Cellular defibrillation: interaction of micro-scale electric fields with voltage-gated ion channels

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Abstract We study the effect of micro-scale electric fields on voltage-gated ion channels in mammalian cell membranes. Such micro- and nano-scale electric fields mimic the effects of multiferroic nanoparticles that were recently proposed [1] as a novel way of controlling the function of voltage-sensing biomolecules such as ion channels. This article describes experimental procedures and initial results that reveal the effect of the electric field, in close proximity of cells, on the ion transport through voltage-gated ion channels. We present two configurations of the whole-cell patch-clamping apparatus that were used to detect the effect of external stimulation on ionic currents and discuss preliminary results that indicate modulation of the ionic currents consistent with the applied stimulus.

Keywords Ion channel gating · Patch-clamping · Multiferroic nanoparticles · Microscale electric fields

1 Introduction

The research presented in this article relates to a new concept of remote stimulation of ion channels in mammalian cells [1]. This concept involves two essential components: multiferroic nanoparticles and an interaction of voltage-gated ion channels with micro-scale electric fields.

There are many potential biomedical uses of magnetic nanoparticles currently under investigation [2–4]. One of the most developed applications is magnetic separation. Magnetic nanoparticles are functionalized with antigens that attach to specific proteins in cells or to DNA and the labeled cells are dragged by the gradient of the magnetic field away from

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unmarked cells. Spintronic biochips using magnetoresistive sensors can be used in vitro for biorecognition, or detection of magnetic labels that bind to specific proteins or DNA due to DNA–DNA hybridization, antibody–antigen recognition or ligand–receptor binding [5]. External magnetic force is also used in magnetic drug targeting to guide magnetic microspheres loaded with toxic drugs to be released in a specific location or organ.

The first clinical application of nanoparticles was for enhancing contrast in magnetic resonance imaging. In this application, the local magnetic field from nanoparticles affects relaxation times of protons in water molecules leading to a negative contrast. Some other applications use the detection of magnetic nanoparticles by means of magnetic sensors instead of MRI. The magnetic sensing of nanoparticles can be combined with targeted drug delivery.

Other extensively studied applications of magnetic nanoparticles regard nanomagnetic gene transfection and magnetic fluid hyperthermia. Magnetic particles exposed to alternating magnetic fields with frequencies ranging from 20 kHz to 1 MHz increase their temperature due to the frictional rotational motion in a viscous liquid (Brownian mechanism) and the magnetic hysteresis losses (Néel relaxation). When functionalized nanoparticles attach to cancer cells, the heat produced in their vicinity has a damaging effect on these cancer cells.

The most recent research on magnetic nanoparticles explores the effects of mechanical actuation of magnetic particles on cell surface receptors. Magnetic nanoparticles bind to specific receptors via antigens and cause changes in their conformation when pulled by an external magnetic field. Depending on the strength, the actuation of receptors can lead to the production of a specific tissue matrix, initiation of cell division or to cell apoptosis.

A new proposed application of nanoparticles [1] involves magnetic nanoparticles coated by a ferroelectric or piezoelectric layer. These multiferroic core-shell nanoparticles respond to external magnetic fields by creating a mechanical stress that is transferred to the piezoelectric layer and results in a localized electric field. Thus, such nanoparticles are capable of generating a local electric field when exposed to an alternating magnetic field due to stress-mediated conversion of magnetic to electric energy. The local electric fields generated by the nanoparticles are expected to modify functions of voltage-gated ion channels in mammalian cells and thus to control transmembrane ion transport, as well as to affect other voltage-sensing biomolecules. Macroscopic-scale electric fields have found applications in defibrillators and Taser guns. Although these applications provide indirect evidence of the effect of external electric fields on voltage-gated ion channels, the voltage used in these cases is over 1000 V and is applied globally, affecting many organs. Therefore, it is difficult to conclude anything about the interaction of the electric field with individual cells. This article describes experimental procedures and initial results that reveal the role of the electric field, highly localized in close proximity of cells, on the ion transport through voltage-gated ion channels in mammalian cell membranes.

The electric fields produced by electrodes used in our experiments are intended to mimic the action of electric fields generated by multiferroic nanoparticles. The change of polarization of the ferroelectric shell in multiferroic nanoparticles is due to the piezoelectric properties of the shell, which is strained by the deformation of the magnetostrictive core in the applied magnetic field [6]. Multiferroic nanoparticles with magnetoelectric coefficient α_E subjected to a change of magnetic field H are expected to generate electric field $E = \alpha_E H$, which for nanoparticles with the size of d translates into voltage of about $V = d \alpha_E H$ across the particle. For example, nanoparticles with $d = 100$ nm and magnetoelectric coefficient $\alpha_E = 5 \text{ V cm}^{-1} \text{ Oe}^{-1}$ subjected to a field of 500 Oe will generate electric field $E = 2500 \text{ V/cm}$ and an electric potential difference of about 25 mV over the particle size. This potential difference

is commonly applied across the cell membrane to trigger action potentials or opening of ion channels. However, the application of external floating potentials is yet to be tested. Because nano-sized particles are difficult to detect in biological experiments, their number in the vicinity of a cell, their locations, and distances from the cell are difficult to determine. Therefore, the use of patterned stimulating electrodes with a precisely known location with respect to the cell and known magnitude of voltage between them seems to be more appropriate to test the effect of floating potentials on ion-channel currents.

2 Materials and methods

2.1 Electrode deposition

A design of a set of triangular electrodes with rounded tips prepared using AUTOCAD software and a photolithography mask of chromium on thick glass was made. The radius of the smallest electrodes was $2.5\text{ }\mu\text{m}$ and the distance between them was $5\text{ }\mu\text{m}$. Other electrodes had distances of 10 and $20\text{ }\mu\text{m}$, comparable to a typical size of mammalian cells, which is around $10\text{ }\mu\text{m}$. To ensure good adhesion of the electrodes, a 5-nm Cr film was deposited on a thin glass slide using magnetron sputtering. Subsequently, a 20-nm layer of Au was deposited on top of the Cr film and spin coating was used to cover it with PMMA photoresist. The photoresist was exposed to UV light using a Deep UV exposure station and a mask aligner (Newport) and a pattern of electrodes was developed in the photoresist. An ion milling system (AJA International) was used to remove the film from the parts of the glass not covered by the photoresist. The final step in preparation of the electrodes was the removal of the photoresist with acetone and cleaning the electrodes in alcohol. An optical image of the electrodes immersed in the recording medium with cells is presented in Fig.1.

Fig. 1 A set of triangular Cr/Au electrodes patterned on a thin glass slide used in the experiment. *Inset* displays details of the electrodes separated by $5\text{ }\mu\text{m}$

2.2 Electrophysiology

The method of stimulation of ion channels in cell membranes with electric fields from patterned electrodes was applied to voltage-gated Shaker K⁺ channels, which are closely related to the human K_v1.x family. The Shaker K⁺ channels were stably expressed in tsA201 cells (gift from D. Hanck). The cells were cultured in 35-mm Petri dishes (Corning) in DMEM medium (ATCC, Manassas, VA) supplemented with 10% FBS (ATCC), 1% penicillin/streptomycin (Gibco BRL, Gaithersburg, MD) and 100 µg/ml Zeocin (Invitrogen, Carlsbad, CA) at 37 °C in 5% CO₂ in a CO₂ incubator (Fisher Scientific, Pittsburgh, PA). The whole-cell ionic currents were recorded in voltage-clamp mode using an electrophysiology apparatus consisting of an Axopatch 200B amplifier with a CV 203 BU headstage (Molecular Devices LLC, Sunnyvale, CA), a Nikon TS100 inverted microscope (Nikon Instruments Inc., Lewisville, TX) and a MP-285 micromanipulator (Sutter Instrument Co., Novato, CA). The experiments were performed at 12 °C, maintained by a TS-4 temperature controller (Physitemp Instruments Inc., Clifton, NJ) and the apparatus was mechanically isolated by a TMC vibration isolation table (TMC, Peabody, MA). Patch-clamping pipettes were pulled from borosilicate capillary glass (Warner Instruments LLC, Hamden, CT) on a P-97 pipette puller (Sutter Instrument Co., Novato, CA) and polished on a MF-830 microforge (Narishige International USA, Inc., East Meadow, NY). The voltage in the patch-clamping circuit was controlled from Patchmaster software (HEKA Instruments, Bellmore, NY) and converted to analog form by an ITC18 AD/DA converter (HEKA Instruments). The ionic currents were digitized at a rate between 50 and 200 kHz and stored on a hard disk. The cell-stimulating circuit consisted of electrodes patterned on a glass coverslip, as described in the previous section and mounted on the inverted microscope stage. Stimulation was provided from a DS345 function generator (Stanford Research Systems, Sunnyvale, CA) or a Digimeter DS2A Constant Voltage Isolated Stimulator (Warner Instruments LLC, Hamden, CT). The details of the stimulation method are described in the Results section.

3 Results

Voltage-gated ion channels have a voltage sensor that moves within the molecule in response to variations in the electric field and causes conformational changes of the molecule that may shift it from one of the closed configurations to an open configuration and vice versa. The purpose of our experiment was to detect any changes in ionic currents through ion channels resulting from changes in the external electric field applied through micro- and nano-scale electrodes. The measurements of ionic currents were made using the whole-cell patch-clamping technique, as described in the Methods section. In the whole-cell mode, the total ionic current through an ensemble of ion channels (all channels present in the membrane of a given cell) is measured. The stimulating electrodes were deposited on a glass coverslip as described in the Methods section and the cells were placed on this coverslip for the purpose of patch clamping. Once a gigaseal was formed, the cell could be repositioned over the electrode gap using a micromanipulator.

Multiphysics micromodeling software COMSOL was used to determine the distribution of the electric potential and electric field strength around the patterned electrodes. The potential distribution between −5 and +5 V in the space between electrodes is represented in Fig. 2a by the color variations. The plot of the strength of the electric field in V/cm versus vertical

distance from the midpoint between the electrodes separated by 5 μm is shown in Fig. 2b. It is important to notice that although the distance between the electrodes is in the micrometer range, the strength of the field applied in the experiment is comparable to that which can be generated by the multiferroic nanoparticles. Another important observation is that the field strength due to a dipolar-type electric field decreases rapidly with the distance from its source (electrodes or nanoparticles). In the recording configuration described in the preceding paragraph, only a certain proportion of the channels will be exposed to sufficiently strong electric fields and there is a limited control of the orientation of the electric field relative to the ion channels. Hence, it is difficult to estimate the scale of the modulation of the ionic current that the external fields might cause.

The ionic current in the whole-cell mode is typically described as:

$$i(t) = g_0 g(V(t)) (V(t) - V_r) P_{\text{open}} \quad (1)$$

where $i(t)$ is the current, $V(t)$ is the transmembrane voltage, V_r is the reversal potential at which the ionic charge transport ceases for a given type of cell and channel, P_{open} is the proportion of the ion channels present in the membrane that are in an open state. In the case of voltage-gated ion channels, this proportion depends on the strength of the electric field sensed by the voltage sensor. Finally, g_0 expresses the overall conductance of the cell membrane (determined by the number of channels present in the particular cell and the conductance of a single channel of a given type) and $g(V)$ represents any additional non-linear effects of voltage on the conductance (such as blockage by divalent ions). This formula shows that there are two principal causes for the changes in the ionic currents: the instantaneous response of the current to changes in voltage (Ohm's law) and the change in the total membrane conductance caused by opening or closing of ion channels in response to changes in the electric field. From the point of view of potential applications of the method described in this paper, a detection of changes of the latter kind is much more interesting.

To that end, we tested two experimental configurations that provided external electrical stimulation of a cell while avoiding direct changes of the reference bath potential. A schematic diagram of the experimental setup is shown in Fig. 3. For the purpose of this paper, we refer to the patch-clamping circuit consisting of the pipette electrode, bath (ground) electrode, and the amplifier as the recording circuit while the electrodes deposited on the coverslip and the stimulating device are called the stimulating circuit.

Fig. 2 **a** Three-dimensional image of the electric potential in the gap between tips of the electrodes separated by 5 μm with a potential difference of 10 V. **b** Electric field strength variation with vertical distance from the electrodes

Fig. 3 Diagram of the experimental apparatus. Solid lines represent wired connections. Dashed lines represent control stimuli and experimental data sent through AD/DA converter

3.1 Configuration 1

In this configuration, the recording circuit and the stimulating circuit are physically decoupled, i.e., when one is active the other is not. We achieved this by introducing a relay between the pipette electrode and the headstage (see Fig. 4). The relay was triggered by a signal generated from the Patchmaster software and synchronized with the voltage pulse the software provides to the recording circuit.

The voltage protocol is shown in Fig. 5. It consists in a series of typical activation pulses (voltage steps from the holding voltage at -70 mV to a series of voltages from -50 mV in 16 mV steps) preceded by an 80-ms-long prepulse. This sequence was repeated three times. The first repetition was a standard recording with the voltage protocol applied through the voltage clamp. During the second repetition, the relay

Fig. 4 Photo of the headstage with the relay box mounted. Inset shows open relay box

Fig. 5 Voltage protocols used in configuration 1. The control voltage in the recording circuit (*top*), the trigger voltage for the relay (*middle*), and the trigger voltage for the stimulating circuit (*bottom*)

was triggered for the duration of the prepulse, thus disconnecting the recording circuit. For the third repetition, in addition to triggering the relay, the coverslip electrodes were stimulated during the prepulse. This stimulation was provided by a function generator (FG) connected to the coverslip electrodes and triggered from another DA channel in Patchmaster. For the FG stimulation, we tested square and sinusoidal waves of frequencies 25, 100, and 1000 Hz and amplitudes varying between 10 mV and 5 V. This method is similar to the one used in [7] and uses the activation series as a diagnostic tool to reconstruct the initial probability distribution that is not otherwise measurable. This initial probability distribution for the activation series is also a final distribution resulting from the applied voltage prepulse. In this experiment, the three repetitions of the activation series were used to detect any differences in the probability distribution resulting from the three different prepulses.

We compared the activation currents for the three repetitions of the protocol. The expectation was that if the stimulation changes channel gating, the third repetition would show a different time course of the rising current. As Fig. 6 shows, the relay closure caused current oscillations that resemble contact bounce in typical relays. However, we observed similar effects when a solid-state relay with no moving parts replaced a mechanical relay; hence, these turned out to be the electrical oscillations in the recording circuit. With this configuration we did not observe any differences in the activation ionic currents with or without the external stimulation. The effect of the stimulation (if any) was of such magnitude that it was entirely masked by the artifacts.

3.2 Configuration 2

In the second configuration, the external stimulation was applied during the recording of the ionic currents. We used three different types of voltage protocols as shown in Fig. 7. In all of them the timing of the external stimulus was controlled by a trigger from a DA channel in Patchmaster. The stimulus trigger is also shown in Fig. 7. In protocols A and B, the external stimulus was applied during the activation voltage steps and it was expected to modify the activation currents. Protocol C was similar to the method used for configuration 1 where the external stimulus was used during a prepulse followed by the activation series. Again, the effect of the external stimulus was expected to show as an altered rising phase of the activation currents.

This configuration was first used with a function generator as a source of external stimulus. In order to avoid direct interference between the two circuits, the recording and stimulating circuits, we first used coverslip electrodes coated with an elastic PMMA polymer photoresist and silica, which provided electrical resistance exceeding the range of a typical ohmmeter. However, in that configuration we did not observe any effect of the stimulation. This in fact ought to be expected, since in such a case the entire voltage drop between the two coverslip electrodes occurs across the insulating layer and no field penetrates the conducting liquid. Next, we used coverslip electrodes in direct contact with the conducting extracellular solution and stimulated using a function generator (FG). However, we observed an unmanageable level of electrical noise in the recording circuit, due to ground loops that the coverslip electrodes connected to the FG created. Therefore, we used an isolated stimulator. The constant voltage isolated stimulator (IS) uses a battery-powered stimulating circuit optically isolated from a

Fig. 6 Ionic currents for the activation voltage protocols and three different prepulse conditions: **a** typical resting potential of -70 mV, **b** pipette electrode disconnected from the recording circuit by opening the relay, **c** same as **b** but with external stimulation applied to coverslip electrodes. The applied stimulus was a square wave of frequency 25 Hz and amplitude 20 mV. *Inset* shows an enlarged part of the current during the prepulse in **c**

triggering circuit to provide a floating voltage between the coverslip electrodes. This eliminated all ground loops and the resulting noise previously observed. The timing of the stimulus was again controlled from the Patchmaster software. We applied the IS stimulus to all three voltage protocols. All recordings were performed using the P/4 method available in Patchmaster software for eliminating capacitive currents.

Figure 8a shows a sample ionic current obtained with the IS stimulus applied during the steady-state (plateau) phase of the channel activation. The corresponding voltage stimulus (the control and stimulating voltages) is shown in Fig. 7b. A similar effect on the ionic current was observed in other voltage protocols across a range of applied frequencies and amplitudes of the stimulating waveform. The range of current fluctuations was proportional to the amplitude of the voltage variations. These current variations appear for two reasons. First there are instantaneous changes following any voltage step that indicate the change in current flow through open channels. Secondly, there is a delayed non-linear change in current due to changes in membrane conductance resulting from channel gating. For comparison with current variations caused by external stimulus, we recorded ionic currents (see Fig. 8b) from the same cell for protocols where the controlled voltage in the voltage-clamp circuit was modified in a manner resembling the external stimulus. This protocol is depicted in Fig. 8c and shows a square wave superimposed on the usual activation protocol in the second half of the pulse. The ionic currents show both an instantaneous stepwise change in current (reflecting the conductance of open channels) and a non-linear change due to channel gating. The latter is more pronounced in the sweeps corresponding to a lower steady-state voltage value, since these

Fig. 7 Voltage protocols used with configuration 2: **a** external stimulus triggered during the rising phase of the activation current, **b** external stimulus applied during the steady-state phase of the activation current, and **c** external stimulus used as a conditioning prepulse. Both the control voltage (applied in the recording circuit) and the stimulating trigger (triggering floating potentials from the IS – the values were varied using the IS control panel) are shown on a common time base

Fig. 8 **a** Typical ionic currents recorded with a coverslip electrode stimulated with floating voltage from the IS. Data shown are for protocol B (cf. Fig. 5) with a stimulus frequency of 25 Hz and an amplitude of 2 V. **b** Ionic currents recorded in a regular patch-clamping configuration (no external stimulus) with the control voltage showing square-wave fluctuations during the same segment as the external stimulus in **a**. The voltage protocol is shown in **c**

values fall well within a typical channel activation range. Similarly, both types of current changes are observed with external IS stimulus. However, the delayed non-linear change seems more pronounced than in Fig. 8b and is present even for traces with the largest steady state control voltage. This may indicate that the IS stimulus has more effect on the channel gating than the changes in the control voltage. The goal of our experiment was to test if floating potentials applied across two electrodes (in a manner similar to how multiferroic particles would generate electric fields) could be an effective way to control ionic currents through ion channels. These preliminary results suggest that the proposed method of modifying the gating of voltage-sensitive ion channels could be successfully applied; however more studies are in progress.

4 Conclusions

A “cellular defibrillation” experiment was devised to test the effect of localized microscopic electric fields on ion transport through voltage-gated ion channels. In this experiment, the stimulation of ion channels in cell membranes is achieved with external electric fields from micro-size electrodes. We recorded whole-cell ionic currents in patch-clamping experiments on Shaker potassium channels with the external stimulation in the form of square and sinusoidal waves of frequencies 25 Hz, 100 Hz, and 1 kHz, and a range of voltages between

10 mV and 5 V applied to electrodes deposited on a glass coverslip. We observed a measurable effect of the external floating potentials in the form of modulation of the ionic current measured in whole-cell patch-clamping experiments consistent with the applied stimulus. The effect of such external floating potentials has been compared to modulation of the transmembrane potential controlled in voltage-clamp experiments. The results indicate that the external stimulus is capable of modifying the channel gating. However, further study is in progress to determine the magnitude of this effect. It is worth noting that in the voltage-clamp experiments, the transmembrane voltage was applied across the entire cell membrane while the stimulating potentials from the coverslip electrodes were applied only to the part of the membrane closest to them.

The ultimate goal is to use multiferroic nanostructures in the form of piezoelectric nanodots deposited on a magnetostrictive substrate or multiferroic core-shell nanoparticles to induce these types of electric fields. The strength of the electric field used in the experiments corresponds to that of multiferroic nanoparticles of diameter 100 nm with a magnetoelectric coefficient of 5 V/(cm Oe) that are subjected to magnetic field pulses of 500 Oe. The computer simulations demonstrate that the dipolar-type fields decay rapidly with distance. Therefore, efficient interaction between ion channels and nanoparticles is expected when the particles are in close proximity to the cells. Preferably, they should be functionalized and attached to the proteins in the membrane in a similar way as demonstrated by Kozissnik and Dobson [4]. This would enable remote stimulation of voltage-sensing biomolecules, such as ion channels, using externally applied magnetic fields and multiferroic nanostructures as localized converters of electric to magnetic fields.

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