Altered Ion Channel Conductance and Ionic Selectivity Induced by Large Imposed Membrane Potential Pulse

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ABSTRACT The effects of large magnitude transmembrane potential pulses on voltage-gated Na and K channel behavior in frog skeletal muscle membrane were studied using a modified double vaseline-gap voltage clamp. The effects of electroconformational damage to ionic channels were separated from damage to lipid bilayer (electroporation). A 4 ms transmembrane potential pulse of −600 mV resulted in a reduction of both Na and K channel conductivities. The supraphysiologic pulses also reduced ionic selectivity of the K channels against Na^+ ions, resulting in a depolarization of the membrane resting potential. However, TTX and TEA binding effects were unaltered. The kinetics of spontaneous reversal of the electroconformational damage of channel proteins was found to be dependent on the magnitude of imposed membrane potential pulse. These results suggest that muscle and nerve dysfunction after electrical shock may be in part caused by electroconformational damage to voltage-gated ion channels.

INTRODUCTION

For more than two decades, electrical field pulses have been used to introduce pores or pore-like structures within cell plasma membranes to facilitate transmembrane exchange of materials. In general, these studies were concerned with gene transfection or cell fusion induction on small, nonexcitable cells. Within the past few years, electroporation has been postulated as an important mechanism in electrical trauma (Lee and Kolodney, 1987).

Electrically, a biological cell can be viewed as an insulating shell containing a conducting cytoplasm usually surrounded by an extracellular conducting fluid. The conductivity of cell membranes is about six to eight orders lower than that of cytoplasmic and extracellular fluids. In a uniform extracellular electric field, the cell membrane experiences an induced voltage drop that scales with cell size. The effective field-strength across the cell membrane can be many orders of magnitude higher than the external field-strength applied to the cell. The larger the cell, the larger the magnitude of the induced membrane potential. Therefore, electrically larger cells are more susceptible to damage than smaller cells (Gaylor et al., 1988). Study of electrical trauma has thus focused on physically and electrically large cells, such as skeletal muscle and nerve cells. Recently, studies of membrane damage that results from high voltage electrical shock have focused on electroporation mechanisms, cell viability, and pharmacological means to seal pores and to restore the integrity of plasma membranes (Lee, 1990; Lee et al., 1992; Chen and Lee, 1992; O'Neill and Tung, 1991).

Victims of electrical shock often experience loss of skeletal and nerve function, despite little or no changes in the appearance of their skin (Lee, 1990). Uses of controlled electrical fields to manage cardiac arrhythmia is a well established common clinical practice. However, undesirable side effects often accompany these defibrillation treatments. Arrhythmia, refibrillation immediately post-defibrillation, and signs of contractile dysfunction of the heart cells have been reported (Jones and Jones, 1982). Among the effects that have been observed is the depolarization of membrane resting potential after defibrillation shocks (Winegrad, 1979; Jones and Jones, 1982). The currently accepted explanation attributes this reaction to microlesion of cell membranes, and consequent nonselective ionic leakage (Jones et al., 1987).

One of the pioneering studies of supraphysiologic transmembrane potential pulses on membrane proteins was conducted by Tsong and Teissie (1980). Using red blood cells, these investigators showed that leakage currents across the cell membrane of blood vessels induced by electrical shock can be partially reduced by ouabain. They attributed the partial leakage to formation of pores in Na^+/K^+ pumps. Yet at this time, functional or structural damage to membrane proteins on muscle and nerve cells has not been completely described. The question we wish to address is, in addition to causing local membrane electroporation, how do high voltage electrical fields affect the structure and function of membrane protein, particular the voltage-gated Na and K channels, which are known to determine the membrane resting potential and action potential.

Recently, we suggested a modification of the double vaseline-gap voltage clamp technique using cut fibers to study the effects of high voltage electrical pulses on skeletal muscle cell membrane (Chen and Lee, 1994). This technique allowed us to impose large transmembrane potential pulses using voltage clamp to measure the transmembrane current both during and after exposure to the electrical pulse, and to distinguish field-induced effects on different contributions to the total transmembrane current.

Using this technique, a series of experiments were carried out to investigate pulse-induced conformational damage to

Received for publication 18 October 1993 and in final form 20 May 1994.
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0006-3495/94/08/603/10 $2.00

the voltage-gated Na and K channel proteins in frog skeletal muscle membrane. The objective was to answer such basic questions as: the differences between the membrane potential threshold for electroporation and that for damage to ionic channels; the interaction between cell membrane leakage and channel protein conformational damage during a large imposed membrane potential pulse (Chen and Lee, 1994); the effects of a high voltage electrical pulse on neurotoxin binding to channel proteins; post-shock functional changes in voltage-gated Na and K channels; and imposed pulse-dependent reversibility of the damaged channel proteins. This report describes some results of these investigations.

MATERIALS AND METHODS

Skeletal muscle fiber preparation

The protocol we followed for muscle fiber preparation has been used in other laboratories (Kovacs et al., 1983; Irving et al., 1987; Chen and Hui, 1991). Single twitch fibers, *semitendinosus* from English frogs, *Rana temporaria*, were hand-dissected and mounted in a custom-made, double vaseline-gap chamber. The width of the central pool of the chamber was 300 μm, and each partition was 100 μm wide (Chen and Lee, 1994).

The muscle fiber was held by clips at the two endpools and was spread across the two partitions with vaseline seals to electrically and chemically divide the chamber into three distinct pools. The three pools were connected by six agar bridges to three ponds filled with 3 M KCl which, in turn, were connected to the voltage clamp via six Ag/AgCl pellets. To permeabilize the cell membrane, each muscle fiber segment in the two end pools was soaked for 2 min in 0.01% saponin and was then thoroughly rinsed with a relaxation solution. The experimental solution in the two endpools was Cl^--free internal solution with 20 mV EGTA. The fiber was stretched to a sarcomere of length of 3.5 μm to eliminate fiber contraction during experiments. All experiments were performed at room temperature (24°C).

Composition of solutions

Two different external solutions were used in the central pool, depending on the purpose of each experiment: external solution I containing tetraethylammonium (TEA^-) and Rb^+ (Chen and Lee, 1994) was used to block K channel currents; external solution II was normal Ringer's. 1 μM tetrodotoxin (TTX) was added to the external solution in experiments involving the blocking of Na channel currents.

Contents of the solutions are as follows. (1) Relaxing solution: 120 mM potassium glutamate, 1 mM $MgSO_4$, 0.1 mM K_2EGTA, 5 mM K_2PIPES, pH 7.0. (2) Internal solution: 45.5 mM potassium glutamate, 20 mM Tris-creatine phosphate, 20 mM EGTA, 5.5 mM Na_2ATP, 5 mM glucose, 5 mM K_2PIPES, 6.8 mM $MgSO_4$. (3) External solution I: 120 mM TEA-Cl, 2.5 mM RbCl, 1.8 mM $CaCl_2$, 2.15 mM Na_2HPO_4, 0.85 mM $NaHPO_4$, pH 7.1. (4) External solution II: 120 mM NaCl, 2.15 mM Na_2HPO_4, 0.85 mM NaH_2PO_4, 1.8 mM $CaCl_2$, pH 7.1.

Voltage-clamp configuration

We modified a traditional voltage-clamp to investigate the electroconformational damage of protein channels (Chen and Lee, 1994). In the traditional configuration of voltage-clamp (Hille and Campbell, 1976; Irving et al., 1987; Chandler and Hui, 1990; Hui and Chen, 1992), the ratio of the membrane potentials at the two edge of the central pool has been estimated by calculation to be 8:1 for an *electroporated* cell membrane (Chen and Lee, 1994). Therefore, it is not possible to use the traditional voltage-clamp to quantify the membrane potential threshold or the voltage dependence of cell membrane damage. In contrast, the newly configured voltage clamp with significantly improved membrane potential uniformity in the central pool is well suited for such studies.

In the modified voltage clamp, a series resistance compensation circuit has been incorporated to compensate for the voltage drop across the intracellular resistance. The positive feedback circuit can follow changes in transmembrane current and, thus, can compensate for voltage drops across the intracellular resistance. Therefore, membrane potentials at the edges of the central pool more closely conform to the command voltage. The comparison of membrane potential distribution between the two configurations and the compensation procedure are described in greater detail in a previous paper (Chen and Lee, 1994).

Electrical recording

The recorded transmembrane currents in response to an imposed electrical pulse consist of different components, as follows. (1) leakage current underneath the vaseline seals; (2) membrane capacitance current; (3) normal membrane leakage current; (4) pulse-induced electroporated leakage current; and (5) ionic channel current. The first three components can be easily removed from the total response current by subtracting a template current because these components are proportional to the magnitude of the imposed electrical pulses (Chen and Lee, 1994).

The following procedure was used to generate the template current: Before exposure to each stimulation pulse, the cell membrane was held at -110 mV for 20 ms, then a group of N pre-pulses was delivered in a magnitude of $1/N$ and the same duration as the following stimulation pulses. The criteria for choosing N is that the membrane potential held by these pre-pulses is lower than the threshold for opening ion channels. The summation of these pre-pulse currents served as a template of the membrane capacitance and resistance currents resulting from the stimulation pulse.

When the stimulation pulse and its pre-pulses are applied before the cell membrane is exposed to a high voltage electrical pulse, the template current consists of only the vaseline leakage current, membrane capacitance and normal resistance currents. However, when the stimulation pulse and its pre-pulses are applied after the high voltage electrical pulse, the resulting template current also includes the electromediated leakage current. In either case, after subtracting the responding template current from the total current, the remainder represents the pure channel-mediated currents. By a comparison of the channel currents taken before and after a electric shock, we are able to show the effects of a high voltage electrical pulse on the voltage-gated ionic channels of a cell membrane. In these experiments, the shock pulse is defined as a supra-physiological membrane potential pulse with a magnitude of 300 mV or higher, and the stimulation pulse as an electrical pulse with a magnitude in the physiological range. To mimic the real situation of a electrical shock, all shock pulses had a fixed duration of 4 ms. A 4 ms duration pulse and a half-cycle sinusoid current at commercial (60 Hz) power frequency with the same RMS value have the same energy. The duration of all stimulation pulses was 30 ms. When no pulse was delivered, cell membranes were always held at a holding potential of -90 mV.

RESULTS

Ion channel damage threshold versus membrane electroporation threshold

The electroporation current studied in this report is a pulse-induced, nonselective membrane leakage current (Tsong, 1991). To focus on the electroporation of cell membranes, shock pulses were negative in polarity, hyperpolarizing cell membranes to avoid opening the Na and K channels. In addition, external solution I with 1 μM TTX was used to block both Na and K channels.

To determine the threshold of membrane electroporation, a sequence of negative shock pulses from -100 mV to -300 mV was applied to the membrane. Immediately before and after each negative shock pulse, a subsequence of 30 ms positive stimulation pulses ranging from +20 to +150 mV

was applied across the cell membrane to open Na and K channels. A sequence of N pre-pulses, each with a magnitude of $1/N$, was always applied before each stimulation pulse to determine the corresponding template current. After the post-shock stimulation pulse sequence, the fiber was relaxed to the membrane holding potential for 5 s, which allowed full recovery from any electroporation current that might have occurred.

A family of traces of total transmembrane currents in response to a positive stimulation pulse sequence applied before a shock pulse is shown in the upper panel of Fig. 1. After

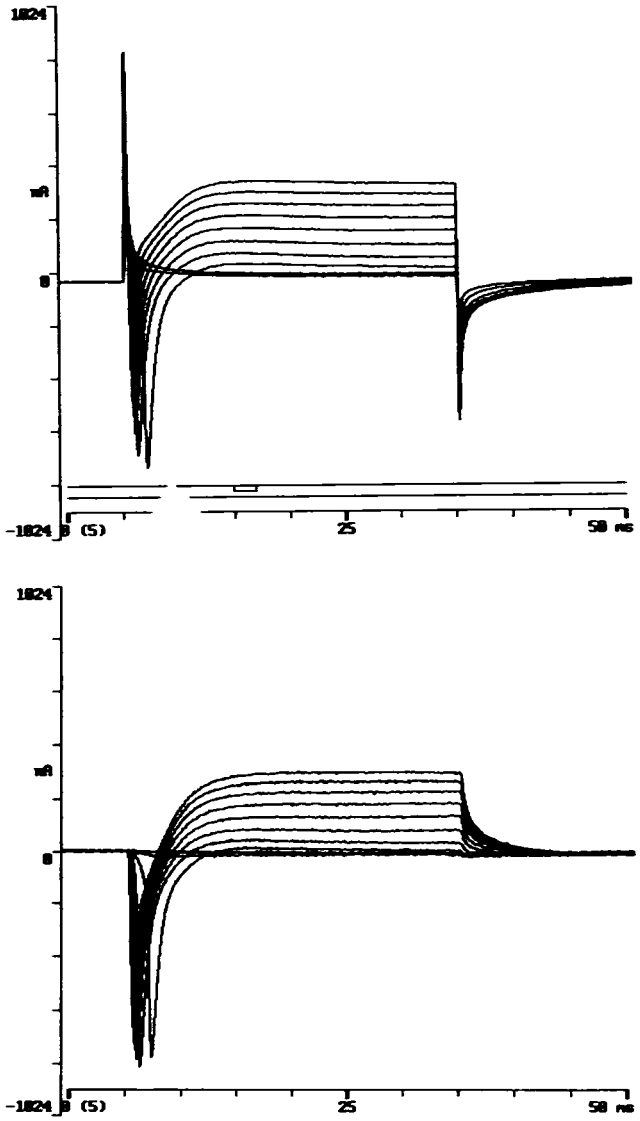


FIGURE 1 The Na and the delayed rectifier K channel currents recorded immediately before exposing to an electrical pulse of 4 ms and −300 mV. The upper panel shows a family of current traces responding to a sequence of 30 ms stimulation pulses ranging from +20 to +150 mV. After subtraction of the leakage and membrane capacitance currents, the Na and the delayed rectifier K channel currents were separated as shown in the lower panel. The solution in the central pool was external solution II. After a 4 ms, −300 mV pulse, the same sequence of stimulation pulses was again applied. The resulting Na and K channel currents were very similar to those shown in the lower panel.

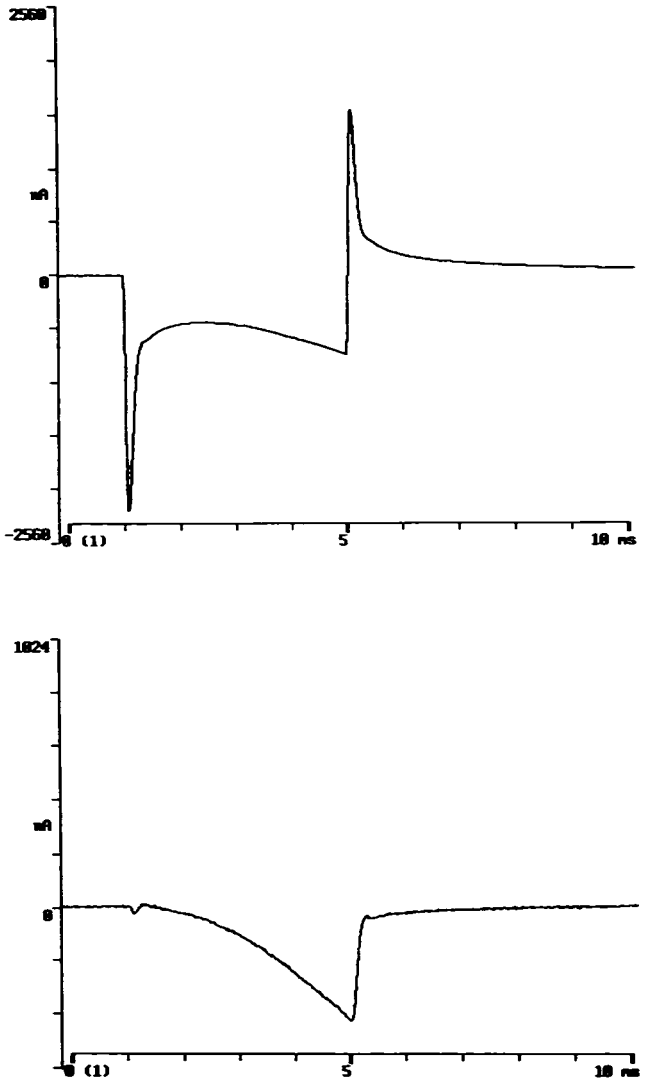


FIGURE 2 Electromediated transmembrane leakage currents recorded during an exposure to a 4 ms, −300 mV shock pulse for the same fiber used for Fig. 1. The external solution in the central pool was normal Ringer's. Upper panels show the total transmembrane current response. Membrane capacitance current and normal leakage current were removed by subtracting the template current which was obtained right before the application of the shock pulse and the remaining electroporated transmembrane leakage currents are shown in the lower panels.

subtracting the template current, the residual currents comprise primarily the channel currents: an inward, quickly activated, then inactivated Na channel currents and a delayed rectifier K channel current, as shown in the lower panel. These currents were deduced to be Na and K channel currents because of their elimination by TTX and TEA, respectively.

The trace in the upper panel in Fig. 2 represents the total transmembrane current in response to a imposed 4-ms pulse of −300 mV. After subtracting the capacitance and normal leakage currents, the electrical pulse-induced leakage current rapidly increased during the shock pulse, as shown in the lower panel, suggesting the formation of electrical field-mediated pores or defect-like structures in the cell membranes (Chen and Lee, 1994). However, the channel-

mediated currents obtained from the family of currents taken immediately after the shock pulse show great similarity as those in Fig. 1 in both shape and magnitude.

The measurement of nonselective leakage membrane current was started when the cell membrane was hyperpolarized by a pulse that holds the membrane potential from −250 to −300 mV. The membrane potential threshold for electroporation of frog skeletal muscle membrane was measured to be about −250 to −300 mV.

A comparison shows very little changes in the voltage-gated Na or K channel currents before and after an electrical shock by a single, 4-ms pulse for membrane potential up to −400 mV, which exceeds the electroporation threshold of cell membranes. Thus, in effect, the membrane potential threshold for damage of voltage-gated channel proteins is higher than that for the damage of phospholipid membrane.

Supraphysiological membrane potential pulse-induced reduction of the K and Na channel conductances and effects on neurotoxin blocking

The upper panel of Fig. 3 shows the total transmembrane current in response to a 30 ms, 120 mV stimulation pulse before an electric shock. The Na and the delayed rectifier K channel currents that remain after subtracting the template current are shown as the trace in the middle panel. After the cell membrane was exposed to a 4-ms pulse of −600 mV, a 120 mV stimulation pulse was again applied to the membrane. Using the same protocol, the channel currents were determined and are shown in the lower panel of Fig. 3. Both the peak inward current and the steady-state outward current were reduced by a single shock pulse. As shown in the lower panel, the peak Na channel currents were reduced from 420 to 350 nA. The steady-state K channel current in this fiber decrease from 450 nA (equivalent to 1 mA/cm^2) to 310 nA. About 20% of the Na channel currents and 30% of the delayed rectifier K channel currents were eliminated by a single 4 ms shock pulse of −600 mV. The surviving transient inward peak current and the steady-state outward current were proven to be Na and K channel currents, because they could be eliminated by TTX and TEA, respectively (shown in the *bottom* panel in Fig. 3).

The next question considered was how a large imposed electrical pulse affected the binding sites for neurotoxins TEA and TTX; what fraction of current recorded after the electric shock (shown in the *lower* panel) can be attributed to the Na and K channel currents? Immediately after the lower panel trace was recorded, the solution in the central pool was changed to the external solution I with 1 μM TTX, and the same stimulation pulse was again applied. After subtraction of the template current, the residual currents were found to be negligible (*bottom* panel). This result indicates that after a single 4-ms pulse of −600 mV, the voltage-gated Na and K channel currents are reduced and the surviving channel currents can still be substantially blocked by TTX and TEA, respectively. In other words, the neurotoxin, TTX and TEA, binding effects on Na and K channels are not affected by the electrical shock studied in these experiments.

Effects on K channel conductance

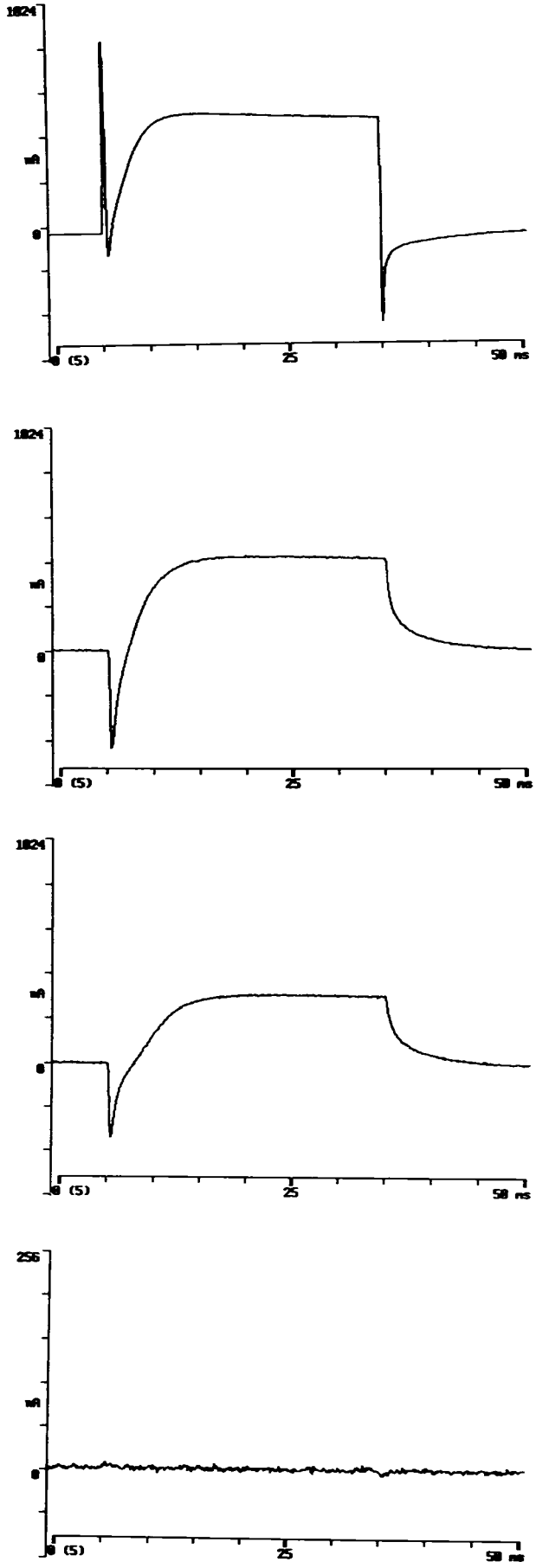
To evaluate the electrical pulse-induced damage to the voltage-gated K channels, we substituted the external solution in the central pool with the external solution II plus 1 μM TTX. A sequence of stimulation pulses ranging from +20 to +150 mV was applied across the cell membrane immediately before and after an electrical shock by a 4 ms, −600 mV pulse. Using the same protocol for subtraction of the template current, families of traces of the pre- and post-shock K channel currents were isolated and is shown in the upper and lower panels of Fig. 4. Experiments were performed sequentially for seven different fibers, and all seven fibers showed a reduction in the K channel currents.

To analyze quantitatively the effects of high voltage electrical pulses on voltage-gated K channels, the steady-state I-V curves shown in Fig. 5 were determined. Each point on the curves represents the mean value of the last 50 acquired data points (out of 512 points for the whole trace) taken from the plateau of each K channel current trace. The hand-drawn upper and lower I-V curves were recorded before and after the application of a 4 ms, 600 mV pulse, respectively. The best-fit for the upper pre-shock curve to the right of the −20 mV, indicates a membrane conductance, g_k, of 3.6 μS. Although the best-fit for the lower post-shock curve shows that the membrane conductance fell to 2.4 μS, slightly higher than 60% of the initial value. Assuming only two possible conditions for the K channels, functional or nonfunctional, nearly 40% of the voltage-gated K channel proteins were damaged by a −600 mV shock pulse.

Reduction in K channel currents is fiber-dependent, which might be related to the voltage drop on the intracellular resistance. We had compensated for the intracellular resistance of the fiber segment in the vaseline-gap area, but could not compensate for the intracellular resistance inside the central pool. The voltage drop on the uncompensated intracellular resistance resulted in a lack of uniformity in the membrane potential distribution in the central pool. The resultant actual membrane potential along the fiber in the central pool varied from fiber to fiber. The average reduction of the K channel conductance for seven fibers is 34 ± 9%.

Effect on ionic selectivity against Na^+ ions of the delayed rectifier K channels

The Golden-Huxley-Katz (GHK) voltage equation (Katz, 1966) defines two empirical measures of membrane permeability: absolute permeability and permeability ratio. When the cytoplasmic fluid and the extracellular bathing solutions contain only one type of monovalent ions on each side of the cell membrane, the GHK equation can be reduced to a di-ionic transmembrane potential equation. By measuring the membrane reversal potential, this simplified di-ionic potential equation can be used to determine the membrane permeability.



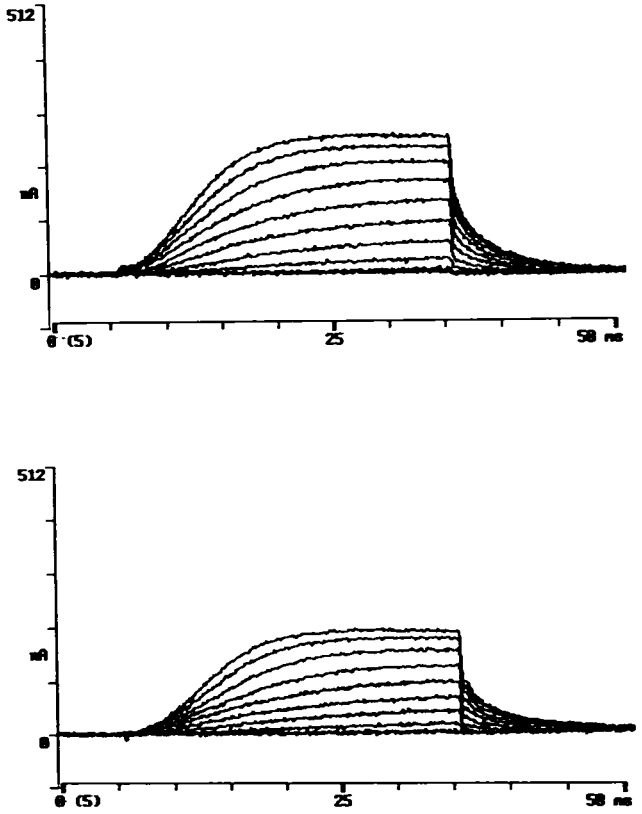


FIGURE 4 A sequence of currents responding to a series of 30 ms stimulation pulses ranging from +20 mV to +150 mV. The traces shown in the upper and middle panels were taken before and after a 4 ms, −600 mV pulsed shock, respectively. The bathing solution in the central pool contained no channel blockers. The solution in the central pool was then changed to the external solution I with 120 mM TEA, and the surviving K channel currents were eliminated by the blockers (not shown).

However, even though the initial compositions of the internal solution are known, it might not possible to measure the actual ionic concentrations in the cytoplasm. The absolute ionic permeability of ion channels is thus difficult to calculate. Therefore we measured changes in the reversal potential instead of the absolute potential. It is not useful to study the ionic selectivity using the traditional method by changing the bathing solution (Hille, 1973; Gay and

FIGURE 3 Effects of a high-voltage negative electrical pulse on the voltage-gated Na and K channel currents. The top panel represents the total transmembrane current response to a 30 ms, 120 mV simulation pulse preceding a high voltage shock. After subtraction of the template of capacitance and leakage currents, the channel currents alone are displayed as the trace in the middle panel. The fiber was then shocked by a 4 ms, −600 mV pulse. Immediately post-shock, the channel currents were recorded using the same stimulation pulse and are shown as the trace in the lower panel. Then the bathing solution was changed to the external solution I with 1 μM TTX. After subtracting the membrane capacitance and leakage currents, the negligible remaining currents shown in the bottom panel in response to the same stimulation pulse (shown in the bottom panel) indicated that primary currents in the lower panel are Na and K channel currents. External solution II was used for this group of experiments.

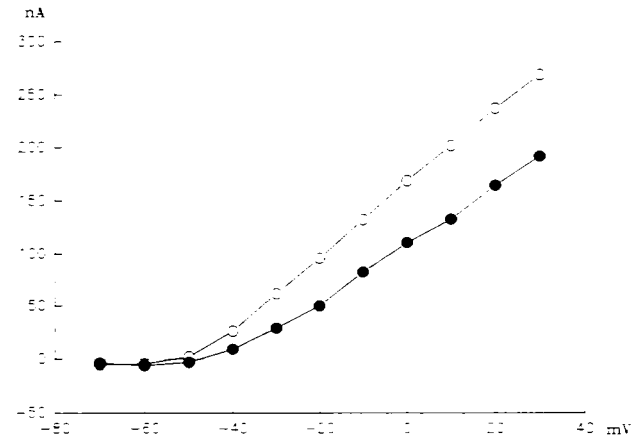


FIGURE 5 Steady-state I-V curves of voltage-gated K channels. The top curve represents the pre-shock K channel current, and the bottom curve represents the channel current after a 4 ms, −600 mV shock pulse. The best-fit curve shows that the channel conductance is 3.6 μS pre-shock and 2.4 μS post-shock.

Stanfield, 1978), because the electroconformational alteration of interest can be transient. Nevertheless, it is important to monitor transient changes in the ionic permeability of ionic channels, especially changes in the permeability ratio of Na^+ over K^+ ions of the K channel. This ratio dictates the membrane resting potential and the kinetics of the falling phase of the action potential. Assuming that the ionic concentrations in the cytoplasm and the bathing solution remain the same before (b) and after (a) a pulsed electrical shock, the change in the reversal potential of the cell membranes can be expressed as

$$\Delta E_{rev} = E_{rev,a} - E_{rev,b} = \frac{RT}{zF} \ln\left\{\frac{(P_{Na}/P_K)_a}{(P_{Na}/P_K)_b}\right\}. \quad (1)$$

where P_{Na} and P_K represent the membrane permeability of the K channels to Na and K ions, respectively. R is the general gas constant, T the absolute temperature, F the Faraday constant, and z the valence of Na^+ and K^+ ($z = 1$). Equation 1 is applicable if Na^+ is the only monovalent cations in the external solution. Any changes in the permeability ratio affected by a high voltage pulse can be determined by measuring the post-shock shift in the reversal potential of the cell membrane.

One fact that complicates the measurement of reversal potential is that when a large ionic current is driven through a cell membrane, a local accumulation or depletion of permeant ions may develop, which might affect the local ionic concentrations. The possible ionic pathways include electropores in the cell membrane and the K channels. Local ionic accumulation and depletion can be avoided if: 1) all of the channel current measurements are performed 10 s after a pulsed electrical shock; 2) the capacitance and leakage currents are subtracted; and 3) only a mean value of the last 50 acquired data points in the K channel current traces close to 20 ms after the rising phase of the stimulation pulses is used to determine the reversal potential.

Experiments were performed using a typical two-pulse protocol as shown in the inset of Fig. 6. The first pulse depolarized the cell membrane up to +10 mV and opened most of the K channels, and the test pulse then held the membrane at different potentials while the transmembrane currents were simultaneously recorded. As shown in Fig. 6, the reversal potential shifted in the positive direction by 11 mV after shocking by a single −700 mV, 4-ms pulse. At room temperature, 25°C, RT/zF = 25 mV. By substituting the shift of the reversal potential (11 mV) into Eq. 1, the K channel ionic permeability ratio of Na^+ over K^+ ions before and after the pulsed electrical shock was computed to be $(P_{Na}/P_K)_a/(P_{Na}/P_K)_b$ = 1.5. Thus, after a single −700 mV pulse, the ionic selectivity of the delayed rectifier K channel against Na^+ ions was reduced by about 50%. If this ionic permeability ratio is substituted into the GHK equation for normal physiological solutions, the membrane resting potential should shift from −90 to −84 mV. The results for five experiments show that on exposure to a single shock pulse of −700 mV and 4 ms, the K channel ionic permeability ratio of Na^+ over K^+ ions before and after the shock was measured to be 1.7 ± 0.4.

Depolarization of the membrane resting potential after a pulsed current-shock

According to the GHK equation, the reduction in ionic selectivity against Na^+ ions of the K channels results in a depolarization of the resting membrane potential. To confirm the occurrence of membrane depolarization, current-clamp experiments were performed. Current pulses were applied to the cell membrane and changes in the membrane potential

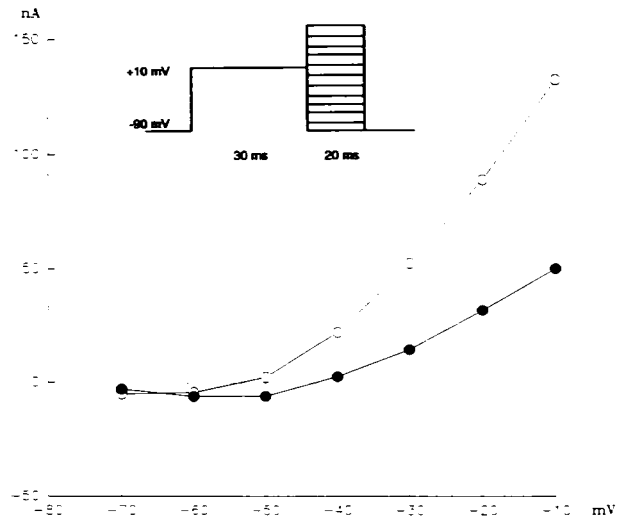


FIGURE 6 Shift in the cell membrane reversal potential caused by a high-voltage electrical shock. The inset represents the two-pulse protocol. The pre-pulse depolarized the cell membrane to +10 mV and opened most of the channels. Then, the membrane potential was held at different levels by the test pulses. The two curves represent the steady-state I-V curves of the K channel before (*top*) and after (*bottom*) exposure to a single 4 ms, −700-mV pulse. The external solution for this group of experiments was external II plus 1 μM TTX to block Na channels.

were monitored. In most fibers, pre-shocked membrane resting potentials ranged from −40 to −70 mV. These membrane resting potentials were similar to the values recorded by a membrane potential-monitoring electrode in voltage clamp mode when the gain was set to at a minimum value (the voltage-clamp now was equivalent to a voltage meter). A large-current square pulse was then delivered to shock the cell membrane, and any transient changes in membrane potential after the pulsed shock were recorded. The membrane resting potential in these studies shifted in the depolarization direction by about 7 ± 4 mV (N = 5) after a single 4 ms, 1 μA shock pulse, which is typical of the transmembrane current after a −700 mV pulse in voltage-clamp mode.

Shock pulse-dependent recovery of pulse-induced damage of voltage-gated K channels

To study the reversibility of the shock pulse-induced reductions of the voltage-gated K channels, a sequence of stimulation pulses ranging from +20 mV to +150 mV were applied three times pre-shock, and then repeatedly post-shock with a time interval of 1 min between pulses. The fiber was placed in the voltage clamp with the membrane holding potential at −90 mV, and the 4 ms shock pulses were delivered with either −600 or −900 mV. All leakage currents were computationally removed from the recorded total current. After a relaxation at the membrane holding potential for 5 min, the K channel current traces were taken again and compared with the traces taken immediately after the shock (0 minute). The K channel current reduction afterg a −600 mV shock pulse appeared to be reversible, with substantial recovery occurring within minutes. In contrast, after exposure to two 4 ms, −900 mV pulses, the K channel current declined significantly and then slowly recovered. After 40 min of post-shock relaxation, the channel current remained stable at about 50% of its initial value (Fig. 7). This result indicates that the recovery of the damaged channel proteins is directly related to both number and strength of the electric shock pulses. The mechanism of the recoverable reduction of K channel conductance might be related to reversible conformational changes, such as the rearrangement and reorientation of the charged group. Permanent damage of the K channels after an electric shock might occur because of dissociation of the charged groups of the channel proteins.

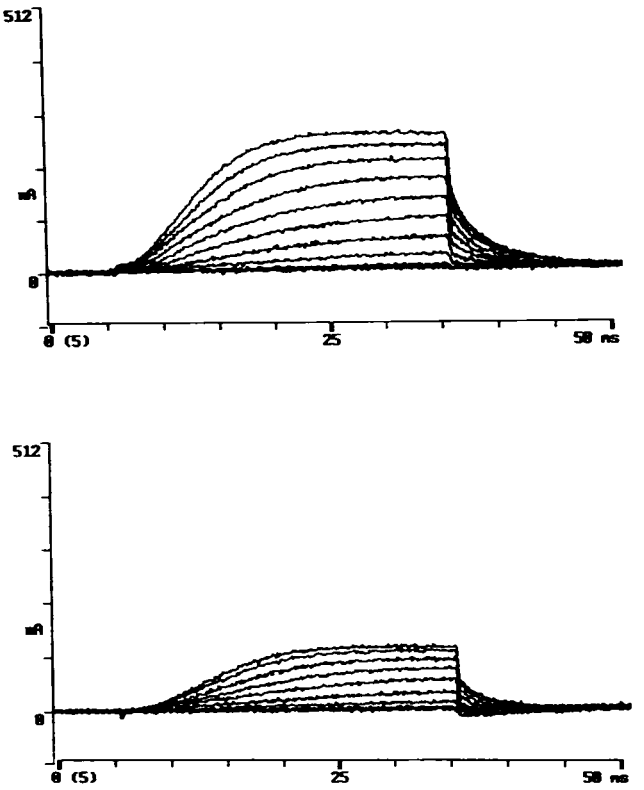


FIGURE 7 K channel currents responding to the same sequence of +20 to +160 mV pulses before and after high voltage electrical pulse shock. The external solution contains 1 μM TTX. (*top*) The family of traces of K channel currents recorded immediately before the shock. After two 4 ms, −900 mV pulses, the channel currents were dramatically reduced followed by a slow recovery. (*bottom*) The family of traces of the K channel current; 40 min later, the channel currents have reached stable values.

DISCUSSION

Membrane potential threshold for electropermeabilization and ionic channel damage

Our experimental results show that the field-induced leakage current could be measured when the membrane potential difference is hyperpolarized to a range between −250 and −350 mV. However, in this membrane potential range neither Na nor K channels conductance was altered.

The results show that both the Na and the delayed rectifier K channel conductances can be reduced when membrane potential is increased to −690 mV for a period of 4 ms. A comparison of neurotoxin blocking effects on channel proteins recorded pre- and post-shock implies that this magnitude of electrical pulse does not affect the neurotoxin binding to either the Na or the delayed rectifier K channels. At present, however, the effects of the shock pulse on the binding sites of the channel proteins during the shock pulse is unknown.

It is interesting to point out that the threshold for electroporation of cell membranes obtained in our laboratory is lower than those obtained by other investigators (O'Neill and Tung, 1991; Tovar and Tung, 1992; Gaylor, 1990; Benz et al., 1980). Studies of frog cardiac muscle using a patch clamp with a microelectrode have shown that membrane electroporation occurred when the membrane potential was held at 400 mV (Tovar and Tung, 1992) to 800 mV (O'Neill and Tung, 1991). The discrepancy between the thresholds might have resulted partly from the differences in the anatomical structures of cardiac and skeletal muscle membranes or the difference in the species of animal used in these studies. It might also have resulted from the far greater access resistance (of the order of megohms) present in patch-

electrode. The conductance of electroporated membrane is much greater than that of normal cell membrane, which results in a much greater voltage-drop on the patch-electrode during a electrical shock pulse. It can be difficult to compensate for the high resistance of patch-electrode during a high-voltage electric pulse because of a large membrane electroporated currents.

Our results demonstrate that the voltage threshold causing damage to voltage-gated Na and K channel proteins is higher than that required to electroporate cell membranes. The reason for the discrepancy is unknown. One explanation might relate to the structure of the phospholipid bilayer as a supramolecular assembly held only by forces of hydration without chemical bonding, whereas amino acids and subgroups of channel proteins are assembled with chemical bonds. Differences in charge densities and distributions might also account for the different electromechanical forces introduced by an external electric field.

It is necessary to note that in the comparison of membrane potential thresholds of ion channel damage and membrane electroporation, electroporation is only attributed to the category of fast recovery pores. There exist at least two kinds of electropores or two stages of pore formation, defined by their recovery time courses (Chen and Lee, 1994). The fast recovery pores resulting in a large leakage current seal within milliseconds, and the slow recovery pores resulting in a small amount of the leakage current take minutes to recover. A small increase in the membrane post-shock holding currents (Fig. 4) implies that after a shock by a 4 ms, −600 mV pulse, the slow recovery electropores were not significant, although the channel currents were substantially reduced.

It is worthwhile to point out that this paper focuses on the effects of high voltage electrical shock on channel proteins of cell membrane by measuring channel currents and channel conductance. No attempt has been made thus far to study changes in single channel conductivity. In the absence of single channel recordings, we can only predict that either the number of functional channels is reduced or each channel protein is partially damaged, or combination thereof.

Two mechanisms related to shifts in the membrane resting potential

According to the GHK equation, a change in the concentrations of Na^+ and K^+ ions and a change in the permeability ratio of the cell membrane can both result in a shift in the membrane resting potential. There are two pathways that can lead to changes in ionic concentrations in cytoplasmic fluid and extracellular environment: ionic channel currents and shock pulse-induced membrane leakage currents. Referring to the ionic currents passing through the K channels, Armstrong and Hille (1972) argued that the inward tail of the current that was sometimes seen after repolarization of the cell membrane after a strong depolarizing pulse resulted from an accumulation of K^+ ions just outside the cell. This tail decays with a time constant on the order of 1 ms. Presumably, the concentration of the accumulated ions falls with a time constant on the order of 1 ms. Although this result was originally obtained by studying the node of Ranvier, Armstrong and Hille's method of studying channel selectivity has also been applied on frog skeletal muscle fibers (Gay and Stanfield, 1978). In fact, in our experiments, the negative high voltage electric pulses hyperpolarized the cell membranes and prevented the K channels from opening. For stimulation pulses ranging from +20 mV to +150 mV, the recorded K channel currents in our experiments did not show any inward tails, (see traces in Figs. 2, 3, 4, and 7). This indicates that the channel-mediated accumulation of ions immediately outside the cell membrane is negligible.

As mentioned previously, two types of electropores or pore-like structures (or two stages of electroporation) can be distinguished in terms of their recovery kinetics: 1) pores that induce leakage current with a value comparable to or slightly higher than the current resulting from opening of K channels. These pores can self-seal within milliseconds post-shock; and 2) pores that induce leakage current with a value several times higher than the normal membrane holding current but much lower than that of open channel current. These pores take minutes to recover (Chen and Lee, 1994). Because the amount of pore-based leakage current during an electrical shock pulse is similar to that of the channel-mediated current, the amount of accumulated or depleted ions should be similar to that caused by open K channels. Therefore, within an order of milliseconds, similar to that caused by open K channels. Measurement pulses applied 10 s after an electrical shock pulse should be able to avoid this transient ionic accumulation. In fact, the electromediated membrane leakage currents responding to a high voltage pulse shown in the lower panels of both columns of Fig. 1 do not show any outward tail in the falling phase of the shock pulse. This indicates that the accumulation and depletion of ions is negligible. Leakage current that decays in several minutes cannot be avoided. However, the much weaker leakage current (usually up to 100 times lower than the leakage current during the electrical shock) minimizes changes in ionic concentration. A small change in membrane post-shock holding currents (Figs. 4 and 7) implies that the electroporated current caused by slow recovered pores was not significant.

It is important to note that our experimental conditions differ from a natural intact fiber by huge volumes of both intra- and extracellular compartments. The large ratio of volume-to-surface encountered in our experiments stabilizes the ionic concentration and allows us to focus on characteristics of cell membranes by minimizing changes in the ionic concentration of cytoplasm or extracellular environment.

Shifting of the membrane resting potential in a depolarization direction after a high voltage electrical pulse

Our results show a reduction in the permeability ratio of Na^+/K^+ ions of voltage-gated K channels, which predict that a single 4 ms, −700 mV pulse applied on a cell membrane could introduce a shift in the membrane resting potential

from −90 to −84 mV. This predicts a shift in the membrane resting potential of about 6 mV in the depolarization direction. This value, derived from the GHK equation, compares well with that from our experiments using the current-clamp mode.

Two mechanisms can underlie the shifts in membrane resting potential: one is the change in ion concentrations, and the other is the reduction in ionic permeability ratio, P_K/P_{Na}, of the cell membrane. We do not know the proportion of the shift in the membrane resting potential attributable to the ionic concentration change and to the channel permeability ratio reduction. However, as mentioned previously, it is reasonable to assume that the concentration of K^+ and Na^+ ions in the central pool and the end pools are relatively stable. If this assumption is true, as in our cut-fiber experiments, damage to membrane proteins plays a major role in shifts in the membrane resting potential after the application of a high voltage pulse.

Ionic selectivity change

Because both Na and K ions are smaller in size than 3 Å of the diameter of the narrowest part of the pore (the selectivity filter of the K channel), our results do not provide information about the changes in the size in the selectivity pores. However, according to the Esinmann theory (Krasne, 1978), a so-called weak-field strength of the selective filter of K channels has been assumed to explain the high selectivity against Na^+ ions. The low-field strength might be a result of protein's low-charge density of the subgroup located at a far distance. Random orientation of each dipole might cancel each other and form a weak electrical dipole. When exposed to a high intensity electrical field, protein electroconformational coupling might move or reorient itself towards the electrical dipole parallel to the external electrical field. The channel protein conformational change might involve one or more of the following stages: randomly orientated electrical dipoles are reorientated under the force of the external electrical field and form a permanent electrical dipole; some charges relocate in closer proximity to one another and form a strong electrical dipole; the direction of the permanent dipole of a protein channel, located at the narrowest part of the pore, changes so that its negative part is directed to the inner pore of the selective filter; or the structure of the subgroup at the filter changes, so that charges on the pore come into close contact with ions. The final result is that the total equivalent site-ion interaction energies are increased, and the ionic permeability ratio of a K channel becomes less dependent on the dehydration energy. Direct result is a reduction in ionic selectivity of the K channel against Na^+ ions.

In summary, the improved double vaseline-gap voltage clamp allows us to distinguish the supraphysiological pulse-induced changes in ionic channel currents and membrane nonselective leakage current. By using this technique, we have presented evidences that high voltage pulse introduces not only electromediated pores or pore-like structures in cell membranes, but also electroconformational damage in channel proteins. Functional changes in voltage-gated Na and K channels were also investigated. These results suggest that muscle and nerve dysfunction after electrical shock is in part caused by electroconformational damage of voltage-gated ion channels. As an example, the field-induced reduction of channel conductance and the ionic selectivity of the delayed rectifier K channels might reveal a mechanism, in addition to electroporation, that contributes to depolarization of the resting membrane potential.

We wish to thank Diane Rudall for her editorial assistance. This research is supported by grants from Electric Power Research Institute and the Empire State Electric Energy Research Corporations.

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