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Effects of Electric Field on the Functions of Cell Membrane Proteins

Zhongsheng Zhang
University of South Florida

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Effects of Electric Field on the Functions of Cell Membrane Proteins

by

Zhongsheng Zhang

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Physics
College of Arts and Sciences
University of South Florida

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Date of Approval:
February 21, 2008

Keywords: channel, pump, synchronization, modulation, kidney

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Dedication

To my wife Hongwei Shang and my son Kevin K. Zhang
Acknowledgments

This dissertation is accomplished under the supervision of Dr. Wei Chen, without whose instruction, guidance and encouragement, many meaningful results would not have been achieved.

Special thanks to chairman Dr. Randy Larsen and committee members, Dr. Dennis K. Killinger, Dr. David Rabson and Dr. Garrett Matthews, for their support, help and suggestions.

NIH and NSF have also supported this work.
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Effects of Electric Field on the Functions of Cell Membrane Proteins

Zhongsheng Zhang

Abstract

The most important and most common channels on cell membrane are voltage-gated Na+ and K+ channels. In so-called “excitable cells” like neurons and muscle cells, these channels open or close in response to changes in potential across the membrane in order to accomplish muscle contraction and transmit signals. By controlling the membrane potential, we observe extraordinary inactivation behaviors of the voltage-gated Na+ channels and the voltage-gated delayed rectifier K+ channels, which shows that electric stimulation pulses can temporarily close the Na+ and K+ channels, just as drugs, like tetrodotoxin (TTX) and tetraethylammonium (ETA), do.

The Na/K pump is essential for living system and is expressed in virtually all cell membranes. The ionic transport conducted by Na/K pumps creates both an electrical and a chemical gradient across the plasma membrane, which are required for maintaining membrane potentials, cell volume, and secondary active transport of other solutes, etc. We use a pulsed, symmetric, oscillating membrane potential with a frequency close to the mean physiological turnover rate across the cell membrane to synchronize Na/K pump molecules. The pump molecules can work as a group, pumping at a synchronized pace after a long train of pulses. As a result, the pump functions can be significantly increased. After the pump molecules are synchronized, the applied electric-field frequency can...
gradually increase in order to resynchronize the molecules to a new, higher frequency. Modulating the pump molecules to a higher frequency leads to a significant increase of pump current. Synchronization and modulation of pump molecules can become a new method to study the function of Na/K pump molecules. This method has huge potential applications in clinic medical treatment.

After single-fiber-level study, the final project is on organ level, the rat kidney, by using synchronization and modulation of Na/K pump molecules on the proximal tubule membrane. Because Na+ re-absorption is directly related to the function of the Na/K pump, the more active Na/K pumps are, the more Na+ ions can be absorbed, which results in an increased potential inside the renal proximal tubule. This project is the first step of synchronization and modulation applied on the level of an organ.
Chapter 1

Introduction

A living cell is an extraordinarily complex, dynamic, and physicochemical system which maintains in or near the steady state by continual entry and exit of materials and energy to finish self-assembling and self-replicating process. The cell boundaries are formed by a dynamic membrane which is consisting of phospholipids, cholesterol, proteins, and carbohydrates, etc. The typical thickness of cell membrane is about 2-6 nanometers, which physically and chemically isolates cells (cytoplasm) from their environments. Cell membrane is essential for the integrity and functions of the cell. The main functions of cell membrane include: (1) Protect the cell from environments (2) Regulate the transport materials in and out of the cell by such methods as pumps, channels, and exchangers, etc. (3) Provide stable binding sites for the enzymes catalysis (4) Allow cells to recognize each other (5) Provide anchoring sites for cytoskeletal filaments and components to group cells together to form tissues [1, 3, 4]. Figure 1.1 shows three different views on cell membrane and basic components of cell membrane [2]. Part (A) is a human red blood cell membrane of electron micrograph in cross-section. Part (B) and (C) are schematic drawings of two-dimensional and three-dimensional views of a cell membrane and its basic components.
Figure 1.1 Three different views and the basic components of a cell membrane [2].

All cell membranes contain lipid bi-layers and proteins: lipid bi-layers provide the basic compartmentalization function of the cell membranes, whereas proteins invest membranes with their specialized functions: signaling, transport, and catalysis, etc. [3, 4]. Membrane proteins are very important to the regulation of cell behaviors. For example, some proteins in cell membrane are receptor proteins, which deal with communication and recognition between cells [12]; and some are transport proteins that regulate the movement of ions and soluble molecules through the cell membrane, like sodium (Na+) and potassium (K+) ions channels [4]. Each type of cell membranes contains specific proteins and lipid bi-layers components that enable them to perform their unique roles for cells functions [1, 4].

The concentration of ions inside the cell is different from that of outside in order to maintain cell living conditions. For example, the cytoplasm of animal cells contains a
concentration of K+ ions as much as 20-40 times higher than that in the extra-cellular fluid. Conversely, the extra-cellular fluid contains a concentration of Na+ ions as much as 10 times greater than that within the cell (Table 1.1) [1]. At equilibrium state, each kind of ion with the concentration difference generates a voltage across the cell membrane. This potential can be calculated by Nernst equation [1], which is derived from basic principles of physical chemistry. For example, the electric potential generated by Na+ ions (Na+ ions’ equilibrium potential) across the membrane can be calculated by the following equation:

$$E(Na) = \frac{RT}{ZF} \ln\left(\frac{[Na]_i}{[Na]_o}\right)$$

where R is the universal gas constant, T is the absolute temperature in degrees Kelvin, z is the charge number of the electrode reaction, and F is the Faraday constant. If $[Na]_i/[Na]_o = 0.12$ (the rate of Na+ ions concentration inside of the cell to that outside of the cell), then $E(Na) = -67$ mV, which shows that the inside is negative with respect to the outside (Table 1.1). In living cells, all different ion types are simultaneously present and contribute to the resting potential across the cell membrane, and the presence of pumps maintains these concentration differences [1]. It is found that the resting potential of the cell membrane is about -70 mV to -100 mV for most animal cells [1]. The direct effect of changing membrane potential is to modulate the transport properties of charged particles across the membrane and affect cell mechanical properties.
<table>
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<th>Extracellular Concentration</th>
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<td>Na+</td>
<td>12 mM</td>
<td>145 mM</td>
<td>-67 mV</td>
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<tr>
<td>K+</td>
<td>155 mM</td>
<td>4 mM</td>
<td>+98 mV</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>0.001 mM</td>
<td>1.5 mM</td>
<td>-129 mV</td>
</tr>
<tr>
<td>Cl-</td>
<td>4 mM</td>
<td>123 mM</td>
<td>+90 mV</td>
</tr>
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</table>

Table 1.1 Ion distributions and Nernst potentials across skeletal muscle membrane [1].

Most ions, like Na\(^+\), K\(^+\), H\(^+\), Ca\(^{2+}\), Cl\(^-\), SO\(_4\)\(^{-2}\), are transported through the cell membranes by transport proteins [1, 3, 4], and each type of protein mainly transports a unique substance in order to maintain the cell compositions, volume and membrane potential. Depending on the transport proteins, transport occurs by different mechanisms - those that do not consume energy in the form of ATP form channels (passive transport) and those that do consume ATP form pumps (active transport) [1]. In order to understand how concentration gradient and membrane potential are formed through the cell membrane by channels and pumps, we have to know which ion is transported across the cell membrane, how the ions are transported and what the different mechanisms between different transport systems are.
Ion channels are pore-like proteins spanning the cell membrane [1, 4]. Ion channels can open simultaneously at both sides of the membrane, and ions move through these channels. Transport through channels is always down electrochemical potential gradient (electric potential and chemical gradients), so membrane potential can change the channel functions and behaviors. Transport properties of a particular ion channel are the result of summation of hundreds and thousands of single channels over time (different ion channels have different density on different cell membrane). There are a lot of kinds of channels among the cell membrane with different functions and different “open and close” mechanisms. The conformational change between closed and open states is called gating system. Ion channels can be classified according to which chemical or physical modulator controls their gating activities. Thus, we have different groups of channels as voltage-gated channels, ligand-gated channels, and second-messenger-gated channels, etc [1]. The most important and most common channels are voltage-gated Na+ and K+ channels [1, 7] (“voltage-gated” means the probability of a channel opening or closing purely depends on the membrane potential). In so-called “excitable cells” like neuron and muscle cells, those channels open or close in response to change in action potential across the cell membrane.

All ion channels show selectivity - prefer certain ions while rejecting others, but none of ion channels have an absolute selectivity for a single ion species [1]. It has been proved that channels, though selectively permeable, could pass many ionic species. Thus, Na+ channel is a channel that Na+ ions normally permeates, but other ions still can go through with much lower permeability than Na+ ions. Similarly, the K+ channel also allows ions other than K+ ions to pass [49]. The sequence of ions to which a channel is
permeable is according to the permeability (the selectivity sequence), which is a characteristic property of channel [1].

A large number of pharmacological agents (neurotoxins) have been identified which can affect ionic channels of electrically excitable cells, called channel blocker. The most important and common agents are Tetrodotoxin (TTX) [5] and Teraethylammonium (TEA) [6], which selectively block Na+ and K+ channels, respectively. Only nano-molar concentration of TTX is required to block 100% Na+ channels, and micro-molar concentration of TEA is required to block most K+ channels. These agents enable investigators to block either Na+ channels or K+ channels in a reversible manner. Therefore, it is possible to study the individual kind of ionic channel.

Channels can reduce the ionic gradients across the membrane because the ions always move from high concentration to lower concentration when channels are opened. The concentration gradients across the membrane are established and maintained mainly by active transport system [13, 14, 130, 131], which process generally requires chemical reactions of binding the transported substances to a protein, then “pumping” the ions in or out of the cell through the cell membrane. The reason that this kind of protein is called “pump” is that the ions are transported against concentration gradient or electric field. For example, Na/K pump proteins, for each loop, extrudes three Na+ ions and intrudes two K+ ions, both from low concentration side to high concentration side [8]. This is one reason why active transport needs a lot of energy from the hydrolysis process of ATP to finish this loop [8, 9, 10]. It has been estimated that roughly 20-30% of all ATP is hydrolyzed by Na/K pumps in a resting human being [11, 13].
There are a lot of kinds of pump proteins with different mechanisms and transporting different substances on the cell membrane. Na/K pump protein is the most important and basic active transport protein which is found in different kinds of cells with a slightly different structure but almost the same functions [9, 10]. Na/K pump can create and maintain trans-membrane ions concentration gradients of Na+ and K+ ions, which then form energy source that is used by cells for a variety of tasks, including several vital functions [1, 9, 13]: (1) It helps establish a potential across the cell membrane with the interior of cell being negatively charged in respect to the exterior. This resting potential prepares for the propagation of action potentials leading to nerve impulse and muscle contraction. (2) The accumulation of Na+ ions outside of cell draws water out of cell and, thus, enables the cell to maintain osmotic balance. (3) In steady state, the passive flows of Na+ ions and K+ ions from high concentration side to low concentration side of the cell membrane are mainly balanced by active Na/K pump transport.

The stoichiometry of Na/K pump appears that one molecule of ATP is hydrolyzed to one molecule of ADP and one molecule of phosphate, three molecules of Na+ are transported outward and two molecules of K+ are transported inward [8, 9, 10]. Under these circumstances, Na/K pump is electrogenic [10, 14], producing a net current that will result in membrane hyper-polarization. Since Na/K pump requires both Na+ and K+ ions and catalyzes hydrolysis of ATP, it has been called Na/K-ATPase.

Contrary to ionic channels operation, (1) Na/K pump proteins are never open to both sides of the membrane simultaneously. (2) Na/K pump proteins are working all the times over the physiological membrane potential till this potential is lower than Na/K pump equilibrium potential which is about -300 mV [1, 80]. Na/K pump protein’s
movement can be explained by a relatively simple chemical cycle that includes several steps: binding ions, conformational change, and releasing ions, etc. Na/K pump protein apparently has two primary conformational states called E1 and E2 [1, 23, 105]. These two conformational states have different affinities for Na+, K+ and ATP. In each cycle of reaction sequence, E1 and E2 alternately bind and release Na+ and K+ ions and catalyze hydrolysis of ATP. The E1 conformation has an ion-binding site that faces cytoplasm and binds Na+ ions, whereas E2 conformation has an ion-binding site that faces extracellularly and binds K+ ions. As of today, the sequence of Na/K pump transport events can be summarized as follows: (1) Pump at E1 conformation state, binding ATP and 3 intracellular Na+ ions; (2) ATP hydrolyzed to ADP and P, with ADP released resulting in an occluded state, E1-P-[3Na+]; (3) Conformational change of Na/K pump to expose the Na+ ions to the outside of the cell membrane, where they are released, resulting in E1-P state. The release of Na+ ions may be a release of individual ions through intermediate reactions [15]; (4) Conformation of E1-P state converts to conformation of E2-P state; (5) Conformation of E2-P binding 2 extra-cellular K+ ions; (6) Na/K pump reoriented. Two K+ ions are released in the cytoplasm side; and (7) Na/K pump in E1 conformation state again and ready for next cycle. Step 3 and step 6 are rate-limiting steps [1, 16, 103]. Because the ions move against the electric potential and concentration gradient by using the energy released from ATP, the time of these two rated-limiting steps takes up the majority of the entire motion time. Changing rate-limiting steps will change the whole cycle time [16, 84, 90, 103].

Na/K pump proteins can be blocked by cardiac glycosides, such as Ouabain [1, 18, 22], applied in extracellular solution with micro-molar concentration. Active efflux of
Na+ ions and influx of K+ ions are blocked in a matter of minutes for muscle cells [1]. Because of its specificity of action, Ouabain has been enormously used to study Na/K pump proteins. In fact, the blockage of the flux components by Ouabain is taken as strong evidence that this component is transported by Na/K pump proteins. The pump current is also called Ouabain-sensitive current.

As described above, membrane potential plays a very important role in the functions of channels and pumps. External electric fields can produce a variety of profound biochemical and physiological changes on the cell membrane proteins [17, 19, 20, 21]. My research focuses on how oscillation electric fields influence the functions of Na+ channel, K+ channel and Na/K pump proteins by changing the membrane potential according to well-designed potential protocols.
Chapter 2

Inactivation of Voltage-Dependent Na+ Channel by Repeated DC and AC Stimulations

Introduction

The macromolecule of Na+ channel protein is the first voltage-gated ion channel which is isolated and sequenced from electric organs of electric eel [24], and later from a number of other tissues including rat skeletal muscle, rabbit skeletal muscle, and human brain, etc [26, 27, 28, 33]. Isolation of Na+ channel macromolecule allows researchers to determine, using methods of molecular biology, the nucleotide sequence of genes encoding the primary structure of Na+ channel protein’s amino acid residues sequence [29, 30, 31].

Na+ channel proteins of different cells have different number of amino acid residues. For example, analysis of Na+ channel sequence of electrophorus electricus electroplax indicates that this Na+ channel protein consists of 1,820 amino acid residues [24]; while the amino acid sequence of a Na+ channel from squid loligo bleekeri contains 1,522 amino acid residues [32]. However, all Na+ channel proteins consist of four domains, I-IV [1], with each domain consisting of eight hydrophobic segments (Figure 2.1) [25]. Six long segments (S1-S6) are $\alpha$ helices that span the cell membrane, and two short segments (SSI and SSII) are inserted in the membrane (face outside of the membrane). The filled circles on the connecting segments between S5 and S6 represent a
TTX binding site. An inactivation “ball” exists between domains III and IV (inside of the membrane). Initial determination of the amino acid sequence reveals that the segment S4 [40, 50] contains four amino acids with positively charged residues (Figure 2.1), which are responsible for sensing the membrane potential.

Figure 2.1 Diagram depicting a Na+ channel protein with four repeating units (domains), each of which consists of eight hydrophobic segments. Six long segments (S1-S6) are α helices that span the membrane, and two short segments (SSI and SSII) are inserted in the membrane. The filled circle on the connecting segments between S5 and S6 represents a TTX binding site. An inactivation ball exists between domains III and IV inside of the membrane [25].

Na+ channel is formed as a tetramer where each domain from I to IV forms a quarter of the channel. The ionic pore has a large aqueous cavity, with a gate close to the
interior and a selectivity filter on the outer vestibule (Figure 2.2) [25]. The mouth of ion channel is found to be about 1.2 nm, narrowing to 0.3-0.5 nm. This narrowing of the channel forms a selectivity filter [33].

Figure 2.2 Cross section of Na+ channel protein with an inactivation gate, selectivity filter and TTX binding site [25].

Voltage-gated Na+ channel is normally closed at resting potential. However, in response to the membrane potential depolarization, channel can change from closed state to open state (Figure 2.3). Then, Na+ channel transits to inactive state automatically [34, 35, 36, 37], followed by a close state after the cell membrane potential becomes re-
polarized. Inactivation is a distinct and major property of voltage-gated Na+ channel, which has a fast decay phase [36]. The time of inactivation is within millisecond range. The reason that Na+ channel can be inactive automatically is that there is a special structure responsible for inactivation of the channel between domain III and domain IV. Once channel is opened, even though depolarization potential is still maintained, conduction stops. This is thought to be the result of docking the region (“ball”) of protein into internal mouth of the channel. Inactivation process is coupled with the opening of channel; that is, those two events are not independent (Figure 2.3). What will happen when depolarization is terminated and membrane potential returns to normal values? Channel can not function fully until the “ball” exit from the mouth of the channel. For example, when the membrane potential is returned to -90 mV, it takes an average of 2-3 ms [36] for all “balls” to exit from the internal mouths of channels. This time has an important consequence because if membrane potential is depolarized again during 2 or 3 ms, not all channels are able to conduct as some “balls” are still in or partially in the inactivation position (channel stays in the refractory period [1]). This is one of the reasons why an action potential can not be followed too closely by another in “excitable cells”.
Figure 2.3 Activation and inactivation processes of Na+ ion channel dependent on the membrane potential.

Although most attention is initially focused on refining knowledge of ionic current, investigations of capacitance current give important new insights into channel mechanisms [38, 39, 40, 44]. The capacitance current is also called gating current or displacement current, which is generated by the motion and redistribution of charges inside membrane that accompanies opening or closing of an ionic channel. Since the gate is in molecular scale, it is subject to the thermal effects. Hence, the gate opening and closing are random; however, its probability of being in open state is increased as membrane potential increases (non-linear relationship) [41]. When the gate is opened, the positive gating charges move from inner surface of membrane to outer surface, which signals conformational change of channel macromolecule. In response to a step of membrane potential, the total gating charges, Q (on), estimated by integrating the gating
current from the onset of membrane potential pulse, are equal to the total gating charges, $Q_{(off)}$, estimated from the offset of the pulse [1]. This result indicates that the redistribution of charges in membrane is reversible [1, 39]. After gating charges are moved, the gate opens, and Na$^+$ ions flow through the channel. So the inactivation “ball” and gating charges are working together to control Na$^+$ channel opening and closing behaviors.

Pharmacological manipulation can not only block conduction of channels (TTX is used to block Na$^+$ channel currents), but also manipulate channel kinetics. For example, proteolytic enzyme pronase, applied at intracellular, removes the “ball” structure that is responsible to inactivation from Na$^+$ channel macromolecule without affecting the part that is responsible for activation of the channel [42]. This result suggests a segregation of function within Na$^+$ channel macromolecule.

**Methods and Materials**

The experimental techniques and recipes of the solutions used in this research are developed previously [43] and widely used in our lab.

(1) Solutions (mM) [43]:

- **Normal Ringer:**
  - 120 NaCl
  - 2.5 KCl
  - 2.15 Na$_2$HPO$_4$
  - 0.85 NaH$_2$PO$_4$. H$_2$O
  - 1.8 CaCl$_2$.H$_2$O

- **Relaxing Solution:**
  - 120 K-glutamate
<table>
<thead>
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<th>External Solution:</th>
<th>Internal Solution:</th>
</tr>
</thead>
<tbody>
<tr>
<td>120 NaCl</td>
<td>45.5 Cs-glutamate</td>
</tr>
<tr>
<td>5.4 KCl</td>
<td>5 Cs₂-PIPES</td>
</tr>
<tr>
<td>4 MOPS</td>
<td>20 Cs₂-EGTA</td>
</tr>
<tr>
<td>1.8 MgCl₂</td>
<td>6.8 MgSO₄</td>
</tr>
<tr>
<td>2 BaCl₂</td>
<td>5 Glucose</td>
</tr>
<tr>
<td>0.2 CdCl₂</td>
<td>5.5 Na₂-ATP</td>
</tr>
<tr>
<td>1 CsCl</td>
<td>20 Tris-Creatine Phosphate</td>
</tr>
<tr>
<td>3, 4 Diminopyridine (DAP),</td>
<td></td>
</tr>
</tbody>
</table>

(2) Voltage clamp

TEV-200 (Two-Electrode-Voltage-Clamp) has been designed as a general purpose voltage clamp with two electrodes for control (Channel 1 and Channel 2) and one electrode for grounding (Figure 2.4). Channel 1 continuously records the actual cell membrane potential and that value is compared to the command potential, which is
generated from either PClamp software or LabView program according to different experiments. When the membrane potential need be clamped, other electrode (Channel 2) continuously passes current to maintain the membrane at the command potential through a feedback loop. In addition, TEV-200 features a virtual current monitor/bath clamp (VCM/BC) head-stage which monitors the sum of channel currents flowing through preparation and simultaneously maintains the bath at zero potential (Grounding).

Figure 2.4 Photograph of the voltage clamp (TEV-200).
(3) Skeletal muscle fiber preparation [43]

Frogs (rana pipiens) are killed by rapid neck disarticulation, in accordance with the protocol approved by Institutional Animal Care and Use Committee at University of South Florida. Skeletal twitch muscles, semitendinosus and illus, are dissected and removed from frog hindlimb, then put into a dish with Normal Ringer solution. This treatment is necessary to depolarize the muscle fiber and prevent cells from contraction during dissection and experimental preparation. After muscle is put into a dish filled with Normal Ringer solution and pinned down, change relaxing solution and wait about 10 min before single fiber dissection. A single muscle fiber with good quality (clear, about 50-150 um diameter and 3-6 mm long) is hand-dissected and transferred to a custom-made chamber [44, 45, 46] (Figure 2.5) filled with the relaxing solution. After that, the fiber is clamped by two plastic clips at both ends. Adjusting the clips allows fiber to be stretched properly to avoid fiber contraction during chemical treatment and electric stimulation. The width of two partitions is about 100 um and the width of central pool is about 300 um. Thin Vaseline and two plastic glass cover slips are used to electrically isolate the three pools from each other. The fiber segments in two end pools are treated by a solution with 0.1% Saponin for two minutes [43] (it makes the two fiber segments electrically and ionically permeable within the two end pools.) and washed out with the internal solution by 3 times. Finally, the central pool solution then is changed with the external solution by 3 times. The whole process time is about an hour.
(4) Experiment setup

Three agar bridges connect three pools (one central pool and two end pools) to three small ponds filled with 3M KCl. The agar bridges are glass tubes with inner diameter of 1 mm filled with agar gel made by 3M KCl solution. Three intermediate ponds are then connected to voltage clamps by three Ag/AgCl pallets to make the pass way resistances as low as possible. Figure 2.6 shows the sketch of whole experiment setup including chamber, voltage clamp and controlling computer.
Figure 2.6 Sketch of the setup for Na+ channel currents measurement including chamber, voltage clamp and controlling computer, etc.

(5) Data acquisition

Both stimulation pulse generation and signal recording are either carried out with a DAQ multifunction system (National Instruments PCI6036E) controlled by LabView programs or PClamp software. PCI6036E is low-cost 16-bit DAQ to deliver reliable performance in a wide range of applications. The advantage of this system is to allow researchers to make different LabView programs according to different purposes of experiments with different parameters. PClamp is a commercialized software package for
voltage clamp and patch clamp, which is very easy to use but has a lot of limitations for our research. An example of interface of PClamp software for Na+ channel currents measurement is shown in Figure 2.7: top panel is the actual voltages which are delivered on the cell membrane and bottom panel is the actual measured Na+ channel currents using the P/4 method.

Figure 2.7 An example of PClamp software interface used in Na+ channel currents measurement with the top figure showing the actual voltages which are delivered on the cell membrane and the bottom figure.

(6) P/4 method

When membrane potential is depolarized, the recorded trans-membrane current generally consists of three major components: (1) Linear leakage current, which is the
current that flows through membrane resistances and membrane capacitances. Ideally, this current scales linearly with the value of depolarization potential. (2) Nonlinear gating current, which is the displacement current associated with the movements of gating charges inside of the membrane [38, 39]. (3) Nonlinear, voltage-activated current [1], which is the current that flows through ion channels when channels open due to change in the membrane potential. The channel current does not linearly depend on the membrane potential.

In order to easily study nonlinear, voltage-activated current component, it is essential to remove the linear leakage current from the record current (gating current is too small in comparison to the channel current). Briefly, a series of scaled-down replicas (sub-sweep) of the stimulation pulses are applied to the cell membrane prior to or after actual stimulation pulses. Then, the accumulated sub-sweep response current is subtracted from actual stimulation response current. The remaining current is nonlinear voltage-activated current. This technique is called P/N subtraction, where N is the number of sub-sweeps that each has 1/Nth of the magnitude of main stimulation pulse (usually, N equals to 4, thus called P/4 method). An example of P/4 subtraction protocol is shown below. In Figure 2.8, the main stimulation pulse consists of a single depolarizing step function with the magnitude of +40 mV compared to the holding potential of –90 mV. There are four sub-sweeps with 1/4 of this magnitude (+10 mV), executed before the main pulse. Sub-sweeps are executed at their own holding level. In this example, the normal holding potential level is –90 mV. It is desirable to execute the sub-sweeps at –110 mV because in this potential the ionic channels can not be opened. After the normal holding potential is changed to sub-sweep holding level, the program
waits a specified setting time at sub-sweep holding level before sending sub-sweeps. After the sub-sweeps are completed, membrane potential returns to the normal holding level (-90 mV) to accomplish the main step pulse function.

Figure 2.8 An example of P/4 subtraction protocol. The main pulse consists of a single depolarizing step function with a magnitude of +40 mV compared to the holding potential of -90 mV. There are four sub-sweeps with 1/4 of this magnitude (+10 mV), executed before the main pulse with the holding potential at -110 mV.
Figure 2.9 Measurement of trans-membrane current without using P/4 method.

Figure 2.10 Na+ channel current after using P/4 method.
Figure 2.9 and 2.10 show the measured current before subtraction (without P4 method, Figure 2.9) and the current after the subtraction of sub-sweeps (P/4 method). The remaining current (Figure 2.10) is the nonlinear voltage-activated current - Na+ channel current.

(7) Electric stimulation pulse trains

In this experiment, two main stimulation pulses are used: one is unidirectional stimulation pulse train with 2000 pulses as Figure 2.11 shows. The duration of pulse is 8 ms and the magnitude of each pulse is +50 mV; the other is symmetric stimulation pulse train as Figure 2.12 shows. The reason to use two different trains is mainly because Na+ channel proteins have different behaviors under the influence of those two kinds of electric stimulation trains.

Figure 2.11 Unidirectional stimulation pulse train consisting of 2000 pulses. The duration of each pulse is 8 ms and the magnitude is +50 mV with the holding potential of -90 mV.
Figure 2.12 Symmetric stimulation pulse train consisting of 2000 pulses. The duration of each pulse is 8 ms and the magnitude is +/-50 mV with the holding potential of -90 mV.

(8) All the experiments are conducted at room temperature including those explained in the following chapters unless otherwise indicated.

**Experimental Results**

When the cell membrane is depolarized, the combination currents, mainly Na+ ions and K+ ions channel currents, can be measured. The early transient current is carried by Na+ ions and later the persistent current is carried by K+ ions. As mentioned before, TEA can block the later current component which is normally carried by K+ ions without affecting the early Na+ current component. In this experiment, in order to identify voltage-gated Na+ channel current, K+ ions inside the cell are substituted by Cs+ ions to reduce outward K+ current. 3 mM of 3, 4 diminopyridine (DAP), which is another K+
channel blocker, is added to the external solution (TEA already in the solution). Since the kinetics of Na+ channel current are much faster than those of K+ channel current, the remaining small K+ channel current does not affect the accuracy of Na+ channel current measurement.

A sequence of 28 stimulating pulses with 8 ms duration holding the membrane potential at a range from -70 mV to -2.5 mV is first applied on the cell membrane (membrane holding potential is -90 mV, which is very close to resting potential in physiological condition). The difference of the potential between two consecutive pulses is 2.5 mV (Figure 2.13). The relaxing time between two consecutive pulses is 2 s. The trans-membrane currents responding to each stimulation pulse are recorded. After subtracting the linear leakage current (P/4 method), Na+ channel currents responding to different membrane potentials are plotted in Figure 2.14.

Figure 2.13 Diagram of a sequence of 28 stimulating pulses with the duration of 8 ms and the potentials ranging from -70 mV to -2.5 mV. The increase between two consecutive pulses is 2.5 mV. The relaxing time between two consecutive pulses is 2 s.
Figure 2.14 Na+ channel currents by using P/4 method responding to the pulses shown on Figure 2.13.

Figure 2.15 Relationship between peak values of currents corresponding to different membrane potentials (Na+ channel I-V curve).
In Figure 2.15, the triangles represent the peak values of each Na+ channel current corresponding to different potentials from Figure 2.14. The crossing point (channel current equal to zero) of Na+ channel I-V curve with X-axis represents the reversal potential. At this potential, the chemical force due to ionic concentration gradient and the electric driving force due to the applied membrane potential are balanced to each other, so there is no Na+ channel current at this potential. In our experiments, both external and internal solutions are designed to reduce reversal potential, which normally is 60 mV for frog skeletal muscle fiber. The result of reversal potential from Figure 2.15 is very close to the estimated number (-15 mV). The slope of Na+ channel I-V curves represents channel conductance.

After Na+ channel I-V curve measurement, the membrane potential is changed according to the designed protocol (Figure 2.11), which is a train of unidirectional stimulation pulses. Potential and responding trans-membrane current are simultaneously recorded during the whole stimulation period. Using P/4 method, the linear leakage current is removed from the raw data. For each pulse, an increasing percentage of channel proteins remain in the un-conductive state (inactivation state or closed state). After 2000 of unidirectional stimulation pulses, almost all individual channel proteins remain in the un-conductive state. This indicates that Na+ channel currents can be blocked by electric method (Figure 2.16).
To illustrate the result more clearly, Figure 2.16 just shows some of Na+ channel currents within 2000 pulses (1\textsuperscript{st}, 10\textsuperscript{th}, 20\textsuperscript{th} \ldots 100\textsuperscript{th}, 200\textsuperscript{th}, \ldots, 2000\textsuperscript{th}). The result indicates Na+ channel currents gradually decrease with the increase of pulses, until almost all the channels reach the un-conductive state. All Na+ channel currents have the same rising phase, falling phase and peak position.

Figure 2.17 shows the relationship between the peak values of each Na+ channel current and the index of the pulse. It is clear that, after 2000 pulses, Na+ channel currents gradually reach zero (< 5%).
Figure 2.17 Relationship between the peak values of Na+ channel currents and the index of the pulses.

Figure 2.18 Na+ channel currents measured under a symmetric stimulation train. The currents do not show distinct effect when 2000 pulses are applied on the cell membrane.
We change the stimulation train to a new protocol (Figure 2.12), which is a symmetric pulse train as compared to the previous protocol. The new train has the same positive magnitude (+50 mV relative to -90 mV) and negative magnitude (-50 mV relative to -90 mV) with 8 ms pulse duration. Interestingly, Na+ channel currents do not show distinguishable effects during the period when 2000 pulses are applied on the cell membrane. Figure 2.18 (1st, 10th, 20th …100th, 200th, …, 2000th) shows some of Na+ channel currents during symmetric stimulation train.

Other experiments are performed to confirm that those phenomena result from Na+ channel function change, not because of the change of local ionic concentration around Na+ channels, which concentration becomes lower and lower with continuous flow of Na+ ions into the cell. We use two different potential trains (like Figure 2.11) on the same fiber: one potential is lower than the reversal potential (-40 mV) and another potential is higher than the reversal potential (-10 mV). In both experiments, the peak current absolute values (Figure 2.19 and Figure 2.20) gradually decease, the results similar to the results that we get before. The crossing points for the same order of two Na+ currents are almost the same in Figure 2.21 (1st, 10th, 20th …100th, 200th, …, 2000th). This result proves that the concentrations of Na+ ions inside and outside of the cell are the same as original values no matter how many pulses are applied on the cell membrane. Quick diffusion of Na+ ions in the external solution provides enough ions flow through Na+ channels for each pulse.
Figure 2.19 Measurements of Na+ channel currents by using a unidirectional stimulation pulse train when this pulse potential is lower than the reversal potential.

Figure 2.20 Measurements of Na+ channel currents by using a unidirectional stimulation pulse train when this pulse potential is higher than the reversal potential.
Figure 2.21 Lines connecting Na+ current peak values of Figure 2.20 and that of Figure 2.21 cross nearly at the same point.

In previous experiments, the duration of pulse potential and the duration of holding potential are both 8 ms. To further illustrate the relationship between the duration of pulse and the duration of holding potential, we finished a series of experiments by using one single fiber with different holding potential time trains. Figure 2.22 shows the stimulation pulse train. We change the duration of T for each experiments (T = 4 ms, 10 ms, 20 ms and 200 ms). Figure 2.23 to Figure 2.26 show the responding results under different holding potential duration trains respectively. With the increase of the holding time, it becomes more difficult to force Na+ ion channel proteins to remain in the un-conductive states. In those figures, the same selected currents (1\textsuperscript{st}, 10\textsuperscript{th}, 20\textsuperscript{th} …100\textsuperscript{th}, 200\textsuperscript{th}, …, 2000\textsuperscript{th}) as before are used.
Figure 2.22 Sketch of unidirectional stimulation pulse trains with different holding potential durations.

Figure 2.23 Na+ channel currents responding to the holding potential of -90 mV with duration of 4 ms.
Figure 2.24 Na+ channel currents responding to the holding potential of -90 mV with duration of 10 ms.

Figure 2.25 Na+ channel currents responding to the holding potential of -90 mV with duration of 20 ms.
Figure 2.26 Na+ channel currents responding to the holding potential of -90 mV with duration of 200 ms.

Figure 2.27 Relationship between the peak values of Na+ channel currents and the index of pulses. The curves represent holding durations of T=4 ms, T=10 ms, T=20 ms and T=200 ms from the top to the bottom.
In Figure 2.27, X-axis represents the index of each pulse. Y-axis represents the peak values of each Na+ channel current. The curves represent a holding duration equal to 4 ms, 10 ms, 20 ms, and 200 ms from the top to the bottom. This result shows that the longer the holding time becomes, the more likely that Na+ ion channel proteins return to normal configuration before the next pulse.

![Graph showing Na+ channel I-V curves](image)

**Figure 2.28** Three Na+ channel I-V curves. One is pre-train (triangle), one is 0.5-ms-later-of-train (diamond), and one is 2-min-later-of-train (circle). This figure proves that the stimulation pulses can just temporarily keep Na+ channel proteins in an un-conductive state.

Finally, another three Na+ channel I-V curve measurements are conducted by applying the same step pulses (Figure 2.13) to the cell membrane before and after 2000 pulse train stimulation. There are two I-V curves after the train stimulation with two different relaxation times (0.5ms and 2 min.). The three Na+ channel I-V curves are shown in Figure 2.28. One is pre-train (triangle) curve, one is 0.5-ms-later-of-train
(diamond) curve and one is 2-min-later-of-train (circle) curve. The 2-min-later-of-train I-V curve is almost identical to the pre-train I-V curve, which proves that indeed electric stimulation pulses can just temporarily make the Na+ channels stay in the un-conductive state.

**Discussion and Modeling**

There are two possible explanations for this research results. The first theory is based on the influence between the charge movement and the ball movement. The second theory is based on the gating charges’ “tiredness” under the electric stimulation pulse train.

1. Under a single pulse (Figure 2.3), Na+ channel proteins will open due to the gating charge movement, which takes about 1-2 millisecond. After the channels are open, the inactivation balls will move into the mouth of the channels to block Na+ channels automatically and the channels stay in inactive state. Na+ channels inactivation process follows a single exponential decay [48] because only one parameter determines the whole process - the ball’s movement. The time constant of this single exponential curve is the mean time for balls to move from resting positions to inactive positions, which is within millisecond range. Figure 2.29 shows the result - two curves fit as expected (one is data from Na+ channel current measurement; one is a single exponential fitting curve). The time constant is about 0.5 ms in this measurement. One important thing to point out is that there is a delay between the charge movement (opening door) and the ball’s movement (inactivation), so those two events are hard to influence each other.
Figure 2.29 Two curves fitting, with one being the real data from Na+ channel currents measurement and the other is the fitting data of single exponential decay.

When the cell membrane potential returns to the normal holding potential, Na+ channel deactivates and the balls will exit from the internal mouths of channels to the resting positions and the gating charges will move back to the closing positions at the same time. Will the ball’s movement still fit the same single exponential decay curve with the same time constant? Now the two movements will influence each other because in this condition there is no delay between the charge’s movement and the ball’s movement. The ball’s movement may not follow the same single exponential curve when leaving the inactive positions. Just like the charge’s movement current, “on” and “off” are not the same curves, despite the total gating charges, Q (on) is equal to the total
gating charges, \( Q \) (off) \[39\]. The open door charge’s movement curve is totally different from the closed door charge’s movement curve \[39\].

Because there is no direct way to measure the curve of refractory period, an indirect method is applied. We use two consecutive pulses (conditioning depolarization) on the cell membrane to measure channel currents. When conditioning depolarization is applied, after first pulse most of channels stay in inactivated state, and membrane potential must return to normal (-90 mV) or negative value (<-90 mV) to restore their ability to conduct again. We use variable intervals between those two pulses. Na+ channel current measured from the second pulse indicates the percentage of channels that can still conduct or the percent of balls that still stay in the mouths of Na+ channel proteins. The recovery result shows that if the restore voltage is negative (Figure 2.30 Left), it is a very fast recovery process compared to the recovery using the holding potential (Figure 2.30 Right). From this result, we can explain why the symmetric pulse train just has a little effect or no effect on Na+ channel protein functions. For unidirectional pulse train, the recovery time is relatively longer. When continual unidirectional pulse train is applied on the cell membrane, after the first pulse, some balls leave the mouths of the channels. When the second pulse arrives, some balls are still in the inaction positions, which results in the second Na+ channel current smaller than the first channel current. The same situation occurs on the third, fourth, fifth currents, so on and so forth until finally almost all channels stay in un-conductive state.

The process involves two exponential curves: one is the movement of charges and the other is the movement of balls (as we mentioned before, charges movement and balls movement have different time constant). Figure 2.32 shows the sum of two exponential
curves matching the experimental data, a fitting result much better than that in Figure 2.31 (a single exponential current fitting). Two time constants from Figure 2.32 have a difference of one order (Figure 2.32).

![Graph showing recovery times after channels are opened.](image)

Figure 2.30 The recovery times after channels are opened. When the restore voltage is negative (left side) compared to the holding potential (right side), the recovery process is faster than that using the holding potential.
Figure 2.31 Single exponential curve fit experimental data.

Figure 2.32 Double exponential curves fit experimental data.
(2) The second possible theory to explain this result is the immobilization and mobilization of the gating charges. We know for each pulse the gating charges (mobilized part) will physically move from the inside of the membrane to the outside of the membrane to control Na+ channels opening and closing. In Armstrong and Bezanilla’s paper [47], they point out that there are two kinds of charge groups existing on the cell membrane. (1) The charge that is mobilized in inactivation process forming the fast component which is presumably related to the gating activation process. (2) The charge that is immobilized in inactivation process forming the major part of the slow component. If before the full recovery of the charges movement from the opening position to the closing position, the second pulse is applied on the membrane, resulting in part of mobilized charges becoming immobilized. The same occurrence appears for subsequent pulses. As a result, less and less Na+ channel proteins can conduct with the increase of the number of pulses.

Under the train stimulation, most of the charges are immobilized temporarily. We call it “tiredness” of gating charge. In the same paper [47], the authors also point out if the holding potential returned to the negative potential, it is easy to detect that the mobilized charges go back to the initial position. This is why it is hard to block Na+ channels under symmetric oscillation train stimulation. Because the movement of charges controls the door behaviors, the “tiredness” of gating charges means the door will not conduct any ions under a continual pulse train. This explanation just deals with the charge movement under the influence of electric field rather than the ball movement.
Conclusion

Na+ channels are trans-membrane proteins that allow Na+ ions flow in or out of cell due to different membrane potential. In our present work, we observe some extraordinary behaviors (un-conductive state) of voltage-gated Na+ channel proteins, which show that unidirectional electric stimulation pulses can temporarily close Na+ channels.
Chapter 3

Inactivation of Voltage-Gated Delayed Rectifier K+ Channel by Oscillation Electric Field

Introduction

The voltage-gated K+ channel has different mechanisms of activation and inactivation from those of the Na+ channel because of the different configuration of protein structure [51, 52]. Several K+ channel genes have been cloned and sequenced [52, 53, 54, 55]. The different amino acid sequencing of different K+ channels can explain the differences in behaviors (i.e. kinetics) of these K+ channels. Voltage-gated K+ channels [52] comprise of a family of at least 50 different isoforms ranging from delayed rectifier [53] (slow activation, very slow inactivation) to A-type K+ channel [54] (fast activation, fast inactivation). In this chapter we will focus on voltage-gated delayed rectifier K+ channel protein’s [55] behaviors under the influence of oscillating electric field (all “K+ channels” below are considered voltage-gated delayed rectifier K+ channels unless otherwise indicated).

Like the Na+ ion channel, the K+ ion channel also consists of four homologous domains, each of which has six membrane spanning regions and some of those regions have been identified with specific channel functions [60]. Four domains form a pore through which K+ ions can permeate across the cell membrane. K+ channel protein is also tetrameric symmetry like Na+ channel protein. Thus the subunits are arranged
around a central four-fold axis that is coincident with the axis of the central pore [60]. The extra-cellular mouth leads into a narrow selectivity filter. Beyond this there is a central water-filled cavity that can accommodate a single K+ ion. There is then a narrow hydrophobic region, which forms the main channel gate [60, 63]. The region of amino acid sequence associated with the selectivity filter is highly conserved between different K+ channels. All K+ channels are thought to share the same main structure. They are different in presence/absence of additional helices, non-membrane domains or subunits that control their gating behaviors. The structure of K+ channel main domain is revealed by x-ray diffraction studies of a bacterial K+ channel, KcsA [62].

Each segment 4 (S4) contains a sequence of 5 to 8 positively charged amino acids. Experimental evidences [50, 61] prove that this region is involved in sensing the membrane potential and therefore controlling channel opening and closing. When any of four S4 segments still stays in resting position (inside the membrane), the pathway for K+ ion conduction is still blocked. The only way to make K+ channel conductive is to move out all the four S4 segments toward outside of the membrane. In the resting potential, most of time S4 segments are in resting position (closing door) and very infrequently all four S4 segments may be, for a brief period of time, in active position due to a thermal effect which produces a brief ionic current through the K+ channel. If the membrane potential is suddenly depolarized, there is a period of time before all four S4 segments move into active position (opening door); this will produce an initial delay in the opening of the channel. K+ ions always flow from high concentration region (inside cell) to low concentration region (outside cell). The K+ channel usually will function till the
membrane potential falls back to the resting potential. This is why people call this kind of K+ channel “voltage-gated delayed rectifier K+ channel”.

One of the common properties of voltage-gated channels is their ability to inactivate in response to the membrane potential depolarization. Two general mechanisms of inactivation have been characterized. The first mechanism is usually characterized by relatively fast inactivation during a sustained membrane potential depolarization, effected at molecular level by the ball. Molecular mechanisms of this type of inactivation, called N-type inactivation [64, 65, 66], have been studied in detail in Na+ and K+ channels. The ball part responsible for this type of inactivation is localized at the cytoplasmic side of channel. For example, in Shaker K+ channel, the N-terminal region of the channel plays the role of the ball, which occludes the inner part of the pore after channel activation and thus closes the channel. This kind of inactivation mechanism includes Na+ channels discussed in chapter two. The second type of inactivation process, usually with significantly slower kinetics, has been observed in a number of channels. This type of inactivation is called C-type [67, 68, 69] or slow inactivation. Although the detailed mechanisms of slow inactivation processes are incompletely understood, there exist two explanations: one is the rearrangement of the pore; the other involves relatively localized charges in conformation of the residues near the external mouth of the permeation pathway, rather than motion of a region (the ball) of the channel. Most commonly, membrane depolarization opens these channels and closes automatically very long time later. Voltage-gated delayed rectifier K+ channel belongs to this group. Researchers thought at first that this kind of voltage-gated K+ channel did not have the inactive process, but later found out it does, but just very slow inactivation process.
Methods and Materials

(1) Skeletal muscle fiber preparation (see chapter two)

(2) Solutions (Unit mM):

Normal Ringer: (see chapter two)
Relaxing Solution: (see chapter two)
External Solution: 120 NaCl
                   4.25 KCl
                   2.15 Na$_2$HPO$_4$
                   0.85 NaH$_2$SO$_4$
                   1.8 CaCl$_2$
                   1.5 RbCl$_2$
                   1.5 BaCl
Internal Solution: 45.5 K-glutamate
                   5 PIPES
                   20 EGTA
                   6.8 MgSO$_4$
                   5.5 Na$_2$-ATP
                   20 Tris-Creatine Phosphate
                   5 Gluocse

(3) Voltage clamp (see chapter two)

(4) Data acquisition (see chapter two)

(5) Electric stimulation pulses
Figure 3.1 shows the unidirectional stimulation train which includes 200 pulses. All pulses have the same magnitude (-10 mV) and duration (10 ms). The symmetric stimulation pulse train with 200 pulses (like Figure 2.13 in chapter two) has a magnitude of -10 mV/-170 mV (holding potential is -90 mV as usual) and pulse duration of 10 ms.

![Unidirectional Stimulation Pulse Train](image)

Experimental Results

First, a sequence of 28 stimulation pulses with 25 ms duration holding membrane potential at a range from -70 mV to -14 mV is applied on the cell membrane. The increasing potential of two consecutive pulses is 2 mV (Figure 3.2). The relaxation time between two successive pulses is 10 s, which assures that the fiber can relax back to the initial condition before next pulse. The trans-membrane currents responding to each
stimulating pulses are recorded. After subtracting the linear currents, K+ channel currents corresponding to different potentials are plotted in Figure 3.3. It shows the main characteristic of the so-called “delayed rectifier” K+ channel, which is that the channel needs about 10-15 ms to complete opening processes, and all currents are outward currents with unobservable inactivation. So unlike Na+ channel current measurement, the duration of stimulation pulse for K+ channel currents measurement is relatively longer, which permits K+ ions currents to reach saturation state (Figure 3.2).

Figure 3.2 A sequence of 28 stimulation pulses with a 25 ms duration with the membrane potential changing from -70 mV to -14 mV is applied on the cell membrane to measure K+ ion channel currents.
Figure 3.3 K+ channel currents corresponding to different membrane potentials. It shows the major characteristic of the so-called “delayed rectifier” K+ channel, which is that the channel needs about 10-15 ms to complete the opening process, and all currents are outward currents with unobservable inactivation process.

The values of K+ channel saturation currents are plotted as a function of the membrane potentials (shown on Figure 3.4). The triangles represent K+ channel saturation currents and the solid line is a fitting straight line for last ten data points. The line slope represents channel conductance and the crossing point with X-axis is the theoretical turning point, the open-door “threshold”. When the membrane potential is higher than this “threshold”, (there is not a real threshold in K+ channels due to thermal effect) theoretically K+ channel conductivity is constant just like a normal resistor. In this experiment, the conductance of this K+ channel is about 6.7 uS. When the channel is opened, K+ ions are transported from the high electrochemical potential side to the low
electrochemical potential side, and channel currents depend on membrane potential and concentration of ions (this is why it is a straight fitting line on K+ channel I-V curve). Below this “threshold”, there is still a small amount of K+ currents. Due to the thermal effect, there is still a small probability that K+ channel can open no matter what the cell membrane potential is.

Figure 3.4 K+ channel saturation currents are plotted as a function of the membrane potentials. A fitting straight line is plotted also. The line slope represents the channel conductance, and the crossing point with the X-axis is the theoretical turning point, K+ channel open door “threshold”.

After measuring the K+ channel I-V curve, the stimulation train (Figure 3.1) is delivered on the cell membrane by using a LabView program. Two signals of voltage and current are recorded and shown in Figure 3.5 and Figure 3.6. Figure 3.5 shows the “real”
voltage signal that is applied on the cell membrane during experiment, which has a very
good comparison to Figure 3.1 except the edge. Figure 3.6 shows K+ channel current
signal. It is clear that K+ currents decrease with the number of pulses increased. Both
figures just show part of the whole stimulation train (First 1000 ms).

Figure 3.5 Measured voltage signal that is applied on the cell membrane during the
experiment (The first 1000 ms), which is similar to the designed potential.

By cutting the long current signal and putting all pieces together, Figure 3.7
shows the same result as Figure 3.6 but with a different view. It shows clearly the
decrease of K+ channel currents with an increase of the number of pulses. Figure 3.8
demonstrates the relationship between number of pulses and the corresponding K+
currents. After 200 pulses, there is still 18% K+ current remaining in this experiment.
Figure 3.6 Recorded K+ channel current (The first 1000 ms).

Figure 3.7 Recorded K+ channel current (all 200 pulses) viewed from another perspective compared to Figure 3.6.
Figure 3.8 Demonstration of the relationship between the number of pulses and K+ channel saturation currents under each pulse.

If an electric field indeed can inactivate K+ channel proteins, experiment results should be independent to the intracellular concentration of K+ ions. We performed other experiments to confirm this assumption. Three internal solutions with 20, 40, 70 mM concentration of K+ ions are used separately in three fibers with the same experiment procedures. The measured K+ channel currents are shown in Figure 3.9. The top curve represents K+ ions concentration of 70mM inside of the cell, the middle curve 40 mM and the bottom curve 20 mM. These three curves have similar characteristics with different absolute values. Figure 3.10 shows that the three curves match each other after normalization, which indicates the independent inactivation mechanism of K+ channel proteins by electric stimulation.
Figure 3.9 Three different internal solutions with 20, 40, 70 mM concentrations of K+ ions are used in three fibers through the same experiment procedures. The top curve represents a K+ ions concentration of 70 mM, the middle 40 mM and the bottom 20 mM, respectively.

Figure 3.10 Three curves (Figure 3.9) closely match each other after normalization.
In Figure 3.8, there is still 18% K+ current left behind after K+ channel currents reach saturation state after 200 pulses. By using different magnitude and duration trains to re-accomplish this experiment, the lowest remaining K+ current is about 8%, which is still higher than Na+ channel current by electric stimulation train.

The last step of this experiment is to change the resting potential duration. The result is just like what we expected that when resting time is longer, more channel proteins can return back to the initial position and more K+ channel proteins can conduct in second pulse. This result is similar to Na+ channel experiments. Figure 3.11 shows the same muscle fiber experiment result, with top curve having double resting time compared to the bottom curve.

![Graph](image)

Figure 3.11 K+ channel currents measurement (same muscle fiber) under two electric stimulation trains, with the top curve having twice resting time than the bottom curve.
The symmetric stimulation pulse train (like Figure 2.13, chapter two) with magnitudes of -10 mV/-170 mV and duration of 10 ms is applied to the muscle fiber after unidirectional stimulation pulse train (Figure 3.1). There is no difference between symmetric train and unidirectional train experiment results which are totally different from Na+ channel inactivation experiments. For Na+ channel inactivation, the restoration potential is very important for the balls to return from the inactive position to the resting position. Because there are no balls existing on the K+ channel protein structure, so the two experiments (symmetric and unidirectional trains) have similar results.

**Discussion and Modeling**

The central event of K+ channel opening is the movement of S4 trans-membrane segments which carry numerous positive charged residues [61]. This process is energetically favored to the depolarizing membrane potential, but how this S4 segments movement is actually coupled with the opening of the pore is still unclear. However, we do know that when all of the four S4 segments physically move to the opening configuration, the K+ channel is conductive with an initial delay.

C-type inactivation is present in many voltage-gated K+ channels and probably related to “slowing” inactivation in Ca$^{2+}$ channels also. Thus, C-type inactivation is a general gating mechanism with application to a broad number of channels. For a long time, researchers thought that there were no automatic inactivation processes in voltage-gated delayed rectifier K+ channels, but later found out that there is an inactivation but with a very long time-constant [68, 71, 73]. Under membrane-potential depolarization,
the external mouth of channels is slowly occluded which process involves conformational change between the four domains and has a relatively long time-constant.

Many details of the basic channel electrical activities resulted from a series of experiments in the early 1950’s, which led to the award of the Nobel Prize to English physiologists A. L. Hodgkin and A. F. Huxley who applied Ohm’s law to ionic channel current [70]:

\[
I_k = g_k n^4 (V - V_k) \\
I_{Na} = g_{Na} m^3 h (V - V_{Na})
\]

where \( I_{Na} \) and \( I_k \) are Na+ and K+ ion channel currents, \( g_{Na} \) and \( g_k \) are constant. \( V \) is the membrane potential, \( V_{Na} \) and \( V_k \) are the Na+ and K+ equilibrium potential, and “m”, “n”, and “h” are voltage-dependent parameters. The “n” parameter for K+ channels is similar to the “m” parameter for Na+ channels (activation parameters); both increase with the increase of the potential. The “h” parameter in Na+ channels decreases with the increase of the potential which represents the inactivation part. For K+ channels, there is no “h” parameter to represent the inactivation. So modification of the H-H model is necessary to explain the automatic inactivation of K+ channels with a very long time-constant.

Some new theories [67, 71] have developed to explain the C-type inactivation by modifying the H-H model. In Gerald Hrenstein and Daniel L. Gilbert’s paper [71], the authors referred to a new parameter ‘k’ to correspond with K+ channel inactivation parameter and proposed that the H-H equation for K+ channels can be modified to
\[ I_k = g_k n^4 k (V-V_k) \]

where 'k' is a voltage-dependent parameter. If this modification is correct, the inactivation should follow a single exponential decay, but Figure 3.12 does not show the expected result. This result suggests something other than a single voltage-dependent first-order transition between open and inactive states of the K+ channel.

![Inactivation curve fitted to single exponential decay](image)

**Figure 3.12** Inactivation curve is fitted to a single exponential decay.

Recently, researchers found out that the inactivation kinetics of K+ channels were approximated by the sum of two exponential components [67, 71, 72] with fast and slow time constants. In our experiments, the outward K+ currents for each pulse decrease in magnitude as a result of the accelerated inactivation of K+ channels. Inactivation curves
from experiments can be approximated by the sum of two exponential decays with time constants differing by almost an order of magnitude (Figure 3.13).

![Graph showing the inactivation curve approximated by the sum of two exponential decay curves.](image)

**Figure 3.13** Inactivation curve can be approximated by the sum of two exponential decay curves with time constants differing by almost an order.

A simple explanation of this behavior is that two voltage-dependent inactivation processes occurred during the train stimulation. A specific case would be that the two inactivation processes are taken to be separate processes for inactivation of either closed or open channels. The rate of inactivation depends upon the state of the channels. The closed channels have a higher probability of inactivation during a voltage step than the open channels. After the first pulse of the train stimulation, the membrane potential returns to the normal holding potential. Most channels go back to the closing configuration, but a small percentage of channels would stay in the un-conductive
configuration. The same situation occurs on the third, fourth, fifth currents, so on and so forth until almost all channels stay in un-conductive state. Finally, the pathway for $\text{K}^+$ ions conduction is almost blocked, which means that the stimulation train forces individual proteins to stay in un-conductive configuration temporarily. However, which two parts of the protein inactivate the channels (there is no ball on the protein structure) and why the channels can not be inactivated 100% are still unknown.

**Conclusion**

Voltage-gated delayed rectifier $\text{K}^+$ channel plays a fundamental role in the excitability of the cell membrane. The channel opens upon depolarization of the membrane potential and closes in a relatively long time. We studied the relationship between electric field and inactivation of $\text{K}^+$ channels, which shows that $\text{K}^+$ channel proteins can be inactivated by electric field. Another feature of this inactivation is that the inactivation curve can be characterized by the sum of two exponential decay curves. These results could help us better understand the underlying mechanism of the inactivation of $\text{K}^+$ channel proteins.
Chapter 4

Synchronization of Na/K Pump Molecules by Oscillation

Electric Train

Introduction

Discovered by J. C. Skou in microsome of crab nerve a half century ago, the Na/K pump protein represents the first enzymatic transport system [74] in nature which is essential for living creatures and is expressed virtually in all cell membranes [9, 10, 89, 97]. The Na/K pump protein is more like a precise complex machine which never stops working [80]. It uses a lot of energy in the form of ATP and brings various “products”. For example, for a resting human being, Na/K pump proteins consume 20–30% of ATP to actively transport Na+ out and K+ into the cell [105]. In nerve cells, approximately 70% of ATP is consumed to fuel Na/K pumps [75]. Ionic transport conducted by the Na/K pumps create both electrical and chemical gradients across the cell membrane which are critical to maintain the membrane resting potential, cell volume, and secondary active transport of other solutes, etc. [1, 76, 91].

The steady-state binding method has been used to determine the density of Na/K pump proteins on different cell membranes, assuming that one molecule of Ouabain binds to one Na/K pump site [1, 77, 78, 79]. It is estimated that pump densities, in unit of pump sites/um², are 500-5000 in nerves and muscle cells, much higher in kidney tubular
cells and heart cells [1], but less than 1 in erythrocyte membranes [1]. Na/K pump proteins may be distributed fairly evenly, or clustered in certain membrane domains.

The Na/K pump protein is the largest protein complex in the family of P-type cation pumps [82]. The minimum functional units are alpha (α) and beta (β) subunits. Currently, four α-subunits and three β-subunits of Na/K pump protein have been identified in mammal cells [83]. The subunits combine to form Na/K pumps that are expressed in either a tissue- or a cell-specific manner. The bigger α subunit (~113 kDa glycoprotein) is the action part - it binds ATP and both Na+ and K+ ions. The smaller β subunit (~35 kDa glycoprotein) is necessary to activate the complex [83]. The α subunit is also the receptor for cardiac glycosides such as digitalis and Ouabain [18, 22]. Binding these widely-used drugs to Na/K pumps inhibits the pumps’ activity.

The Na/K pump mechanism is highly asymmetric [1, 9, 10]. It is activated by intracellular presence of Na+ ions, Mg^{2+} ions, and ATP and by extra-cellular presence of K+ ions [88]. Normally, a single pump cycle involves hydrolysis of one molecule of ATP to one molecule of ADP and one molecule of phosphate, extrusion of three molecules of Na+ ions and uptake of two molecules of K+ ions, a simple relationship of 1:2:3. (ATP: K+: Na+) [8]. Na/K pump is also an electrogenic [86, 87, 90, 98] protein because during a single pump cycle, it transports 3 Na+ and 2 K+ ions in the opposite directions across the cell membrane, resulting in a net positive charge across the cell membrane.

Traditionally, Na/K pump current measurement is a summation of individual pump currents by a step function. This pump current is a net outward current without inward component.
Studies show that the average of Na/K pump’s turnover rate at physiological condition is around 30-100 Hz (at neutral pH and room temperature) [84, 85]. Turnover rates are determined by the membrane potential and changed when the membrane potential is altered. Because most pump molecules work on or around the mean turnover rate and some work very fast while some work very slowly, the turnover rates of pump molecules on a cell membrane should have a bell-shape distribution, which depends on temperature, voltage and ionic concentration [106]. The turning phase of each Na/K pump molecule is random and should be uniform under normal conditions.

As we mentioned before, Na/K pump proteins’ movement can be explained by a relatively simple chemical cycle that includes several steps: binding of ions, conformational change, and release of ions on other side of the cell, etc. The key toward understanding how the enzyme is regulated at the molecular level is to find out the rate-limiting steps of its complex reaction cycle. In each cycle, there are two steps that are rate-limiting steps: Na+ ions are exposed to the outside, and K+ ions are released in the cytoplasm [1, 8, 9]. In order to influence overall activity of the enzyme, the changing time of these steps is the key. Due to the opposite transports of Na+ ions and K+ ions in the cycle, Na/K pump goes through two inverse voltage dependence processes. The electric field will favor one ionic movement and prevent the other [89, 122], so we cannot accelerate two steps simultaneously by depolarization or hyper-polarization of the cell membrane. In this chapter, we use an oscillating electric field to change pump-protein activities. Under well designed electric field, both rate-limiting steps are accelerated, and the pump function is increased significantly.
**Methods and Materials**

(1) Skeletal muscle fiber preparation (see chapter two)

(2) Solutions (Unit mM):

<table>
<thead>
<tr>
<th>Solution Type</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Ringer:</td>
<td>(see chapter two)</td>
</tr>
<tr>
<td>Relaxing Solution:</td>
<td>(see chapter two)</td>
</tr>
<tr>
<td>External Solution:</td>
<td>15 NaCl</td>
</tr>
<tr>
<td></td>
<td>5.4 KCl</td>
</tr>
<tr>
<td></td>
<td>87.6 TEA.Cl</td>
</tr>
<tr>
<td></td>
<td>2.15 Na2HPO4</td>
</tr>
<tr>
<td></td>
<td>0.85 NaH2SO4</td>
</tr>
<tr>
<td></td>
<td>1.8 CaCl2</td>
</tr>
<tr>
<td></td>
<td>1.5 RbCl2</td>
</tr>
<tr>
<td></td>
<td>1.5 BaCl</td>
</tr>
<tr>
<td></td>
<td>3.4 DAP</td>
</tr>
<tr>
<td>Internal Solution:</td>
<td>20 Na-glutamate</td>
</tr>
<tr>
<td></td>
<td>33.5 K-glutamate</td>
</tr>
<tr>
<td></td>
<td>5 Cs2-PIPES</td>
</tr>
<tr>
<td></td>
<td>20 Cs2-EGTA</td>
</tr>
<tr>
<td></td>
<td>6.8 MgSO4</td>
</tr>
<tr>
<td></td>
<td>5.5 Tris-ATP</td>
</tr>
<tr>
<td></td>
<td>20 Tris-Creatine Phosphate</td>
</tr>
</tbody>
</table>

(3) Voltage clamp (see chapter two)

(4) Electric stimulation pulses
Figure 4.1 A step function is used to measure Na/K pump current. The membrane potential jumps to -30 mV with a duration of 30 ms.

Figure 4.2 A sequence of 15 stimulating pulses with 10 ms duration holding the membrane potentials from -120 mV to +20 mV. The time difference between two continuant pulses is 1 min.
Figure 4.1 shows a step function. The membrane potential jumps from -90 mV to -30 mV with 30 ms pulse duration. A step function is a typical function for the traditional measurement of Na/K pump current. Figure 4.2 shows pulses which are used to measure the Na/K pump I-V curve. The duration of each pulse is 10 ms; the step between two pulses is +10 mV; the potential changes from -120 mV to +20 mV; and the time difference between two continuant pulses is 30s.

(5) Pump current measurement

Through voltage clamp, the measured currents include linear and non-linear currents. The linear currents are first subtracted by P/4 method from all currents. Then, the nonlinear currents (including Na/K pump current) in the presence of Ouabain are subtracted from currents in absence of Ouabain. This way, other non-linear currents including charge movement currents are eliminated. The remaining current is Ouabain-sensitive current, or Na/K pump current.

(6) Electric stimulation train

Figure 4.3 shows the stimulation pulse train including a 100 pre-pulse followed by three data acquisition pulses (the current responding to the last three pulses is recorded; we call those three pulses the data acquisition pulses). All the pulses have the same magnitude (-30 mV/-150 mV) and duration (12 ms). These two potentials are symmetric relative to the membrane holding potential (-90 mV). The pulses train is generated either by LabView or PClamp software program.
Figure 4.3 A stimulation electric train which includes a 100 pre-pulses followed by three 
data acquisition pulses, with all pulses having equal magnitudes and durations. 
The positive pulse potential is -30 mV, and the negative pulse potential is -150 
mV, which are symmetric to the membrane holding potential of -90 mV. The 
duration of each pulse is 12 ms.

(7) Protocol

The protocol of this experiment is as follows: data acquisition pulses (3 pulses) 
are first applied on the cell membrane as a control experiment (T0_C). Then, a 
stimulation pulse train (Figure 4.3) is applied on the cell membrane to examine 
oscillating-potential effects (T100_C) on Na/K pump functions. After that, the external 
solution is changed to the same external solution with 1 mM Ouabain to inhibit Na/K 
pump current. This is a vital step in the whole experiment, which requires that the fiber 
must be maintained in the same conditions before and after the change of the external 
solution. The holding current is a good criterion to inspect the success of this process,
which must stay at the same value after the change of the external solution. Ten to twenty minutes later, which is the time for Ouabain to block Na/K pump proteins, the same two stimulations – data-acquisition pulses and stimulation pulses train – are applied to the cell membrane again. The obtained currents are named T0_O and T100_O, respectively. The traditional measurement of Na/K pump currents equals T0_C minus T0_O and the electric-field-influence pump current equals T100_C minus T100_O.

**Experimental Results**

Figure 4.4 shows the Ouabain-sensitive current (Na/K pump current) elicited by a single 30 ms step pulse depolarizing the membrane potential to -30 mV (Figure 4.1). The Na/K pump current shows only an outward current. The transient current peaks in response to the rising and falling phases of the pulse which are also shown in later figures are due to imperfect matching in P/4 subtraction.
Figure 4.4 A Na/K pump current elicited by a single 30 ms step pulse depolarizing the membrane potential to -30 mV (Figure 4.1). Na/K pump current shows only an outward current component.

A sequence of 15 stimulating pulses with 10 ms duration and holding membrane potential at a range from -120 mV to +20 mV (Figure 4.2) is applied on the cell membrane to measure Na/K pump I-V curve. The trans-membrane current responding to each stimulation pulse is recorded. Then the external solution is changed to the same external solution with 1 mM Ouabain to inhibit Na/K pump current and the same sequences are used again. After subtracting linear currents (P/4 method) and non-linear currents (w/o Ouabain), Na/K pump currents responding to different potentials are plotted in Figure 4.5, which is typical sigmoid curve [97, 107, 109]. We have to stress two points on this I-V curve: (1) A positive slope is found between -80 mV and 0 mV; there is a less steep slope at potential smaller than -100 mV, and a negative slope when the membrane
potential is bigger than 0 mV. (2) Because the holding potential equals -90 mV, in this potential, pump proteins can still work (the pump current’s absolute value is not equal to zero at this potential). According to calculation, the Na/K pump should work when the potential is bigger than -300 mV [89, 121], which is much lower than the resting potential in physiological condition (-90 mV). Because of this, the negative currents are relative values to the pump current when the membrane potential is held at -90 mV (the negative currents are not inward currents).

Figure 4.5 Na/K pump currents responding to different membrane potential pulses.

After the measurement of the Na/K pump I-V curve, we use a stimulation train (Figure 4.3) to influence Na/K pump proteins and measure new pump currents. The two pump currents in Figure 4.6 (without the train stimulation) and Figure 4.7 (with the train stimulation), both result from the difference between the current without Ouabain inside and the current after changing the external solution with Ouabain inside. In other words,
Figure 4.6 is the result of the pump current generated by T0_C minus T0_O; and Figure 4.7 is the result of the pump current generated by T100_C minus T100_O.

Figure 4.6 A pump current generated by current T0_C (without Ouabain) minus current of T0_O (with Ouabain), which is the traditional method to measure Na/K pump currents.
Figure 4.7 A pump current resulted from the difference between two data acquisition pulses with pre-train pulses (T100_C minus T100_O), which indicates the influence of the external electric field on pump proteins.

After a train of stimulation, Na/K pump currents (Figure 4.7) become significantly different from those without the train of stimulation (Figure 4.6). This indicates the functional change of Na/K pump proteins by electric stimulation. First, Figure 4.6 shows that the pump currents are unidirectional outward current which responds largely to the positive parts of the pulses, and whose response to the negative parts is minimal and can be neglected. Figure 4.7 shows that the pump currents respond to data acquisition pulses with relatively big positive and negative components. Second, the outward currents in Figure 4.7 are relatively 2-3 times higher than the outward currents shown in Figure 4.6. Third, the ratio of the outward currents over the inward currents in Figure 4.7 is close to 3:2, which is the stoichiometric number of the Na/K
pump protein. The electric stimulation train can separate the outward and inward current components, which makes it very easy to measure the stoichiometric number of Na/K pump. Traditionally, researchers have to measure radioactive-ion concentrations to get this number [8] which is a very different process. Through our new method, it is easy to verify this number. The separation is a strong sign of synchronization of Na/K pump proteins: in positive half pulses, all pumps extrude Na\(^+\) ions out of the cell, and in negative half pulses, all pumps uptake K\(^+\) ions into the cell.

![Graph showing pump currents](image)

Figure 4.8 Pump currents elicited by the first 20 synchronization pulses. Initially, the inward pump currents responding to the negative half-pulse are very small. After a few oscillating pulses, the inward currents start to be distinguishable and increase with the number of pulses. Both inward and outward pump currents become larger and larger.

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Figure 4.9 Pump currents elicited by the last 20 synchronization pulses become saturated and the magnitude ratio between the outward and the inward pump currents is close 3:2.

Figure 4.8 and Figure 4.9 show another view of the changes of pump currents in response to the synchronization pulse train. The pump currents shown in Figure 4.8 are elicited by the first 20 synchronization pulses, while those shown in Figure 4.9 are elicited by the last 20 pulses. Clearly, as the number of oscillating pulses increase, the magnitudes of pump currents increase significantly. Initially (Figure 4.8), the inward pump currents responding to the negative half-pulse are very small. After a few oscillating pulses, the inward currents start to be distinguishable and increase with the number of pulses, and both inward and outward pump currents become larger and larger.
Figure 4.9 shows that pump currents become saturated after 100 oscillating pulses, showing 3:2 ratio of outward pump currents over inward pump currents.

A certain number of oscillating electric pulses is necessary to synchronize Na/K pump molecules and reach the saturated state. Figure 4.10 shows outward currents as a function of a number of train pulses. During the period of 100 pulses, the outward current reaches a saturation state, which indicates that 100 pulses are needed to synchronize Na/K pump molecules with oscillating membrane potentials from -30 mV to -150 mV and 10 ms duration. Similar results are received for several different pulse potentials and durations as well.

Figure 4.10 Outward pump currents as a function of a number of train pulses, which indicates that 100 pulses are needed to synchronize pump molecules with an oscillating membrane potential from -30 mV to -150 mV and 10 ms duration.
It is necessary to point out that a 10 ms duration for both positive and negative pulses is very close to the average physiological turnover rate [84, 85]. To understand the dependence of the pump currents on the frequency of pulses, different train pulse durations have been studied. We apply a different duration of pulses from 2 ms to 25 ms with the same magnitude to the same fiber using the same process mentioned before. The outward parts of the pump currents are plotted as a function of pulse durations or electric field frequencies (Figure 4.11). When the pulse duration is 10 ms (frequency is 50 Hz), close to the physiological turnover rate, the oscillation train has the highest effect. When oscillating frequencies are much different from the physiological frequency, less effect is observed on the Na/K pump currents. This experiment shows the dependence of the synchronization effect on electric field frequency. Pump molecules whose turnover rates are the same as or close to the electric-field frequency can be eventually synchronized. Pump molecules with a turnover rate beyond this range are not easily synchronized. If the oscillating electric field frequency is too high or too low, a small number of pump proteins is synchronized in comparison to the frequency close to the physiological turnover rate.
Figure 4.11 Outward parts of Na/K pump currents are plotted as a function of the stimulation train pulse durations.

There is an underlying distribution of the turnover rates of Na/K pump molecules within the membrane in physiological condition; however, the characteristic of the distribution is unknown. Our results imply that the turnover rate of the pump molecules is distributed as a bell-shaped curve with a large amount of pump molecules close to the center - the physiological turnover rate; and only a small amount of pump molecules are pumping very fast or very slowly. Chen’s paper [106] gives us a reasonable understanding of the distribution of pump molecules’ pumping rates, which depend on temperature, voltage and ionic concentration.

To further prove that we have synchronized Na/K pump molecules, we did several other experiments. In the first experiment, after 100 pulses with duration 6 ms are applied on the cell membrane, the membrane potential still held on –150 mV for 50 ms
before return to the holding potential (Figure 4.12). Without the oscillating field, pump molecules should change from their synchronized state to a random state after the last pulse of the train. The pump current should go back to zero even when membrane potential is still held at –150 mV. Figure 4.13 shows that after 3 data acquisition pulses, without stimulation pulses, Na/K pump current drops to zero as expected. This decay in the inward pump current signifies that pump molecules return to a random pumping pace.

**Figure 4.12** New stimulation protocol: a 100-pulse train with a duration of 6 ms followed by a membrane potential of –150 mV for another 50 ms before return to -90 mV.
Figure 4.13 After 3 data acquisition pulses, without stimulation pulses, Na/K pump current drops to zero during 6 ms.

Figure 4.14 Stimulation train is the same as that in Figure 4.12 except that the duration is 12 ms instead of 6 ms.
Figure 4.13 clearly demonstrates that there is a time period in maintenance of the inward pump current after cessation of the oscillation on the membrane, which is almost equal to pulse duration (6 ms, point out with an arrow in Figure 4.13). If the electric field has synchronized pump molecules, the time to keep inward current before decay should be exactly the same as the pulse duration of the synchronization train (half-cycle of synchronized pumping loop). Thereafter, we repeated this experiment using another modified synchronization train. All parameters of the new pulse train are the same as those shown in Figure 4.12 except that the pulse duration is changed to 12 ms. Again, the oscillating membrane potential is terminated at the value of negative -150 mV. The pump current is shown in Figure 4.14. After the end of the oscillation, inward pump current is kept for another 12 ms (duration of oscillating pulse) before the pump current decays to zero. Both Figure 4.13 and Figure 4.14 consistently show that after the membrane potential is ended at negative half-pulse, the inward pump current remains for another pulse duration before decreasing to zero. These results provide strong evidence that pump molecules have been synchronized by the oscillating pulse train.

To verify this synchronization theory, we conducted another experiment. The magnitudes of electric potential are changed from –10 mV to –170 mV instead of from –30 mV to –150 mV for the last two data acquisition pulses after three data acquisition pulses (Figure 4.15). After pump molecules are synchronized, the pumps’ turnover rates are restricted by the field frequency, and the stoichiometric number of Na/K pumps remains constant in a wide range of membrane potentials, so the pump currents should be independent of the membrane potential which just maintains the functions of the pumps.
If this hypothesis is correct, there should be no change in pump currents by the increase of pulse magnitude but maintaining the same oscillating frequency.

After the train stimulation (Figure 4.16), the negative part of the pump currents’ magnitude does not change and represents the total number of synchronized molecules. In contrast, the positive part of the pump currents’ magnitude shows noticeable increases because both synchronized and unsynchronized pump molecules have contributed to the outward pump currents. Even though the currents generated by the synchronized pump molecules remain the same, those generated by the unsynchronized pump molecules increase because of their voltage-dependence (Figure 4.5). Therefore, the total outward pump currents are increased. The results (Figure 4.16) indicate that the increase in magnitude of the last two data-acquisition pulses only increases the outward pump currents, but has no effects on the inward pump currents. This proves that some pump molecules have been synchronized by the oscillating electric train.
Figure 4.15 New stimulation protocol: the membrane potential is changed from –10 mV to –170 mV instead of from –30 mV to –150 mV for the last two data acquisition pulses after three data acquisition pulses.

Figure 4.16 Na/K pump currents measurement by using Figure 4.15 stimulation protocol.
Discussion and Modeling

Na/K pump transport process is a loop including two directional operations. In each loop, 3 Na+ ions are pumped out of the cell and 2 K+ ions are carried into the cell. Because of structural independence, pump molecules may run at individual pumping rates and random pumping phases. The measured pump currents are the sum of all individual Na/K pump currents: the outward component represents 3 Na+ ions extrusion and the inward component represents 2 K+ ions being pumped in. The pumping out Na+ ions and the pumping in K+ ions can not be distinguished (left side of Figure 4.17 [110]), resulting in one net ion out of the cell in each cycle.

In this research project, the external electric field has significant effects on the steps of the ions which are moved across the cell membrane. As the membrane potential is continuously oscillated with a frequency comparable to the pump natural turnover rate, the magnitude of pump currents is changed gradually. Eventually, the net outward pump currents are separated into two components: outward and inward currents alternatively corresponding to the two half-cycles of oscillating pulses. The magnitude of outward pump currents is increased by about three times and the magnitude of inward pump currents is increased by about two times. The ratio of outward over inward components is about 3:2, which reflects the stoichiometric number of Na/K pump protein. So under the influence of the electric field, the pumping rate and pumping phase are changed accordingly by the designed electric field - synchronization (right side of Figure 4.17 [110]).
Many phenomena of the Na/K pump protein can be expressed by a carrier-mediated model [108, 110], which postulates the existence of a chemical intermediate, a carrier which binds the solutes. The most successful model is the Post-Albers [92, 95, 96] model, which explains the biochemical behavior of the isolated enzyme, and is still the backbone of current biophysical models for electrogenic Na/K pump. The Post-Albers model is designed to accommodate the observed kinetics of enzyme phosphorylation and dephosphorylation catalyzed by Na+ and K+ ions, respectively. According to the model,
enzyme is phosphorylated by ATP (in the presence of Na+ ions and Mg$^{2+}$ ions) in one conformation state (a high-energy intermediate E1 state), and then undergoes a conformational change to a low-energy E2 state, which process is rapidly dephosphorylated in the presence of K+ ions. Figure 4.18 is a modified Post-Albers model adopted from Fonseca’s paper [101]. The Post-Albers cycle in this figure indicates the main stages of conformational changes (E1-E2), ions binding, ions release, ions occlusion, and ATP hydrolysis. K+ ions are released and Na+ ions are bound on the intracellular side, while Na+ ions are released and K+ ions are bound on the extra-cellular side. The arrows of circle indicate forward pump cycle.
(E1 ↔ E2): ion binding, release and occlusion, and ATP hydrolysis. The circle of arrows indicates the forward pump cycle [101].

From the Post-Albers model of Na/K pump, we can observe the following facts. First, the transport steps of both Na+ and K+ ions are voltage dependent due to the ions’ movements. Because charges move across the membrane, the external electric field will influence those steps. Second, Na+ ions and K+ ions transports are in opposite directions; therefore, any membrane potential change will have opposing effects on those two transports. Third, those two ionic transports - the extrusion of Na+ ions and pumping in of K+ ions - are the two slowest steps in the whole loop and have rate coefficients many
folds slower than any other steps in the loop (rate-limiting steps). The change in either step will affect the whole pumping rate. Finally, these two transports do not happen simultaneously, but instead are in a sequential pattern.

In Chen’s paper [110], he simplified the Post-Albers model to an asymmetric 6-state (Figure 4.19) model. “Asymmetry” means that the transporters have different binding affinities to different ions when facing different sides of the cell membrane. All voltage-dependent steps, primarily Na+ and K+ ions transport steps, are incorporated into two voltage-dependent steps. Four voltage-independent steps represent other processes, including binding and unbinding steps. These assumptions make it possible to calculate numerical results because the kinetic differential equations describing the loop functions
now can be simplified to algebraic equations. After all parameters are taken into
calculation, pump flux from the six-state model is:

$$
\phi_1 = C_{ET} \frac{C_3 e^{(A_1-A_2)V} - C_6 e^{-(B_1-B_2)V}}{C_1 e^{A_1V} + C_2 e^{-(B_1)V} + C_3 e^{A_2V} + C_4 e^{B_2V}}
$$

(4.1)[110]

Figure 4.20 Calculated Na/K pumping flux as a function of the membrane potential from
six-state mode [110].

By using equation 4.1, the analytically calculated pumping flux is a nonlinear
sigmoid curve as a function of the increase of the membrane potential, exhibiting a
shallow slope first, followed by a very sharp slope, a saturation state and a negative slope
(Figure 4.20 [110]). These results are consistent with our previous experimental results
(Figure 4.5) and experimental results of others [107, 108]. This calculated curve indicates
that membrane potential depolarization can not significantly increase pump current, and there exists an upper limit of pump current. When the membrane potential is further depolarized, the Na/K pump current will eventually go down.

In equation 4.1, the numerator is a subtraction between two exponential terms where the first term index is \((A_1-A_2)\) and the second term index is \((B_1-B_2)\). When two forward reaction rates and two backward reaction rates are comparable in normal situation, the results of the two subtractions are small. Equation 4.1 shows that the value of the first term cannot be too high, and the value of the second term cannot be too low. As a result, the pumping flux can not be significantly increased even when a large membrane potential is applied to the cell membrane. In order to increase the pumping flux, we need a large value of numerator in equation 4.1, which can be realized by increasing the first term and decreasing the second term. It is impossible to accelerate two opposite voltage-dependent ion-transport steps at the same time by polarized or depolarized membrane potential. In our experimental protocol, we apply an oscillating electric field with both positive and negative potentials relative to -90 mV. During the positive half-cycle, Na+ ions transport is accelerated, and during the negative half-cycle, K+ ions transport is accelerated. Thus, the oscillating electric field can alternatively facilitate both ion-transports, which process we call “synchronization.” Now, equation 4.1 becomes

\[
\phi_1 = C_{ET} \frac{C_5 e^{(A_1+A_2)V} - C_6 e^{-(B_1+B_2)V}}{C_1 e^{A_1V} + C_2 e^{-B_1V} + C_3 e^{A_2V} + C_4 e^{-B_2V}}
\]  

(4.2)[110]
Figure 4.21 Calculated Na/K pumping flux under the influence of an oscillating electric field is increases dramatically [110].

The calculated flux from equation 4.2 is increased dramatically with the increase of the membrane potential (Figure 4.21). As we mentioned before, the two steps of Na+ pumping out and K+ pumping in do not happen simultaneously in the loop. Instead, they work in a sequential pattern. When an oscillating electric field with a frequency comparable to the pumps’ turnover rate is applied on the cell membrane, the field’s two half-cycles match the time courses of two ion-transports. The oscillating electric field will treat individual pumps differently, based on their turnover rates and phases. For pumps whose turnover rate is a little lower than the field frequency, the electric field may facilitate both Na+ and K+ transport steps, alternatively and loop by loop, until the pump’s turnover rate matches the field frequency. For those whose turnover rate is a little higher than the field frequency, the electric field may slow them down until they reach
the field frequency. As a result, the pace of the Na+ and K+ ions transports will be
dominated by the two half-cycles of the oscillating electric field, respectively. In other
words, Na/K pump molecules are synchronized by the oscillating electric field. Na/K
pump currents are separated into distinguishable outward and inward components.
During the positive half-cycle, the pump molecules pump out 3 Na+ ions, which is three
times of that from randomly paced pump currents. Then, the pumps bring 2 K+ ions into
the cells during the negative half-cycle, resulting in an inward pump current. The
magnitude ratio of the outward pump currents over the inward pump currents, 3:2,
represents the stoichiometric number of the Na/K pump.

Figure 4.22 The figure on the left side represents the normal configuration of Na/K pump
proteins; the figure on the right side represents the synchronized
configuration of Na/K pump proteins.
The simplest demonstration of synchronization is shown in Figure 4.22. The left side represents the pumps’ normal configuration; the right side represents the pumps’ synchronized configuration after a long stimulation pulse train.

**Conclusion**

We use a pulsed, symmetric, oscillating electric field with frequency close to the mean physiological turnover rate across the cell membrane to synchronize Na/K pump molecules. The pump molecules work as a group, pumping at a synchronized pace after a long stimulation train, through which the pump functions can be significantly increased. The results clearly show separated outward and inward currents in an alternative pattern. The ratio is close to 3:2, which reflects the predicted stoichiometric number for the Na/K pump loop. Synchronization of pump molecules can become a new method to study the functions of Na/K pump molecules. This method has huge potential applications in medical treatment. Pathophysiology for different diseases differ significantly, such as Heart Failure, Alzheimer’s Disease, Cystic Fibrosis, Diabetes, Hyperthyroidism, Myotonic Dystrophy, Hypertension [80, 81], but all are related to dysfunction of Na/K pump molecules either due to a lack of ATP to fuse Na/K pumps or a low density of Na/K pump molecules on the cell membrane. A lot of researchers are studying how to make Na/K pumps work faster to restore the membrane potential and ions concentration so as to cure those diseases by either electrical force or chemical agents [93, 94, 99, 100, 102, 104]. Our research – synchronization Na/K pump proteins – presents a potential new method to conquer those diseases.
Chapter 5

Synchronization of Na/K Pumps under Na/Na and K/K Exchange

Modes by Electric Field

Introduction

Normally, Na/K pump proteins need Na+ ions, K+ ions and ATP [9, 10, 88] present to achieve basic functions, including pumping 3 Na+ ions outside and pumping 2 K+ ions into the cells to maintain ionic concentrations and the potential across the cell membrane [8]. The motion of Na/K pumps can be separated into two mechanisms. One mechanism depends on the inside concentration of Na+ ions; the other depends on extracellular concentration of K+ ions. Both would depend on the presence of ATP as energy source [1,111]. Changing one of the three parameters (the concentration of Na+ ions, the concentration of K+ ions, and presence of ATP) will change the functions of Na/K pump proteins. For example, if metabolic poisons, such as cyanide, block the production of ATP molecules, the efflux of Na+ ions are reduced [111, 112].

According to other researchers’ results, Na/K pump proteins can have several modes of exchange beside normal Na/K exchange mode[8, 9], like the Na/Na [111, 113, 118, 119] and the K/K [114, 115, 120 ] exchange modes, which all depend on the concentration of Na+ and K+ ions. Some papers [114, 115] suggest that ADP is a necessary factor for the K/K exchange mode. Some papers [117] suggest the K/K
exchange mode is a result of mis-measurements and there are just two modes existing: one is Na/K normal exchange mode, and one is the Na/Na exchange mode.

Na+ efflux depends on the extra-cellular concentration of K+ ions. An increase in the external K+ ion concentration will increase Na+ ion efflux [1]. If extra-cellular K+ ions are removed, the coupled efflux of Na+ ions and influx of K+ ions are reduced to near zero [1]. What will happen when there are some other cations with characteristics similar to those of K+ ions existing in the extra-cellular fluid? In general, it has been found that K+ ions have a number of extra-cellular agonistic ions which compete for activation of Na/K pumps. Evidence suggests that the effectiveness of different extra-cellular cations to activate Na/K pump proteins satisfies the sequence of Tl > K > Rb > Li > Na [1]. Papers written by Tosteson and Hoffman [118], and Garrahan and Glynn [116], show that Na/K pumps on the red blood cell membrane, in absence of extra-cellular K+ ions but with Na+ ions present, exchange Na+ ions between the inside of the cell and the outside of the cell with the ratio of 1:1.

Similarly, the Na/K pump is activated by intracellular Na+ ions also. In absence of intracellular Na+ ions, the coupled efflux of Na+ ions and influx of K+ ions also reduce to zero [1]. But if other ions replace Na+ ions in the intra-cellular solution, different researchers come up with totally different results on the K/K exchange mode. In Simons’s paper [120], he points out that there is a K/K exchange mode carried out by the Na/K pumps in human red cells with intracellular K+ ions instead of Na+ ions. In Kaplan and Kenney’s paper [114], K/K exchange through Na/K pump has been measured as Ouabain sensitive Pb+ uptake in Na-free ghosts with 1:1 ratio. But some researchers did not observe the same result [1, 117]. Their conclusion is that Na/K pumps are activated
by intracellular Na+ ions; at is, Na+ ions can not be replaced by other monovalent cations in the cycle of the Na/K pump. In Feraille and Doucet’s paper, they claim that the requirement for intracellular Na+ ions to active the Na/K pumps is almost absolute except for Li+ ions [174].

In the previous chapter, we use electric field to synchronize Na/K pump molecules and make the measurement of pump currents much easier than the traditional measurement. In this chapter, we will use the same stimulation train to examine the Na/Na exchange mode and the K/K exchange mode. If two modes exist in our experimental condition, we should see separate inward and outward currents for both modes.

**Methods and Materials**

(1) Skeletal muscle fiber preparation (see chapter two)

(2) Solutions (unit mM):

Normal Ringer: (see chapter two)

Relaxing Solution: (see chapter two)

**For the Na/Na exchange mode**

External Solution: 15 NaCl

83 CsCl

87.6 TEA.Cl

1.8 CaCl2

1.5 RbCl2

1.5 BaCl2
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>3, 4 DAP</td>
<td>3</td>
</tr>
<tr>
<td>0.1 EGTA</td>
<td>0.1 EGTA</td>
</tr>
<tr>
<td>Internal Solution:</td>
<td>20 Na-glutamate</td>
</tr>
<tr>
<td></td>
<td>33.5 K-glutamate</td>
</tr>
<tr>
<td></td>
<td>5 Cs2-PIPES</td>
</tr>
<tr>
<td></td>
<td>20 Cs2-EGTA</td>
</tr>
<tr>
<td></td>
<td>6.8 MgSO4</td>
</tr>
<tr>
<td></td>
<td>10 Na2-C.P.</td>
</tr>
<tr>
<td></td>
<td>5.5 Tris-ATP</td>
</tr>
<tr>
<td></td>
<td>20 Tris-Creatine Phosphate</td>
</tr>
</tbody>
</table>

**For the K/K exchange mode**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>External Solution:</td>
<td>15 NaCl</td>
</tr>
<tr>
<td></td>
<td>5.4 KCl</td>
</tr>
<tr>
<td></td>
<td>87.6 TEA.Cl</td>
</tr>
<tr>
<td></td>
<td>2.15 Na2HPO4</td>
</tr>
<tr>
<td></td>
<td>0.85 NaH2SO4</td>
</tr>
<tr>
<td></td>
<td>1.8 CaCl2</td>
</tr>
<tr>
<td></td>
<td>1.5 RbCl2</td>
</tr>
<tr>
<td></td>
<td>1.5 BaCl</td>
</tr>
<tr>
<td>Internal Solution:</td>
<td>40 Cs-glutamate</td>
</tr>
<tr>
<td></td>
<td>33.5 K-glutamate</td>
</tr>
<tr>
<td></td>
<td>5 Cs2-PIPES</td>
</tr>
<tr>
<td></td>
<td>20 Cs2-EGTA</td>
</tr>
</tbody>
</table>
(3) Voltage clamp (see chapter two)

(4) Electric stimulation pulses (see chapter four)

**Experimental Results**

(1) The Na/Na exchange mode (external solution without K+ ions)

Figure 5.1 A single step pulse is used on the muscle fiber to measure Na/K pump currents under the Na/Na exchange mode.

First, a single step pulse (Figure 5.1) is applied on the fiber to measure Na/K pump current under the Na/Na exchange mode condition. Because of the absence of K+ ions outside the cell, Na/K pump proteins should work on the Na/Na exchange mode. For each loop of Na/K pumps, same amount of Na+ ions are transported in and out through the cell membrane, which is no longer electrogenic process. Due to the random phase of
pump proteins’ movement, pump current must be equal to zero under a single pulse. The experimental result proves our expectation (Figure 5.2). The transient current peaks in response to the rising and falling phases of pulses are due to imperfect matching in P/4 method.

![Current vs Time Graph](image)

Figure 5.2 When Na/K pump proteins are working under the Na/Na exchange mode, for each loop of Na/K pumps, the same amount of Na+ ions is transported in and out through the cell membrane. This process is no longer electrogenic. Under a single pulse (Figure 5.1), Na/K pump current equals zero.

Using a stimulation train which is similar to Figure 4.3 in chapter four on the muscle fiber, we can measure pump currents during the whole train stimulation. Five minutes later after the train stimulation, the external solution is replaced by the same external solution except with 1 mM Ouabain, and the same electric train is applied on the same fiber again. After subtraction, we can get an Ouabain-sensitive currents, the Na/K 101
pump currents. Figure 5.3 and Figure 5.4 show the currents during first 300 ms and last 300 ms stimulation. Several characteristics are worth of mentioning in those two figures. (1) Both inward and outward currents greatly increase with an increasing number of pulses. After 100 pulses, the currents reach a steady state, just like the result from synchronization we obtained in chapter four. (2) Results clearly show the separation of two currents: one is positive and the other is negative with almost 1:1 ratio. This directly proves that Na/K pumps work in the Na/Na exchange mode.

Figure 5.3 Pump currents under the Na/Na exchange mode during the first 300 ms of the oscillation pulse train.
Figure 5.4 Pump currents under the Na/Na exchange mode during the last 300ms of the oscillation pulse train.

Figure 5.5 The X-axis represents the number of pulses, the Y+ axis represents the positive pulse pump currents and the Y- axis represents the negative pulse pump currents. The ratio between the positive currents and the negative currents is close to 1:1.
In figure 5.5, the X-axis represents the number of pulses, the Y+ axis represents the positive pulse pump current value, and the Y- axis represents the negative pulse pump current value. From this figure, we can observe two very important results as we mentioned before: (1) Na/K pump currents are saturated within 100 pulses; (2) the ratio of the positive current with the negative current is very close to 1:1 in the whole period of the train oscillation.

(2) The K/K exchange mode (internal solution without Na+)

With the same process, internal solution without Na+ ions is used on a single fiber to verify the K/K exchange mode. Under the same electric stimulation pulses, the measurement of pump currents has not shown significant change during the whole stimulation train. Figure 5.6 and Figure 5.7 show first 300 ms and last 300 ms pump currents. There is maybe a little but no notable effect on pump currents by an electric stimulation train in this experiment.

![Figure 5.6](image)

Figure 5.6 Pump currents during the first 300 ms of the oscillation pulse train under the K/K exchange mode.
Figure 5.7 Pump currents during the last 300 ms of the oscillation pulse train under the K/K exchange mode. Compared to Figure 5.6, there is no significant difference between those two figures.

**Discussion**

Experiments show that in the absence of K+ ions and in the presence of relatively high level of extra-cellular Na+ ions, Na/K pump can operate in a mode where Na+ ions influx and efflux are associated. The experimental results show that the efflux of Na+ ions is approximately equal to the influx of Na+ ions, which indicates a 1:1 ratio of the Na/Na exchange mode. Previously, the stoichiometric numbers under the Na/Na exchange mode have been reported as 1:1 [123], 2:1[124], or varying with the Na+ ions concentration [125]. In our experiment to synchronize pump proteins, we can separate the influx and the efflux currents and clearly observe that the stoichiometric number. The Na/Na exchange mode is no longer an electrogenic process.
Two types of Na/Na exchange reactions of Na/K pumps have been previously reported [126]. The electroneutral exchange is a one-to-one Na$^+$ ions counter-transport mechanism [127]. This mode needs relatively high concentrations of Na$^+$ outside the cell which is similar to our experimental results. The second type of the Na/Na exchange reaction is that the wild-type enzyme uses Na$^+$ ions as a low-affinity K$^+$ substitute to move charges across the cell membrane [128, 129, 135]. This electrogenic exchange occurs in the present of ADP and is generally much smaller than the electroneutral Na/Na exchange mode with a stoichiometric number close to 3:2 [132, 133, 134]. It is clear that the electroneutral Na/Na exchange mode does not require ADP and our research belongs to this type.

On the other hand, in the absence of Na$^+$ ions and in the presence of relatively high level of intra-cellular K$^+$ ions, Na/K pumps should work on the K/K exchange mode according to other researchers [114, 115, 120]. If so, we should get separate K$^+$ currents just like the Na/Na exchange mode by electric stimulation. Even though we have changed different concentrations of K$^+$ ions inside the cell, results can not prove the existence of this “K/K exchange mode”. No separate currents can be measured. The reason is either that there is no K/K exchange mode on this experiment setup, or electric stimulation can not turn on synchronization process of Na/K pump molecules due to a lack of Na$^+$ ions inside the cell.

**Conclusion**

We use an oscillating electric train to synchronize Na/K pump molecules under the Na/Na and the K/K exchange-mode conditions. After a long train stimulation, we are
able to measure the separate outward and inward currents in an alternative pattern under the Na/Na exchange mode. The ratio is close to 1:1, which reflects the fact that Na/K pumps can transport the same amount Na+ ions through the cell membrane in each loop, and the Na/Na exchange mode is not longer electrogenic. For K/K exchange mode measurement, there is no significant change in the measurement of pump currents during a long period of stimulation. No separate currents can be measured. The reason is that either there is no K/K exchange mode on this experiment setup, or electric stimulation can not turn on synchronization process of Na/K pump molecules due to a lack of Na+ ions inside the cell. In other words, internal Na+ ions are the trigger of Na/K pump proteins [1].
Chapter 6

Modulation of Na/K Pump Proteins by Electric Field

Introduction

Previous results indicate that a symmetric, oscillating electric pulse train with a frequency close to the physiological turnover rate can synchronize Na/K pump molecules by using a traditional voltage clamp and can separate the inward and outward pump currents [136]. The field-induced activation of the pumps can effectively reinstate (hyperpolarize) the cell membrane resting potential and ion concentrations [130, 131], which are critical to many cell functions, including controlling cell volume, generating electrical signals, and providing energy for other active transporters. Synchronization of pump molecules provides a new method to get more detailed information about Na/K pump proteins. In this chapter, we further present results of our studies in modulation of pump proteins after synchronization: the external electric field with increased frequency can accelerate turnover rate of Na/K pump molecules.

After Na/K pump molecules are synchronized, the applied electric field frequency is gradually increased in order to synchronize Na/K pump molecules to a new high frequency. There are two basic questions: (1) what is the new frequency after synchronization? (2) How many pulses should be applied for the new frequency? For the first question, it is clear that pump molecules can only follow a gradual change (higher) in electric field frequency. If electric-field frequency jumps too much, the synchronized
pump molecules may not follow the new frequency. For the second question, our experiments have shown that 100 pulses are good enough to synchronize most molecules with pulse potential changing from –150 mV to –30 mV. After synchronization, a new frequency which is slightly higher than the previous frequency will force the synchronized group molecules to move to the new frequency. It is estimated that at most 50 pulses will be needed for the new frequency.

Our goal is to modulate Na/K pump molecules to a higher frequency that should lead to a large increase of pump currents and, therefore, Na/K pump functions by the synchronization and modulation stimulation pulse train.

**Methods and Materials**

(1) Skeletal muscle fiber preparation (see chapter two)

(2) Solutions (see chapter four):

(3) Voltage clamp (see chapter two)

(4) Electric synchronization-modulation stimulation pulse train

The synchronization-modulation pulse train consists of five parts with gradually reduced durations of electric pulses. The duration of pulses initially is 15 ms, followed by 10 ms, 6ms, 4 ms and 3ms. There is no time-gap between any two parts. The first part has 100 pulses; the second to the final parts have 50 pulses each. The magnitudes of pulses are +/- 60 mV on the basis of -90 mV holding potential. The synchronization-modulation pulse train is sketched and shown in Figure 6.1. The last three pulses of each different group are the data acquisition pulses, which are used to generate final results of Na/K pump currents.
Figure 6.1 Synchronization-modulation pulse train, the duration of the first part of which is 15 ms, followed by 10 ms, 6ms.

(5) Protocol

Experimental protocol is as follows: the stimulation pulse train (Figure 6.1) is first applied to the cell membrane five times. After that, the external solution is changed to the solution with 1 mM Ouabain, and the same process is repeated five times. The measured currents from five repeated stimulations are averaged to increase signal/noise ratio. Then, P/4 method is used to elicit linear trans-membrane currents. After that, the current with Ouabain is subtracted from that without Ouabain present to get Na/K pump currents for different pulses.

Experimental Results

We have shown that individual pump molecule working at random paces can be synchronized by an oscillating electric field in chapter four. After synchronization, when
we increase the field frequency by a small step, the electric field is able to re-synchronize the pump proteins to the new frequency. If the frequency step is small enough and the electric field is applied for long enough, which means by gradually increasing field frequency and keeping the Na/K pump molecules synchronized, pumping rates can be accelerated and the Na/K pump molecules can be synchronized to the higher frequencies. In this experiment, the synchronization-modulation stimulation train consists of five parts with the duration gradually reduced from 15 to 3 ms. The equivalent oscillating frequency of the first part is 33 Hz and 167 Hz for the last part of the train.

Figure 6.2 Pump currents when the pulse duration is 15 ms (synchronization).
Figure 6.3 Pump currents when the pulse duration is 10 ms (first modulation).

Figure 6.4 Pump currents when the pulse duration is 6 ms (second modulation).
Figure 6.5 Pump currents when the pulse duration is 4 ms (third modulation).

Figure 6.6 Pump currents when the pulse duration is 3 ms (fourth modulation).
Table 6.1 Areas and magnitudes of pump currents responding to both positive and negative half-pulse under synchronization-modulation electric stimulation train.

<table>
<thead>
<tr>
<th></th>
<th>Oscillation group 1</th>
<th>Oscillation group 2</th>
<th>Oscillation group 3</th>
<th>Oscillation group 4</th>
<th>Oscillation group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integral outward current (nA ms)</td>
<td>43.6</td>
<td>46.5</td>
<td>45.3</td>
<td>41.0</td>
<td>39.4</td>
</tr>
<tr>
<td>Integral inward current (nA ms)</td>
<td>-30.3</td>
<td>-30.4</td>
<td>-30.4</td>
<td>-29.0</td>
<td>-27.4</td>
</tr>
<tr>
<td>Average outward current (nA)</td>
<td>2.90</td>
<td>4.65</td>
<td>7.55</td>
<td>10.2</td>
<td>13.1</td>
</tr>
<tr>
<td>Average inward Current (nA)</td>
<td>-2.02</td>
<td>-3.04</td>
<td>-5.07</td>
<td>-7.26</td>
<td>-9.12</td>
</tr>
</tbody>
</table>

The final results of Na/K pump currents for the five parts are shown in Figure 6.2 to Figure 6.6, respectively (15 ms is considered the middle point for comparison). For each of five different figures, the ratios of outward pump currents to inward currents are similar, close to 3:2. However, the magnitudes of pump currents are progressively increased when the duration is reduced. The magnitudes of pump currents responding to both positive and negative pulses are listed in the third and fourth rows in Table 6.1, respectively. In the first part of the train where the duration is 15 ms, the magnitude of
outward pump currents is only 2.9 nA. In the last part of the train, the magnitude of
outward pump currents increases to 13.1 nA, a little less than a five fold increase
increment from the first part. Similar results can be observed by comparing inward pump
currents. The value of inward pump currents induced in the first part is initially about
2.02 nA and finally reaches a value of 9.12 nA, still a little less than a five-times increase.

The first and second rows in Table 6.1 are the areas of outward and inward ionic
fluxes carried by the Na/K pumps and obtained by the integration of time and current
trace. The first row represents the total number of charges extruded from the cell during
the positive half-pulse. Similarly, the total number of charges pumped into the cell during
the negative half-pulses is listed in the second row. The numbers of charges moved
during both half-pulses are approximately the same for all groups. This similarity is
because of a fixed number of pump molecules being synchronized on the cell membrane
and a fixed stoichiometric number of Na/K pumps. In fact, this similarity is a good sign
indicating that most of synchronized pump molecules have been re-synchronized at each
new frequency.

To further compare frequency-modulation effects on Na/K pump proteins, we
superimposed all the pump-current traces in Figure 6.7. The traces are aligned so that the
reversals of polarity in the current all occur at 15 ms. On the left side are outward
currents responding to the positive half-pulse, and on the right side are inward currents
responding to the negative half pulse. The figure clearly shows that the areas underneath
either outward or inward pump currents remain almost the same regardless of pulse
durations. Both outward and inward pump currents are continuously increased when the
synchronization frequency is gradually increased.
Figure 6.7 All Na/K pump current traces superimposed together shows that both outwards and inwards pump currents are continuously increasing when the field frequency is gradually increased. The areas underneath either the outwards or the inwards pump currents remain the same regardless of the pulse durations.

**Discussion**

When the frequency of the oscillating electric field is increased, Na/K pump proteins are forced to be synchronized to the new frequency if the step is small enough and the electric field is applied long enough. Once the new synchronization is reached, the field frequency can be increased again to re-synchronize Na/K pumps to the next higher frequency. By using this method, Na/K pump molecules can be gradually modulated to higher and higher pumping rates. It has been well documented that the stoichiometric number of the Na/K pumps remains constant over a wide range of
membrane potentials [137]. Therefore, the modulation of pumping rate to a higher value results in an increase in pump currents. This can be seen by comparison of five current traces shown in Figure 6.2 to Figure 6.6 and the measurements listed in Table 6.1. It is clear that by slowly increasing the oscillating electric-field frequency and keeping the pumps synchronized, the times needed for the two rate-limiting ionic transports are reduced step by step, and the pumps’ turnover rates are gradually accelerated. As a result, Na/K pump currents are increased. It is necessary to point out that in all of our experiments the pulse magnitudes remain same. The increase in pump currents results solely from the pumping-rate modulation. In this experiment, we only increase the oscillating frequency up to five times which results in close to a five-fold increase in pump currents. Further increase in field frequency may lead to a progressively greater increase in Na/K pump currents.

**Conclusion**

The previous results indicate that a symmetric, oscillating pulse train with the frequency close to the physiological turnover rate can synchronize Na/K pump molecules. In this chapter, we can accelerate the turnover rate of pump molecules by increasing the electric-field frequency gradually in order to re-synchronize molecules to the new higher frequency. After a synchronization-modulation stimulation pulse train, Na/K pump proteins are modulated to the higher frequency with a large increase of pump currents.
Chapter 7

Increase Lumen Potential in Rat Proximal Tubule by Synchronization and Modulation of Na/K Pumps

Introduction

Mammalian kidneys are vital organs which clean the blood and maintain chemical balance inside of the body [139, 140, 141]. Kidneys are sophisticated reprocessing machines dealing with metabolic waste products such as uric acid, urea, and creatinine, etc. The extra water and waste products flow to the bladder through ureters and become urine eventually. Every day, an adult human’s kidneys process about 200 quarts of blood and squeeze out about 2 quarts of waste products and extra water [139]. However, the role of the kidneys is not only excretion; they are also regulatory organs, controlling and maintaining the volume, composition, osmolality and ionic concentrations of blood within very narrow margins. For example, Na+ ions’ concentration, which is the main factor in biological system to determine the blood pressure [141, 144, 145], is controlled by kidneys.

Figure 7.1 shows the basic structure of kidney [169]. The outer layer of the kidney is the renal cortex, which sits directly beneath the kidney's connective tissue and fibrous capsule. The thin membrane-like coverage is very important to keep water on the kidney surface. The deeper layer of the cortex is called the renal medulla. The renal medullar is divided into many renal pyramids, which together with the associated overlying cortex
form a renal lobe. The tip of each pyramid empties into a calyx, and the calices empty into the renal pelvis, which transmits urine by ureters to the bladder.

Figure 7.1 The basic structure of kidney [169]

1. Renal Vein
2. Renal Artery
3. Renal Calyx
4. Medullary Pyramid
5. Renal Cortex
6. Segmental Artery
7. Interlobar Artery
8. Arcuate Artery
9. Arcuate Vein
10. Interlobar Vein
11. Segmental Vein
12. Renal Column
13. Renal Papillae
14. Renal Pelvis
15. Ureter
The actual filtering and reabsorption processes occur in many tiny functional units inside the kidneys called nephrons. Each kidney contains approximately one million of these units [140]. Each nephron begins in a renal corpuscle, which contains a cluster of blood vessels (glomerulus), surrounded by the hollow Bowman's capsule. Ions, water, and small molecules, except for blood cells, big proteins, and large molecules, can be filtered out of glomerulus by different pressures between in- and out- bloodstreams. After glomerulus of ultrafiltration, resemblant plasma enters Bowman's space. Bowman's capsule leads into a membrane-enclosed, U-shaped tubule, which is divided into four parts, namely the proximal tubule, the loop of Henle, the distal convoluted tubule, and the collecting duct (Figure 7.2). The liquid from the collecting ducts through various nephrons merges together and ultimately flows into the bladder and becomes urine.

Figure 7.2 shows the basic structure of the nephron [138]. Each nephron’s tube has four basic parts according to each segment’s structures and functions as mentioned before. Each part has different functions, especially in reabsorption of different matter [142, 143]. For example, 100% glucose and amino acids and probably 65% Na+ ions are reabsorbed in the proximal tubule (Table 7.1). The loop of Henle can reabsorb probably 25 % Na+ ions.
Figure 7.2 The basic structure of naphron [138]
<table>
<thead>
<tr>
<th>Substance</th>
<th>Proximal tubule</th>
<th>The Loop of Henle</th>
<th>Distal tubule</th>
<th>Collecting duct</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>reabsorption (almost 100%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>amino acids</td>
<td>reabsorption (Almost 100%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>urea</td>
<td>reabsorption (50%)</td>
<td>secretion</td>
<td>-</td>
<td>reabsorption in medullary ducts</td>
</tr>
<tr>
<td>Na+</td>
<td>reabsorption (65%)</td>
<td>reabsorption (25%)</td>
<td>reabsorption (5%)</td>
<td>reabsorption (5%, principal cells)</td>
</tr>
<tr>
<td>chloride</td>
<td>reabsorption</td>
<td>reabsorption</td>
<td>reabsorption</td>
<td>-</td>
</tr>
<tr>
<td>water</td>
<td>-</td>
<td>reabsorption (descending)</td>
<td>-</td>
<td>reabsorption</td>
</tr>
<tr>
<td>bicarbonate</td>
<td>reabsorption (80-90%)</td>
<td>reabsorption (thick ascending)</td>
<td>-</td>
<td>reabsorption</td>
</tr>
<tr>
<td>protons</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>secretion (intercalated cells)</td>
</tr>
<tr>
<td>K+</td>
<td>reabsorption</td>
<td>reabsorption (20%)</td>
<td>-</td>
<td>secretion</td>
</tr>
<tr>
<td>calcium</td>
<td>reabsorption</td>
<td>reabsorption (thick ascending)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>magnesium</td>
<td>reabsorption</td>
<td>reabsorption (thick ascending)</td>
<td>reabsorption</td>
<td>-</td>
</tr>
<tr>
<td>phosphate</td>
<td>reabsorption (85%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 7.1 Re-absorptions of different matters on different kidney segments including the proximal tubule, the loop of Henle, the distal convoluted tubule, and the collecting duct [140].

Kidneys are the richest organ containing Na/K pump proteins [161]. Na/K pump proteins are present in peritubular cell membranes (Figure 7.3), where they play a prominent role in Na+ ion reabsorption [142, 143]. Na/K pumps can generate membrane
potential and concentration differences which are the main driving forces for Na+ ion reabsorption from lumen fluid to blood [149, 151, 160]. Net Na+ ion reabsorption is the major function of renal Na/K pump proteins, and a close relationship exists between Na/K pump molecules’ density and the Na+ ions reabsorption capacity of the different segments of a nephron. Renal Na/K pumps are the driving force not only for Na+ reabsorption, but also for secondary active transport of large amounts of a wide variety of other ions and uncharged solutes.

Figure 7.3 The electric potential difference across the renal tubular cell and the direction of net Na+ ions re-absorption in the tubule.

The potential of the interior of the tubular cell (proximal segment) is negative [156,159] that compared to of the peritubular fluid (assume 0 mV) and the lumen fluid. Figure 7.3 shows typical potentials. The potential of the inside of the tubular cell is proportional to the rate of Na+ ions being pumped out, which depends directly on Na/K pump activity [146, 147, 148, 149, 150]. It is generally agreed that the magnitudes of trans-tubular potential are different in different segments [152, 153, 154, 155]. Smaller
potential differences between the lumen fluid and the peritubular fluid are obtained on the loop of Henle, distal convoluted tubule, and collecting ducts [152].

This project is the first application of the synchronization-modulation method at the organ level after our single-cell study (chapter 4, 5 and 6). The main goal is to use synchronization-modulation electric field to activate Na/K pump proteins and, therefore, increase Na+ and other ions reabsorption speed, which will result in an increase in potential of the inside of proximal tubular lumen. Rat kidney is well suitable for the study of proximal distal tubular functions because the surface of rat kidney is composed almost exclusively of proximal tubules (95%) and distal convoluted tubules (5%).

**Experiment Setup and Methods**

(1) Animal preparation

All animal experiments are carried and processed in accordance with provisions of National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved protocols by the Institutional Animal Care and Use Committee at the University of South Florida.

Male rats (250 g to 380 g) fed with standard rat chow and provided with free access to water are used for all experiments. After being anesthetized by intraperitoneal injection of thiobutabarbital sodium (Inactin, 100mg/Kg), the rat is placed on a constant-temperature (37°C) pad which connects to a TP 500 heating pump (heat pump supplies a temperature controlled flow of heated water through a set of hoses to a pad which is then applied to the rat). After adequate waiting time (10 min to 15 min) to make sure the rat has fallen deeply asleep, the left kidney is exposed by a flank incision. Connected tissue
and fat around the kidney are carefully removed, but without touch and harm to the “membrane” of the kidney. Then, the kidney is placed to a plastic cup which connects to a magnetic stand to minimize the vibrations from the rat heart while blood vessels and ureter are still in the normal functional condition and connected to the kidney. The empty space around the kidney is covered with absorbent cotton. The surface of the kidney is covered by the paraffin oil to keep surface water from evaporating.

(2) Solutions (mM)

<table>
<thead>
<tr>
<th>Solution One</th>
<th>110 NaCl</th>
<th>2.5 KCl</th>
<th>2.15 Na₂HPO₄</th>
<th>0.85 NaH₂PO₄. H₂O</th>
<th>1.8 CaCl₂. H₂O</th>
<th>10 NaHCO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution Two</td>
<td>120 NaCl</td>
<td>2.5 KCl</td>
<td>2.15 Na₂HPO₄</td>
<td>0.85 NaH₂PO₄. H₂O</td>
<td>1.8 CaCl₂. H₂O</td>
<td></td>
</tr>
</tbody>
</table>

(3) Microelectrodes for the lumen potential measurements

Micropipettes (40-60 mm) are pulled by a heat puller (Model PB-7, Narishige) from 1.0 mm I.D. to 2-5 um on the tip. There are three setups involving the solutions to prepare microelectrode in experiments (1) Micropipette is filled with Solution One, which is used to measure the lumen potential without blockage of the tubule. (2)
Micropipette is first filled with tiny Sudan black mixed castor oil in the tip and later Solution One (Figure 7.4). The mixed oil is used to block the tubule segment. (3) The same process is used as (2) except Ouabain is solved in the Solution One to block Na/K pump proteins.

After the required solution is prepared, the micropipette is inserted into a connector while a silver wire is put into the solution inside of a glass pipette. The next step is to connect it with channel stage one in a voltage-monitor-mode of TEV-200 voltage clamp (Figure 7.5 shows a sketch; Figure 7.6 shows a real picture). The connector has a side-open-hose to join a pipe which can control the pressure inside the connector.

Figure 7.4 Photograph of a micropipette tip (microelectrode), which is filled with tiny Sudan black mixed castor oil and measurement solution.
Figure 7.5 Schematic diagram of a microelectrode and a connector setup.

Figure 7.6 Photograph of a microelectrode and a connector setup.
(4) Micropuncture and microinjection

Micropunctures are finished by using a high-magnifying lens on a microscope stand. Proximal tubular cells are punctured by a micropipette with the tip about 2-5 um diameter which is filled with mixed oil and measurement solution. After the tip is pushed into the proximal tubule lumen on the kidney surface, the mixed oil is first injected into the lumen controlled by a high pressure which is generated by a pumping machine. After the oil injection, a tiny solution is also injected into the tubule by carefully controlling the pressure and making sure that the oil is pushed away and stays at the two sides of the solution while the silver wire remains inside of the solution. Through this way, we create an independent tubular segment and cut off all feedback system inside the tubule (Figure 7.7). The measured potentials inside of the tubule reflect directly the tubular cell reabsorption process.

The success rate of this process is very low. Quite often, the following situations will occur: (1) The tiny glass tip is blocked by big particles in the mixed oil or during micropuncture through the kidney “membrane” and the tubular cell membrane; as result, the mixed oil can not be pushed into the lumen space by the high pressure. (2) The tip is broken due to the micropuncture through the “membrane” or due to the kidney’s vibration after successful micropuncture. (3) The mixed oil is swept away by the tubular fluid or during the solution injection by the high pressure. There is no independent tubular segment for the measurement.
After micropuncture and oil injection, a tiny solution is also injected into the tubule. Through this way, an independent tubular segment is created and the tubule feedback system is cut off.

(5) Synchronization-modulation electric field and electrodes

Synchronization-modulation electric stimulation trains generated by the MatLab computer program are applied. The stimulation train frequency is gradually increased from 10 Hz (pulse duration is 50 ms) to 500 Hz (pulse duration is 1 ms). Each following pulse frequency is increased within 2% compared to the previous one. Each frequency has 50 pulses except that the first one (10 Hz) has 100 pulses and the last one (500 Hz) has 1000 pulses. The trains have the same magnitudes of +1 to -1 V peak to peak.

The stimulation trains are delivered by two pin-shaped electrodes which are carefully inserted into the two sides of the kidney. The connecting wires are tied to the stand; otherwise, if electrodes slide from the original positions, the blood from the kidney is hard to stop.
Experimental Results

(1) Control experiments

In the first control experiment, we use a micropipette without oil in the tip (filled with Solution One only) to test the system. After micropuncture process, in order to ensure that the tip of electrode is in the lumen space, a small amount of solution is injected to see whether the solution flows along proximal tubule in a very short time. Because the kidney still stays in normal functional condition after micropuncture, the potential inside the tubule should not change with time. Figure 7.8 shows one of the results.

Figure 7.8 The potential inside the tubule in normal functional condition does not change with time.
After testing the system, we use a micropipette filled with mixed oil and Solution One for a series of experiments. After successful microinjection of the oil and the solution, the lumen potential is recorded immediately. With the tubule blocked and Na+ ions, water and other ions reabsorbed, the lumen potential increases until it reaches saturation (Figure 7.9) with a time constant of 297+/−41 s. For different individual rats, the voltages increase differently, and the range of the lumen potential increase when reaching a saturation state is 3-8 mV.

Figure 7.9 With reabsorption of ions and water, the lumen potential is increased until it reaches the saturation state after the tubule is blocked. The time to reach the saturation state is about 297+/−41 s.
(2) Experiments with blockage of proximal tubule and under electric field stimulation

After control experiments, the next step of the experiment is to find out how an electric field influences Na/K pump activities and the lumen potential. In our previous chapters, we have experimentally proved that (1) when a symmetric, oscillating membrane potential train with a frequency close to the mean physiological turnover rate is applied across the cell membrane; the Na/K pump molecules can be synchronized. The Na/K pump molecules work as a group, pumping at a synchronized pace after a long train stimulation. (2) After synchronizing the Na/K pump molecules, we can accelerate the turnover rate of pump molecules by increasing the electric field frequency gradually in order to re-synchronize molecules to a higher frequency. After a synchronization-modulation stimulation pulse train, the Na/K pump proteins are modulated to the higher frequency with a large increase of pump currents, and the membrane resting potential can be hyperpolarized [130, 131] as a result. In Jorgensen’s paper [148], Jorgensen pointed out that the turnover rate of the Na/K pumps on proximal cells is about 20-90 Hz in normal physiological condition, which is similar to the turnover rate of the Na/K pump proteins on the muscle cell membrane which is used in our previous paper [136]. Our synchronization-modulation method should work on the kidney’s Na/K pumps as well.

Micropipettes filled with oil and Solution One are used as described before. After the lumen potential reaches a constant value, synchronization-modulation stimulation electric train is applied to the kidney. In this experiment, two different trains with a slight difference are used: in the first train, the potential of the last pulse of the train changes from -1 V to 0 V, and in the second train, the potential of the last pulse changes from 1 V to 0 V. Two examples of the results are shown in Figure 7.10 and Figure 7.11. The lumen
potential reaches the highest value after the stimulation train stops in both experiments. Then the potential gradually (the time constant is about a range of 50 s to 150 s) returns to a new saturation state; however, the value of the potential is higher than the potential obtained without a train stimulation. The potential increases about 3-7 mV which is about 25%~35% increase over the original lumen potential.

Figure 7.10 After the lumen potential reaches a constant value, the stimulation electric field train (synchronization-modulation) is applied to the kidney. The new build-up potential is increased about 3-7 mV.
Figure 7.11 Same as Figure 7.10 except that the potential of the last pulse of the stimulation train changes from -1 V to 0 V instead of from 1 V to 0 V.

In Figure 7.12 and Figure 7.13, the detailed information of the last pulse for both experiments is shown. We obtain the same result no matter if the potential changes from 1 V to 0 V or from -1 V to 0 V. The lumen potentials have similar shape and magnitude after the trains are finished. The highest lumen potential reaches around zero voltage, which is probably due to the fact that most of Na+ ions are absorbed by the tubular cells.
Figure 7.12 Detailed information of the last pulse in Figure 7.10
(3) Confirm experiments

To confirm that the potential increase inside the lumen is caused by the high activities of Na/K pumps and Na+ ions reabsorption process due to the electric stimulation train, we conducted several experiments.

In the first experiment, high concentration Ouabain (20 mM) is used in Solution One in order to block most Na/K pump proteins, pursuant to Jorgensen’s paper [148]. With high concentration Ouabain inside of the solution, the potential of the lumen after
synchronization-modulation electric stimulation train does not show a significant increase (Figure 7.14). This experiment proves that the increase of the lumen potential is resulted from the Na/K pump activities.

![Graph showing voltage changes over time.](image)

**Figure 7.14** When high concentration Ouabain is resolved in the injected solution, the lumen potential does not show significant change after stimulation train.

In the second experiment, only two electric stimulation pulses are applied to the kidney (Figure 7.15 and Figure 7.16) to see whether the lumen potential increases with a short electric field stimulation. Figure 7.15 shows that the train changes from -1V to 0V during the last pulse; and Figure 7.16 shows that the potential changes from 1V to 0V during the last pulse. The results reveal that the lumen potential does not change significantly under those two stimulations. This experiment proves that the potential increases only under long synchronization-modulation electric stimulation train.
Figure 7.15 The top of the Figure shows two electric stimulation pulses which are applied to the kidney. The bottom of the Figure shows the lumen potential responding to the pulses.
Figure 7.16 Same as 7.15 except that the potential of the last pulse changes from 1 V to 0 V instead of from -1 V to 0 V.

In the third experiment, 95% of the Na+ ions are replaced by K+ ions in Solution One, which is injected into the tubule after the mixed oil injection. Under electric stimulation, the potential of the inside of the lumen changes slightly (the result is, therefore, omitted here. See Figure 7.14 for similar result) compared to the result in the previous experiment. The driving force of Na+ ions’ reabsorption is based on the concentration difference across the membrane generated by Na/K pump proteins; and the driving force of other ionic transport systems is based on the reabsorption of Na+ ions. Actually, in kidney, more than 99% of the transported electrolytes are coupled with the
primary active Na/K pumps [157]. In this experiment, only a very small amount of Na+ ions are available to be transported inside of the lumen, so it is a reasonable result that the potential does not change too much inside of the lumen. The experiment proves that Na+ ions play an indispensable role in the increase of the lumen potential.

In proximal tubule, Na+, HCO_3^- H+ and Cl^- ions are the most important ions to contribute to the lumen potential change. In the fourth experiment, Solution Two (without HCO_3^- ions) is used instead of Solution One. After electric stimulation train, the result shows that the lumen potential can still be increased, but with less value (1 mV to 4 mV, Figure 7.17). This experiment proves that HCO_3^-, like Cl^- ions, contributes partially to the increase of the lumen potential.

![Figure 7.17](image)

Figure 7.17 Solution Two (without HCO_3^- ions) is used instead of Solution One. After electric stimulation train, the lumen potential can still be increased, but with less value compared to Figure 7.10.
The above four confirm experiments prove that the lumen potential increase results from high Na/K pump activity generated by synchronization-modulation electric train. The high Na/K pump turnover rate will require more Na+ ions to be absorbed. Na+ ions and others ions, such as HCO₃⁻, H+ and Cl- ions, work together to increase the lumen potential.

(4) Experiments without blockage of proximal tubular segment and under electric field stimulation

This group of experiments is to test the lumen potential without oil blockage of the segment. If electric field can generate potential difference in the insulating segments as mentioned before, it should affect Na/K pump proteins even without oil blockage. The only difference is that after stimulation, the lumen potential cannot stay at the new potential level, because the kidney will return to the normal condition after the stimulation train. Figure 7.18 confirms that the potential changes in a way as we expected under the synchronization-modulation electric stimulation train. Within 100-150 s after the stimulation train stops, the lumen potential gradually decreases to the original value.
Figure 7.18 After the stimulation train is finished, the lumen potential gradually decreases to the original value when the tubule is not blocked by oil.

**Discussion**

The Na/K pumps in a renal tubular cell are exclusively located in the peritubular membrane and are considered to play a major role in ions transport across the cell membrane through hydrolysis of ATP to generate electro-chemical gradients. The most important substrates in proximal tubule cell transport system which can affect the potential are Na+, H+, Cl- and HCO3-, each of which may involve passive or active transports. But almost all ions reabsorption process is based on the Na+ ion reabsorption which is determined by Na/K pump activity.
In the previous chapters, we have experimentally demonstrated that synchronization-modulation electric train can accelerate Na/K pump turnover rate, which forces Na/K pumps to follow the train frequencies, resulting in a large increase in pump currents. In Chen and Dando’s paper [130,131], the same method is applied to intact fibers. The membrane resting potential can be hyperpolarized. In this research, the tubule cells, under the high activities of the Na/K pumps resulting from the influence of an electric field, can absorb more Na+ ions from the lumen fluid to the peritubular fluid. The process of Na+ and other ion reabsorption and secretion will result in an increase of the lumen potential. In Jorgensen’s paper [148], the author reached the same conclusion: the lumen potential changes positively due to anion transport from lumen to peritubular fluid or due to secretion of cation from cytoplasm to lumen. We already confirmed that the potential increase inside the lumen is caused by the high activity of Na/K pumps and Na+ ions reabsorption process due to the electric stimulation train.

However, what is the mechanism of the potential increase? In order to explain the potential change resulting from high Na/K pump activity due to the influence of an electric field, Na+, H+, HCO3- and Cl- ions transports have to be taken into consideration, which make the transport process a very complex secretion and reabsorption system in proximal tubule.

The reabsorption of Na+ ions is a major event in proximal tubule transport system which is combined with other ions transport system. We will consider two reabsorption and secretion groups: (1) Na+ / H+ / HCO3- and (2) Na+ / Cl- / HCO3-, to find out how the lumen potential can be increased under high activities of the Na/K pump by the
influence of the electric field stimulation, although those two groups are not totally independent processes.

(1) Proximal tubule cells have a relatively high density of Na/H exchangers at both sides of the cell membrane. The Na/H exchanger results in net Na+ ions absorption. The Na/H exchanger serves as a pathway for cellular influx of Na+ and is a major mechanism of extrusion of intracellular H+ ions to the lumen, which is a main factor to absorb the HCO3- ions. The secretion of H+ ions is opposed to reabsorption of HCO3- ions. Results from other researchers \[163, 164\] demonstrate that the increase of the Na/K pumping rate requires an increase of intracellular Na+ ions which can be achieved by increasing Na+ influx through increased Na/H exchangers.

There is an indirect way to couple HCO3- ions reabsorption and Na+ ions reabsorption on the renal reabsorption system (Figure 7.19). First, Na/H exchangers transfer H+ ions to the lumen fluid. There, the H+ ions are used to combine HCO3- ions and convert to CO2 and H2O. These two products (CO2 + H2O) enter the proximal tubular cells through water single-ports which assist in diffusion of H2O and may also play a role in CO2 uptake. Inside the cell, CO2 and H2O are converted to H+ (which is extruded into the lumen by Na/H exchangers) and HCO3- again.

Sym-ports on the membrane between the tubular cells and peritubular fluid operate with a stoichiometry of 1:3 of Na/HCO3- \[163, 171\] (export three HCO3- ions to peritubular fluid with each Na+ ion). As a result, two net negative charges (HCO3- ions) cross the plasma membrane. This process will affect the potential difference between the lumen and the peritubular fluid and make the lumen potential more positive. Under synchronization-modulation electric train, more Na+ ions are transported from the lumen
and more HCO3- ions are transported across the tubular cells which result in the increase of the lumen potential. In his paper [148], Jorgensen came to the same conclusion.

![Diagram of Na+ / H+ / HCO3- ions transport system](image)

Figure 7.19 A sketch of Na+ / H+ / HCO3- ions transport system in the proximal tubule segment.

(2) In the reabsorption process of Na+ and HCO3- ions as we described before, water molecules are also reabsorbed when HCO3- ions are transported into the cell, which will increase the concentration of Cl- ions inside of the lumen from 1 to 1.2-1.4 [167, 168]. According to Karol’s paper [167], the reabsorption process of HCO3- ions is preferred over Cl- ions in the proximal-tubule reabsorption system. In John and Edward’s
paper [168], experimental results indicate that the absorption process of HCO$_3^-$ ions can partially inhibit net Cl- ions’ absorption in the early proximal tubule system and when luminal Cl- concentration is raised, the lumen potential becomes more positive [168,170].

Although HCO$_3^-$ ions dominate when HCO$_3^-$ and Cl- ions are present at the same time, the Cl- ions still can be transported into the peritubular fluid. Because the mechanism of H+ ions’ secretion favors the reabsorption of HCO$_3^-$ over that of Cl- ions, tubular flow’s pH and HCO$_3^-$ concentrations both decrease [148,151], whereas net Cl-absorption increases [167]. The luminal fluid acidification will reduce the efficiency of the Na/H exchangers.

Cl- ions transport across membrane is also coupled to Na+ ion uptake (Figure 7.20). Experimental evidence indicates that approximately 50% of Cl- ions’ reabsorption is dependent on the presence of Na+ ions, and this sym-ports absorption is electroneutral since Na+/Cl- = 1. The other 50% of Cl- ions is diffused into the peritubular fluid (Figure 7.20). Cl- ions transport systems in proximal tubule are both active and passive and the process depends on Na+, H+ and HCO$_3^-$ ions as well as acid-base metabolism [174]. The whole process of Cl- ions’ reabsorption is electrogenic since the net negative charges cross the membrane, which results in the increase of the lumen potential.

Na+ ions working together with other ions (Cl-, H+ and HCO$_3^-$) can generate the positive potential increase in the proximal tubule lumen by high activities of Na/K pumps due to the electric synchronization-modulation stimulation trains
Conclusion

In the previous chapters, we have proved the synchronization-modulation method at the single cell level, which can effectively build up a new potential difference across the cell membrane by high activities of Na/K pumps. For this research, the same synchronization and modulation protocol is applied to the rat kidney, which is the first step to apply this theory at the organ level. Under the influence of electric field, more Na+ ions are reabsorbed, and the potential of the inside of the tubule is more depolarized. The increase of the potential is a result of the complex combination of Na+, H+, HCO₃⁻ and Cl⁻ ions secretion and reabsorption processes inside of the proximal tubule.
Chapter 8
Conclusion and Future Study

A large number of pharmacological agents (neurotoxins) have been identified that can affect electrically excitable cell channel currents. The most important channel blockers are Tetrodotoxin (TTX) and Teraethylammonium (TEA), which selectively block Na+ and K+ channels. These agents enable investigators to block either Na+ current or K+ current in a reversible manner and make the study of individual ionic current more convenient.

In first part of my research, we successfully used well-designed electric stimulation pulse train to block Na+ and K+ channels and found out that almost all of individual Na+ and K+ channel proteins are forced to stay in un-conductive state under electric field. Those results indicate that Na+ and K+ channel proteins can be blocked by an electric method, instead of using chemicals such as TTX and TEA. This research not only introduces a new method in basic science research but also has great clinical potential.

Numerous diseases and disorders are directly related to the functions of Na/K pump proteins, like heart failure, hypothyroidisim, McArdle disease, diabetes and cystic fibrosis. Failures of Na/K pump molecules can be grouped into two categories. In some diseases, patients have very low density of Na/K pump molecules on the cell membrane. In other cases, patients have normal density of pump molecules, but they face a shortage
of nourishment or oxygen which results in a lack of ATP molecules to fuel Na/K pump molecules. A common phenomenon of these diseased cells is a reduction in ionic concentration gradient or depolarization of the membrane potential. In order to restore these features, a possible fast and economic option is to activate Na/K pump proteins.

Significant efforts have been made to study Na/K pump proteins. We recently developed a new approach: dynamic entrainment of Na/K molecules. In this approach we consider molecules energy absorption as a procedure of electrical synchronization and modulation of pump molecules, instead of transient event. In the first step, we organized pump molecules together at the same pace by electric train. The pumping rate of synchronized pump molecule is then further gradually modulated to a higher value by a well designed oscillating electric field. Our experiments in skeletal muscle fibers have demonstrated synchronization and modulation phenomena of Na/K pump molecules and up to several fold increase in pump currents.

After single cell level study, we move to the organ level – rat’s kidney because kidney has plenty of Na/K pump molecules on the cell membrane to reabsorb ions in all tubular segments. Active Na/K pumps directly increase the rate of ions reabsorption and the potential inside the lumen. This research proves that the synchronization-modulation method can work at the organ level as well.

Our research has huge potential in clinical applications, but we have to figure out how to non-invasively put electric stimulation train on the project not only kidney but also other organs like heart, brain and skin, etc.
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List of Publications


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