Non-Linear Microscale Alterations in Membrane Transport by Electropermeabilization

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ABSTRACT: The purpose of this study was to quantify the changes in cell membrane conductance in response to electropermeabilization, which may elucidate the mechanisms of tissue injury resulting from high-voltage electrical shock. A high-speed, space-clamp and voltage-clamp experimental configuration was used. The pulse parameters of an imposed transmembrane potential that are instrumental in membrane properties alteration were precisely controlled. The dynamics of the non-linear electroporation response was characterized. Keywords - electroporation, muscle membrane, non-linear, voltage clamp, membrane conductance, electrical injury.

INTRODUCTION

Membrane permeabilization occurs when a strong electric field disrupts the structural integrity of the membrane and causes transmembrane defects or gaps. Electropores, as these openings are commonly called, are believed to occur on the sub-microsecond time scale and expand in size during the electrical exposure. For low magnitude and short duration pulses, the pores shrink and disappear following the removal of the pulse and thus the electrical breakdown of the membrane can be reversed. In case of a strong electric field, the pores continue to conduct long after the removal of the pulse leading to irreversible damage of cell membrane.

Recently, Winterhalter et al. showed that the initial process of pore formation in planar lipid bilayers starts a few microseconds after the onset of the pulse, and the breakdown of the membrane occurs within a millisecond. O’Neill and Tung used voltage clamp measurements of membrane patches from frog ventricular cells to show large step increases in membrane conductance that occurred on a time scale of less than

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This work was supported in part by grants from Electric Power Research Institute, Commonwealth Edison, Empire State Electric Energy Research Company, New York State Electric and Gas Company, Niagara Mohawk Power Corporation, Northeast Utilities Services Company, Public Service Company of Oklahoma, Shell Oil Foundation and Amoco Oil Foundation.
30 μs at transmembrane potentials of 0.1-1 V/msec. Chen and Lee\textsuperscript{17} showed that the increase in membrane conductance in response to a positive pulse is 3 to 4 times larger than in response to a negative pulse of the same magnitude. The dynamics of the underlying molecular events that cause an increase in membrane conductance are not well understood.

The purpose of this study was to precisely characterize the changes in cell membrane conductance in response to electroporabilization using a high-speed, space-clamp and voltage-clamp experimental configuration.

**MATERIALS AND METHODS**

A double Vaseline gap voltage clamp was used to study the changes in membrane properties resulting from supraphysiologic transmembrane potentials imposed by an external electric field. The voltage clamp technique involves clamping a constant voltage across a cell membrane and simultaneously measuring the transmembrane current. This method also makes it possible to differentiate the transmembrane leakage current due to membrane electroporabilization from current passed through ion channels. The voltage clamp technique enables us to study the rapid dynamics of electroporabilization as transmembrane current can be measured both during and after an electrical pulse with μsec time resolution. Thus, changes in membrane conductance can be calculated during different phases of electroporabilization and relaxation.

The electric field mediated transmembrane leakage current was measured over a range of transmembrane potentials. An improved voltage clamp configuration with electrically connected end pools\textsuperscript{18} was used, which helped to eliminate transient overshock, provide a uniform potential distribution across the length of the fiber and, thus, enable the measurement of transmembrane current under well defined transmembrane potential conditions. It should be noted that there is a negligibleovershock even when the space constant decreases substantially following electroporabilization.

![Schematic illustration of the voltage clamp arrangement. CP and EP represent the central pool and end pools, respectively. VS represents the Vaseline seal partitioning the central and end pools. Voltage monitoring and current injection occur at both end pools.](image)

**Figure 1:** Schematic illustration of the voltage clamp arrangement. CP and EP represent the central pool and end pools, respectively. VS represents the Vaseline seal partitioning the central and end pools. Voltage monitoring and current injection occur at both end pools.
A schematic of the double-vaseline gap voltage clamp used to measure transmembrane current is shown in Fig. 1. The experimental chamber is divided into three partitions. The middle partition, central pool (CP), is 300 μm in width while each adjacent partition, end pool (EP), is 100 μm wide. An isolated muscle cell was mounted in the notches of the two partitions. Agar bridges were used to connect the pools to electrodes. The cell was held in place, spanning the central pool, by two Delrin clips attached to the bottom of the two end pools using high vacuum grade stop-cock grease. Thin vaseline seals and two glass cover slips were used to electrically isolate the three pools from each other. The two end pools were electrically connected and constrained by a command pulse. The command pulse was used in a feedback mechanism to adjust the injected current (I) in order to maintain or clamp the transmembrane potential at a prescribed level. The potential difference between each end pool and the central pool is defined as the transmembrane potential of the membrane over the central pool.

**Preparation of Muscle Cells**

A single muscle fiber from twitch skeletal muscle, the semitendinosus of an English frog (*Rana temperoria*) was hand-dissected in a high K+ relaxing solution. The fiber was mounted in a chamber filled with a relaxing solution and isolated across the three distinct pools by two Vaseline seals. Clips at the bottom of the chamber held the fiber in place. The cell membrane of fiber segments in the two end pools was chemically permeabilized by treatment with 0.01% saponin for 2 minutes in order to ensure that electrical and ionic transfer could take place between the interior and exterior of the cell. The solution in the two end pools was then replaced by an "internal solution" which mimicked the composition of the cytoplasmic fluid. The central pool was filled with normal Ringer's solution containing both Na+ and K+ channel blockers, tetrodotoxin (TTX) and tetraethanoammonia (TEA). Six agar bridges and six Ag-AgCl pellets were used to connect the solutions in the chamber to the external circuit.

The following solutions were used in preparing the muscle cell for voltage clamp experiments:

- **Relaxing solution**: 120 mM potassium glutamate, 1 mM MgSO₄, 0.1 mM K₂ EGTA and 5 mM K₂ PIPES (Piperazineethanesulfonic acid) (pH 7.0).
- **Internal solution**: 45.5 mM potassium glutamate, 20 mM Tris-creatine phosphate, 20 mM EGTA, 5.5 mM Na₂ ATP, 5 mM glucose, 5 mM K₂ PIPES and 6.8 mM MgSO₄ (pH 7.0).
- **Normal Ringer's**: 120 mM NaCl, 2.5 mM KCl, 2.15 mM Na₂HPO₄, 0.85 mM NaH₂PO₄·H₂O, 1.8 mM CaCl₂·2H₂O, 1 μM TTX (pH 7.1).

**Voltage Clamp Set Up**

A Total Clamp 8800 (Dagan Co., Minneapolis, MN) was used in all the voltage clamp measurements. The control commands were transmitted by an IBM personal computer-based digital-to-analog converter. A Data Translation AC/DC general purpose interface board (GPIB) was used as the computer interface. The shape, magnitude and duration of the electrical pulses were specified using a software interface. The transmembrane current was filtered at 3 kHz by an electronic filter (902LPF) and digitized by a digital oscilloscope (Tektronix 11401, Beaverton, OR).
Pulse Sequence

Cell membrane was stimulated by a series of square wave pulses ranging from -150 to -400 mV in steps of 10 mV. Simultaneously, the resulting transient transmembrane current was recorded.

Transmembrane current recorded in response to an electrical pulse consists of several components: (1) leakage current beneath the vaseline seals, (2) the membrane capacitance current, (3) the normal membrane leakage current, (4) the pulse-induced electroporated leakage current and (5) the ion channel current. In all our measurements, channel blockers were used to prevent channel currents from contaminating electric field mediated current through lipid membrane. To distinguish the pulse-induced leakage current from the total recorded current, a template current consisting of components (1), (2) and (3) was generated using the following procedure. Before exposure to each high voltage pulse, a group of \( N \) prepulses was delivered each with the same period as the subsequent high voltage pulse (Fig. 2). The magnitude of each prepulse was \( I/N \) that of the main pulse. The number of prepulses, \( N \), was chosen such that the membrane potential produced by prepulses was lower than the threshold for opening ion channels. Therefore the transmembrane current responding to a prepulse consisted of only the membrane capacitance and normal leakage currents. The sum of these prepulse currents served as the template for the membrane capacitance and normal leakage currents with respect to the subsequent high voltage pulse current. Subtracting the template current from each high voltage pulse current yields the electric field mediated membrane leakage current.

The stimulation pulses were of 4 msec duration in order to mimic the effects of a half-cycle of the commercial powerline. A pulse of duration 4 msec at the RMS value and a half-cycle sinusoidal current at commercial power frequency (60 Hz) have the same energy. Also to mimic physiologic situations, the cell membrane was held at -90 mV except during the stimulation period. The stimulation pulse polarity was such as to hyperpolarize the cell membrane in order to prevent the voltage-gated ion channels from opening.

The series resistance of intracellular region induces a voltage drop which makes the measured membrane potential different from the command voltage. The feedback gain of the series resistance compensation current was adjusted prior to each experiment to compensate for the drop across the series resistance. A transmembrane potential of -20 mV pulse was applied and the gain was adjusted to be slightly below the level which causes a ringing or overshoot of the capacitance transient.

![Diagram of pulse sequence](image)

**Figure 2:** Pulse sequence used in voltage clamp measurements. A set of four pre-pulses were used prior to the high voltage pulse in order to estimate the membrane capacitance and vaseline leakage currents.
RESULTS

The transmembrane current in response to a series of square wave pulses was measured. The dynamics of membrane conductance changes was characterized using the transmembrane current data. Under resting conditions, the transmembrane current was found to be approximately $2.5 \pm 0.03 \text{nA}$ corresponding to a membrane conductance of $27.9 \pm 3.5 \text{nS}$.

Transmembrane Current Response

Transmembrane current traces were recorded for a sequence of applied 4-msec pulses ranging from -200 to -300 mV. The transmembrane current recorded in response to each pulse is shown in Fig. 3 (top). The sharp rise followed by an exponential decay represents the charging current of membrane capacitance. A similar sharp spike of opposite polarity occurs when the pulse is turned off. At low transmembrane potentials, the transient capacitance current decays to normal leakage levels. The decay time constant of transient capacitance current depends on membrane capacitance and series intracellular resistance. Both steady-state leakage current and the capacitance current vary linearly with transmembrane potential. At higher transmembrane potentials, the capacitance current and leakage current are augmented by an additional component. This new component is electric field mediated and results from the creation of non-specific pathways in cell membrane.

Figure 3: Total transmembrane current (top) in response to a series of 4-msec square wave pulses from -200 mV to -300 mV in steps of -10 mV. The total current includes a transient capacitance current, normal leakage current, and an electric field mediated leakage current. Using the pre-pulse method, the electric field mediated transmembrane current (bottom) was isolated. These current traces (bottom) represent transmembrane current through non-specific pathways that result from the elevated transmembrane potential during each pulse.
The linearity of capacitance current and normal leakage current was exploited in subtracting these two components from the total transmembrane current using the prepulse method described above. The resulting transmembrane current (Fig. 3, bottom) represents the leakage current through membrane pores. The transmembrane current increases from approximately -2.5 nA to -550 nA when the membrane potential is increased from -90 mV to -300 mV. The changes in membrane that occur in this range of transmembrane potential (-200 to -300 mV) are transient and fully reversible. An increase in membrane potential beyond -400 mV leads to stable alterations in membrane electrical properties.

**Transmembrane Potential Threshold for Electroportation**

Theoretical models of electroporation predict a strong dependence of transmembrane potential on the magnitude of change in membrane conductance. In our experiment, this dependence was estimated by measuring the transmembrane current in response to a series of pulses over a range of transmembrane potentials. Fig. 4 shows the transmembrane current in response to stimulation pulses of different transmembrane potentials from -200 mV to -440 mV. The transmembrane current increased at a rate dependent on the imposed transmembrane potential. When the field was turned off, the current relaxed back to the resting value. Relaxation kinetics was generally describable by a single time constant which was independent of the amplitude of the previously applied potential.

An applied field of amplitude less than -240 mV caused a negligible increase in transmembrane current. At higher transmembrane potentials, the transmembrane current increased significantly above its resting value when the field is on. The peak current during the pulse reached over 700 nA at -440 mV compared to the resting value of 3 nA. Following a low-voltage pulse (< 300 mV), the transmembrane current relaxes back to the pre-pulse level. However, when the transmembrane pulse was elevated to 400 mV or greater, an elevated transmembrane current of approximately 20 nA persisted 4 msec after the pulse was turned off. This indicated that recovery was not complete.

**Non-linear Alteration of Membrane Conductance**

A direct effect of the formation of aqueous pathways in the cell membrane by an applied electric field is an increase in membrane conductance. These non-specific pathways reduce the energy barrier for the passage of charged particles across the membrane leading to increased conductance. The large increase in transmembrane current shown in Fig. 4 relates to a corresponding increase in membrane conductance. The non-linear relationship between membrane conductance and the membrane potential directly relates to the dynamics of a heterogeneous population of pores in the membrane.

The peak increase in membrane conductance resulting from transient electroporation by a 4-msec pulse, normalized to the conductance at resting potential, is shown in Fig. 5 (top). The non-linear dependence of membrane conductance on membrane potential is evident from these results. Following electroporation by a 4-msec square wave pulse over -300 mV in amplitude, the peak conductance, measured just before the pulse was turned off, increased by over two orders of magnitude compared to its resting value. The absolute value of membrane conductance increased from the resting value of 0.001
mS/m² to 0.2 mS/m² for a -440 mV, 4-msec pulse. Following the pulse, membrane conductance relaxed back to the resting value indicating the sealing of membrane pores. However, electroporation by a pulse over -400 mV in amplitude caused a sustained elevation in membrane conductance long after the pulse is turned off. The residual membrane conductance was approximately 0.005 mS/m² 4-msec after a -440 mV pulse was turned off (Fig. 5, bottom), corresponding to a five-fold increase in membrane conductance from its resting value.

![Figure 4](image)

**Figure 4**: Transmembrane current as a function of time in response to a 4-msec square wave pulse at different transmembrane potentials (inset). Significant transient electroporation results only at transmembrane potentials higher than -280 mV.

**Electroporation and Relaxation Dynamics**

Events leading to large changes in membrane conductance as shown in Fig. 5 involve realignment of the cell membrane lipid bilayer to permit aqueous pores. The rate of increase in transmembrane current relates to the dynamics of membrane lipid movement. In order to estimate the dependence of dynamics of these molecular events on applied transmembrane potential, the time constant of transmembrane current rise during a 4-msec pulse and its subsequent relaxation were calculated over a range of transmembrane potentials. Both current rise during the pulse and its relaxation after the pulse could not be fit to single exponentials. The electroporation time constant presented here is the time taken by the current to reach e⁻¹ of the peak current. Similarly, the relaxation time constant is the time taken by the current to decay to e⁻¹ of the peak current after the pulse is turned off.
The rate of current rise during the stimulation pulse (filled squares) increased for large transmembrane potentials. The transmembrane current reached $c^{-1}$ of its peak value within 0.25 msec after a -440 mV pulse was turned on. The corresponding time for a -300 mV pulse was over 0.6 msec. When the field was turned off, there was an abrupt decrease in transmembrane current followed by a slower decline over the next few milliseconds. The relaxation process appeared to be governed by molecular diffusion and interfacial energy and was largely independent of transmembrane potential as seen in the figure. The difference between the kinetics of electroporation (during the pulse) compared to the kinetics of relaxation (following the pulse) is evident from the figure. Following a 4-msec pulse, transmembrane current decreases with a time constant of around 0.16 msec. This asymmetry is significant at low transmembrane potentials where the time constant of electroporation is over 4 times higher than the relaxation phase.

Figure 5: Membrane conductance, normalized to conductance at resting membrane potential, as a function of transmembrane potential. Peak change in transmembrane conductance (top) occurred just before the pulse was turned off and the residual change (bottom) was measured 4 msec after the pulse was turned off.
DISCUSSION

The purpose of this study was to characterize the changes in cell membrane transport properties before and after exposure to supraphysiologic transmembrane potential pulse, which may allow us to better understand the mechanisms of tissue injury resulting from high-voltage electrical shock. We utilized a skeletal muscle cell model because the large size of these cells make it experimentally feasible to impose various transmembrane currents or potentials and monitor the response in real-time. These data are important for interpreting theoretical models in the literature.

Our results clearly indicate that alteration of membrane properties is governed by the parameters of an imposed transmembrane potential. These parameters include transmembrane potential pulse amplitude and duration. Both in designing bio-technology tools and in treating electric field mediated membrane injury, it is crucial to understand the role of pulse parameters on membrane alteration.

Effect of Pulse Amplitude on Membrane Conductance

Using voltage clamp measurements of transmembrane current, we showed that the onset of transient electroporation occurs at a transmembrane potential of approximately -240 mV in a frog skeletal muscle cell. When the amplitude of the applied field is less than this threshold, the field contribution to pore energy decrease is very small. Thus, a large energy gradient that governs the expansion of membrane pores, favors their spontaneous resealing, which results in a very small increase in transmembrane current. When the transmembrane potential is raised, the pore energy reduces, leading to the creation of more pores and to the pore expansion to larger radii. Due to the shift in pore population toward higher number and larger radii, the transmembrane current increases by over two orders of magnitude above its resting value at transmembrane potentials over -400 mV.

The transmembrane potential threshold for electroporation that we observed is lower than those reported for other types of membranes. It is low in comparison with transmembrane potentials exceeding 0.5-1.0 V required to cause pore formation in artificial lipid membranes. In cardiac cells, the onset of electroporation was found to occur at approximately 300-400 mV for 5-10 msec pulses. According to Hibino et al., the membrane was porated and started to conduct current when the transmembrane potential reached a critical value of about 1V following an external electric field application.

The lower threshold for electroporation observed in the voltage clamp experiments may be due to the experimental design. We used a double Vaseline gap voltage clamp wherein the cell was held at a spatially uniform and accurately measured transmembrane current. In the traditional configuration of voltage clamp, a large part of the cell experiences a potential that is lower than the applied potential. Thus, a larger membrane potential is required to cause the same level of electroporation as with our voltage clamp setup. In studies that used external electrodes to apply an electric field, transmembrane potential was estimated from analytical expressions. The actual membrane potential may differ considerably from theoretical estimates and may be spatially non-uniform.

Non-linear Increase in Membrane Conductance

The transmembrane current is the sum of current through all membrane pores created after exposure to an electric field. An increase in transmembrane potential during the
pulse causes a shift in the pore population. The distribution of membrane pores is a non-linear function of transmembrane potential. Thus, the membrane conductance also has a non-linear dependence on transmembrane potential. By considering the pores of different radii in the membrane as being electrically in parallel, Lee and Prakah-Asante showed that the electrical properties of cell membrane are strong non-linear functions of transmembrane potential. Using voltage clamp measurements of transmembrane current, we have shown a similar non-linear relationship between membrane conductance and transmembrane potential. The membrane conductance transiently increases by over 400 times above its resting value when the membrane potential is increased to -450 mV. In contrast, for a -200 mV pulse, the increase is less than 20-fold of its value at -90 mV.

Both the rate of pore creation and the energy required to expand existing pores are non-linear functions of transmembrane potential. An increase in transmembrane potential favors both creation and expansion of pores causing an increase in the fraction of membrane area occupied by pores and consequently the membrane conductance, because higher transmembrane potentials overwhelm diffusion-limited process of pore contraction. The consequent shift in pore distribution causes an increasingly rapid rise in transmembrane current at higher transmembrane potentials. This is evident from the decrease in electroporation time constant from 0.65 msec to 0.25 msec when the transmembrane potential is increased from -300 mV to -440 mV. Theoretical models of electroporation describe pore creation in terms of an Arrhenius function of transmembrane potential suggesting a non-linear change in pore distribution with changes in transmembrane potential. Our measurements show that both peak conductance during the pulse and residual conductance following the pulse are non-linear functions of transmembrane potential.

Dynamics of Lipid Reorientation Following Electroporation

An applied electric field causes lipid molecules of the membrane to reorient while creating hydrophilic pores. The molecular dynamics of these events involve changes in conformation of lipid molecules and rearrangement of the lipid bilayer. Under quiescent conditions, membrane defects fluctuate in time scales of picoseconds to minutes. The highly cooperative structural rearrangements of lipids occur in milliseconds to minutes, while the rotation of lipid molecules occurs in microseconds. However, in the presence of an electric field, the rate of reorientation of an electric dipole associated with the lipid molecules is enhanced. When the external field is turned off, the lipids return to their initial configuration. The relaxation of transmembrane current following the pulse occurs with a time constant of 0.16 msec. This value relates to a diffusion coefficient of lipids which is two orders of magnitude lower than that for reorientation of lipids in pure bilayer lipid membrane. This suggests that the resealing process is not completely determined by lateral lipid movement as is the case in a pure lipid bilayer membrane. One possible explanation is that the reoriented lipid molecules of a pore wall may form a quasi-stable complex with membrane proteins when pores expand during a pulse. Lipid molecules in such a complex may find it difficult to re-orient after the pulse, which leads to sustained conduction through aqueous pathways.

We observed that the relaxation of transmembrane current following the return of transmembrane potential to the resting level does not follow a single exponential decay. A similar behavior was observed by Benz and Zimmermann, albeit in artificial bilayer membranes at much higher temperatures. They report that the time course of the resealing process cannot be fit to a single exponential curve in the temperature range of 30 to 60°C. The resealing process, following a 50 μs pulse, was shown to have a rapid phase with a
time constant of 350 ns and a slow phase with a time constant of approximately 2 μs. The fast phase could not be measured accurately because of the limitation on the sampling frequency. In addition, the time constant of decay of transmembrane potential to the resting value was approximately 30 μs, which limited the accuracy of measuring the rapid phase of the decay of transmembrane current. The drop is essentially due to the abrupt resealing of small pores when the driving force is removed. The second phase of relaxation occurs because of the current through larger pores that continually shrink when the pulse is removed. Note that the membrane potential is returned to the resting value of -90 mV following the pulse and not set to zero. This provides the necessary driving force for ionic transport through these pores. In addition, following the application of the pulse, the holding current increases. This contributes to an additional component of current through the pores, and to a slower relaxation of the transmembrane current following the pulse.

The characteristic time constants measured in our experiments augment a wide range of characteristic times of pore resealing that have been reported in the literature.59 The large variation in reported sealing time constants can be partially attributed to the size of the tested molecules. Use of large probe molecules in permeability studies does not account for the presence of small pores in the membrane. Measurements using a large probe molecule only provide estimates of the first or second stages of relaxation in the case of reversible electroporation. Another factor that governs the kinetics of re-sealing is the temperature of the medium: the higher the temperature, the more rapid the resealing. The time constant varies by an order of magnitude from 4°C and 20°C, and another order of magnitude from 20°C and 37°C.30

CONCLUSIONS

The cellular membrane can be described as a structured fluid with imbedded interlinked proteins. Transmembrane properties are very sensitive to structural alterations. Small molecular defects lead to rapid changes in order and subsequent major changes in molecular permeability. The kinetics and magnitude of responses to structural alterations have not been well detailed. This paper uses the most controlled experimental techniques to characterize the complex transport responses to electrically induced molecular alterations.

REFERENCES