticidin (10 μg/mL) for 2 weeks. Several single colonies of cells were then passaged, from which a clonal line showing normal growth was selected and used in subsequent measurements (N2a-kir cells).

3. Results

3.1. Characterization of $\Delta\Psi$ in a normal N2a cell

We used undifferentiated N2a cells as a cell platform because almost spherical cells can be easily prepared as shown below. While $\Delta\Psi$ has been experimentally characterized in various types of cells, $\Delta\Psi$ in an N2a cell has not been reported (to our knowledge). Also, strictly, actual $\Delta\Psi$ is dependent on the specific experimental conditions. We therefore started with a system calibration experiment of $\Delta\Psi$ in an N2a cell under our experimental setup using an organic voltage-sensitive dye, Di-4-ANEPPS [32]. Similar calibration experiments in a CHO cell have been visually described previously [33].

It is generally known that $\Delta\Psi$ reaches steady-state rapidly (~ microseconds), and is linearly related to the field strength unless exceeding the limit that stimulates electroporation or membrane breakdown [22–29]. We first confirmed such basic properties in a single N2a cells (Fig. S1). To establish a quantitative measurement, we then focused