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The modified Synchronization Modulation technique revealed mechanisms of Na,K-ATPase

Pengfei Liang
University of South Florida, liangpengfei161@gmail.com

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The modified Synchronization Modulation technique revealed mechanisms of

Na,K-ATPase

by

Pengfei Liang

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

Major Professor: Wei Chen, Ph.D.
Defense Chair: Wenxiu Ma, Ph.D.
Bin Xue, Ph.D.
Jianjun Pan, Ph.D.
Ghanim Ullah, Ph.D.

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Dedication

This dissertation is dedicated to my brilliant, beautiful and selfless wife, Linyu Yu, who inspires me and supports me through this long journey.
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First of all, I would like to specially thank my major professor, Dr. Wei Chen, for his guidance, patience, and support throughout my study and research. It is hard to imagine that I could complete my dissertation without his help. His spirit of research has a positive and lifelong impact on my professional development.

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Abbreviations

SM . . . . . . Synchronization Modulation
TEPD . . . . Transepithelial Potential Difference
ECC . . . . . Electroconformational Coupling
Abstract

The Na/K pumps are essential for living system and widely expressed in all eukaryotic cell membranes. By actively transporting sodium ions out of and potassium ions into the plasma membrane, Na/K pumps creates both an electrical and a chemical gradient across the plasma membrane, which are crucial for maintaining membrane potential, cell volume, and secondary active transporting of other solutes, etc.

Previously, oscillating electric field with a frequency close to the mean physiological turnover rate was used to synchronize and modulate the Na/K pump molecules. Results showed that the turnover rate of Na/K pumps can be accelerated by folds. However, this what we called first generation synchronization modulation (SM) technique can only synchronize sodium and potassium translocations into their corresponding half cycles. The detailed location of each sodium extrusion and potassium intrusion can not be determined. As a result, the synchronized pumps were uniformly distributed, generating steady-state macroscopic currents.

Based on these studies, Dr. Chen developed a new generation synchronization modulation technique. The waveform of original SM by adding an overshoot pulse at the end of each half cycle. This overshoot pulse has a function of energy barrier which will force all of the Na/K pumps into the same state in the pumping cycle until the membrane polarity change. As a result, Na/K pump molecules are not only synchronized into half cycles of oscillating electric field, but individual steps of the pumping cycle. Accordingly, transient pump currents or so called ‘pre-steady state’ pump currents are generated, from which some detailed information about the mechanism of Na/K pumps can be dissected.

In this dissertation, we firstly characterized the synchronized pump currents by modified SM. The results showed that transient currents were induced at the beginning of each half cycle as expected. The ratio between positive and negative transient currents was close to 3:2, stoichiometric number of Na/K pump. Moreover, the transient currents were significantly reduced in the presence of ouabain in a time dependent manner. In addition, by gradually increasing the frequency of SM electric field in a step-wise fashion, the synchronized pump current can be modulated to the
corresponding level. Next, we utilized this technique to study some detailed mechanisms of Na/K pump, including single channel configuration in transmembrane domain and extracellular D$_2$O effect on the turnover rate.

Lastly, we extended our study to applications of this new technique and found that the modified Synchronization Modulation technique can significantly hyperpolarize the membrane potential of skeletal muscle fiber in both physiological and high $[K]_o$ conditions. During intensive exercise, the interstitial potassium ions are accumulated and temporarily reach a high level, which will attenuate the contraction force and induce muscle fatigue. Na/K pumps are crucial in the maintenance of skeletal muscle excitability and contractility by restoring the Na and K concentration gradients. By accelerating the turnover rate of Na/K pumps, SM can efficiently re-establish the membrane potential and enhance skeletal muscle contractivity, which unleashes its potential in improving certain pathological conditions, such as exercise-induced hyperkalemia.
Chapter 1  Introduction and literature review

1.1 Introduction of Na,K-ATPase

Most if not all of the living cells maintain a similar and stable ionic composition on the cytoplasmic side, such as low sodium, high potassium, low calcium, etc.[1] When ionic concentration gradients are significantly altered, continuous neuronal firing or intensive muscle contraction for instance, it requires active transport activity to maintain the homeostasis of intracellular ions. Na, K-ATPase also known as Na/K pump, an active transporter, could fulfill the needs. In each pumping cycle, three sodium ions are extruded followed by intrusion of two potassium ions and thus one net positive charge is exported, hyperpolarizing the membrane potential. Na/K pumps are critical to many enzymatic functions, such as controlling cell volume and maintaining the resting potential, etc. In addition, large amount of energy is transferred from ATP hydrolysis free energy to electrochemical potential energy across the plasm membrane by Na/K pump, which is vital for fueling the secondary transporters or exchangers, such as Na/Glucose transporter and Na/Ca exchanger. The regulation of Na/K pump in variety of tissues have been reported. Herein, we emphasis the functional significance of Na/K pumps in kidney, skeletal muscle and neural system because they are highly expressed in these organs.

In kidney, the Na, K-ATPase is highly demanded. It has been reported that there is up to 50 million pumps per cell compared with a few hundred to a few thousand pumps in non-polarized cells [2]. Na/K pump established the Na concentration between lumen and epithelial cells which provides energy for secondary co-transporters for filtering metabolic waste from blood, reabsorbing amino acids, glucose and other large molecules into the blood and balancing PH [3, 4]. Moreover, the dysfunction of Na/K pump would result in multiply kidney diseases and may potentially cause kidney failure [5]. Recently, people found that impaired Na,K-ATPase signaling in renal proximal tubule contributes to hyperuricemia-induced renal tubular injury [6].

In the neuronal system, Na, K-ATPase consume up to 60 percent of metabolic energy that
is required for the maintenance of the ionic gradients that underlie resting and action potentials for nerve impulse propagation. Na/K pump was first identified as key element for K and Na homeostasis [7, 8]. More recently, people found that the sodium-potassium pump may serve as an information processing element in brain coding and computation upon cerebellar Purkinje neurons [9, 10]. Also, Na/K pump activity can also influence neuronal firing and regulate rhythmic network output [11]. The inhibition of Na/K pump with ouabain increased the frequency and decreased the amplitude of drug-induced locomotor bursting. Moreover, investigators found that the Na/K pump function in neurons from alternating hemiplegia of childhood (AHC) patient was impaired, which would result in significantly depolarization of potassium equilibrium potential as well as resting membrane potential in AHC neurons compared with control neurons [12]. A reduction in Na\(^+\),K\(^+\)-ATPase expression and function is associated with depressive disorders in humans [13, 14] as well as in animal models [15, 16].

The regulation of Na/K pump on skeletal muscle contractility has been well documented in multiply excellent reviews [17, 18]. The skeletal muscle excitation is elicited by a rapid influx of Na ions through Na channel, followed by a similar efflux of K ions across sarcolemma and t-tubular membranes. So during intense work, the interstitial K would be accumulated and temporarily reach a relative high level [19, 20]. In the presence of large number of Na/K pumps located in the sarcolemma and the t tubules of skeletal muscle, Na and K concentration gradients are quickly re-established [21, 22]. Large number of evidences showed that Na/K pumps are crucial in the maintenance of skeletal muscle excitability, contractile force recovery and cell membrane repolarization [23].

1.2 Downregulation of Na/K pump in certain pathological conditions

In certain diseases or disorders, the ouabain binding sites are severely less than normal situation. For kidney, a diffuse loss of Na/K pump sites is seen after adrenalectomy of kidney and it is more pronounced in the outer medulla than in cortex [24, 25]. In addition, massive evidences show that there is downregulation of Na/K pump in ischemic acute kidney injury and after ischemia/reperfusion injury [26, 27].

For neuron system, Na\(^+\),K\(^+\)-ATPase activity in rat brain is significantly reduced during aging [28]. In addition, there is an announced decrease of the Na/K pumps in brain in patients with
Alzheimer disease in comparison with age matched controls, particularly in the cerebral cortex [29, 30]. Also, when the middle cerebral arteries of rats were occluded by cannulation with a nylon suture which produced ischemia, Na, K-ATPase activity was significantly decreased [31].

For skeletal muscle, studies suggested that the contents of ouabain binding sites were 3-6 folds lower in skeletal muscle samples from patients with myotonic muscular dystrophy than control group [32, 33]. As a result, muscles from patients with muscular dystrophy show significant membrane depolarization, which may contribute to impairment of muscle contraction and physical disability [34]. Also the age-dependent decrease in Na-K pump content in rat soleus was associated with a considerable impairment of endurance and force recovery [35].

These observations lead to the first question: How could we upregulate the function of Na/K pumps to compensate the lost in these pathological conditions? Massive efforts have been made to modulate the pump function, among which the oscillating electric field is discussed here due to its potency [36, 37, 38]. The development of studying the oscillating electric fields activation effect on Na/K pump is based on the fact that ion translocations through the Na/K pump are sensitive to membrane potential. The detailed mechanism is discussed in the following.

### 1.3 Oscillating electric fields activate Na/K pumps-ECC model

In each reaction cycle, the Na/K pump transports three Na ions to extracellular across the cell membrane and import two K ions. The one net extrusion of positive charge generates outward membrane current. Historically, voltage dependency of Na/K pumps has been investigated by electrophysiological and spectroscopic approaches. The results have been demonstrated on different tissues and cells including nerve [39, 40], muscle [41, 42], heart [43, 44] and oocyte [45, 46], etc. Briefly, the pump I-V curve is sigmoid with a "foot" at large negative potentials, following with positive slope in a very wide range and reaching maximum at around 0-20 mV (Fig. 1.1) [47]. Based on this biphasic voltage dependence to the pump, people proposed that there are at least two voltage-dependent steps in the pumping cycle that are oppositely affected by the membrane potential. Consistent results have been obtained in the presence of voltage sensitive dye RH421 [48, 49].

As more detailed structure information of Na/K pump is available from the crystalized structure, hydrophilic paths also called access channels are found on each side of cell membrane. People
suggested that most of the voltage dependence of Na/K pump originates as ions move along these access channels sensing the electric field across the membrane. The voltage dependency of sodium ions extrusion [50, 51] and potassium ions intrusion [52] has been investigated separately. Even though there is no final conclusion of the mechanism related to voltage dependency, it is well known that any step that involves net charge movement through the membrane must have voltage-dependent transition rates. Therefore, the Na/K pump can potentially be activated in designed electric field.

Effects of applied electric fields on Na\(^+\),K\(^+\)-ATPase activity have been reported by multiple groups. Pioneering work is done by Tsong. His group described an ouabain sensitive accumulation of rubidium and secretion of sodium, mediated by the Na\(^+\),K\(^+\)-ATPase in red cells, that was stimulated by alternating currents (AC). Moreover, the voltage-stimulated Rb\(^+\) uptake is frequency dependent and completely inhibited by ouabain. They suggest that the AC field is capable of polarizing the membrane potential, which can provide energy required for the inward movement of Rb\(^+\) or K\(^+\). After a simple energetic consideration, author suggested that an enzyme conformational change must have also occurred during the AC stimulation. More interestingly, people found that the stimulated pumping of ions against concentration gradients appeared to have derived energy from the AC field since no excess consumption of ATP was detected [53].

To explain these experimental data, an electroconformational coupling (ECC) model was proposed [54, 55, 56]. The model hypothesizes that a membrane enzyme with several functional states of different charge distributions or electric moments, will undergo conformational changes in an electric field. If the field is oscillatory, it will enforce the conformational oscillation of the enzyme within its catalytic cycle. This field-enzyme interaction will enable the enzyme to utilize the electrical energy for performing chemical work [57].

Later, Xie, etc. reported that a random-telegraph fluctuating (RTF) electric field consisting of alternating square electric pulses with random lifetimes can stimulate the Rb\(^+\) uptake in the Na\(^+\), K\(^+\)-ATPase [58]. They suggested that Na/K pump can recognized an electric field, either in regular oscillating mode or in random fluctuation mode for energy coupling. Also, the same group reported that a Gaussian-RTN-electric field, or a field with amplitude fluctuating according to the Gaussian distribution also activated the Na/K pumps in human erythrocyte [59]. In 2002, Tsong, etc. reported that electric fields could induce the conformational fluctuation without ATP
consumption, where a theory of electro-conformational coupling (TEC) that embodies essential features of the Brownian motion was proposed [60]. To further explain the underlying mechanism of activation of Na/K pump by electric field, an adiabatic pump model have been further postulated [61].

![Figure 1: I-V curve of Na/K pump](image)

1.4 Oscillating electric fields activate Na/K pump - Synchronization Modulation

Previously, I-V curve of Na/K pump is exhibited with a sigmoid shape and saturation behavior, indicating that the pump molecules are not particularly sensitive to the membrane potential, and the pump current has an upper limit. The low sensitivity to the membrane potential is mainly due to the opposite ion translocations, Na-extrusion and K-intrusion. Any membrane potential change, either depolarization or hyperpolarization, can only facilitate one transport but hinder another, and consequently, it cannot significantly increase the pumping rate. To resolve this issue, Dr. Chen and his lab further considered using an oscillating electric field whose frequency is comparable with the Na/K pumps turnover rate to alternatively facilitate both limbs of Na and K transport [47]. This technique is called Synchronization Modulation. To explain the mechanism of this technique, authors constructed the energy profile for the two cations within positive and negative half cycle of the electric field. For skeletal muscle fibers, the intracellular and extracellular Na concentrations
are about 4.5mM and 120 mM [62, 63], respectively. Na equilibrium potential of +60 mV can be easily calculated from Nernst-Planck equation,

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

(1.1)

Where, $[Na]_o$ and $[Na]_i$ are extracellular and intracellular Na concentration, respectively. R is the ideal gas constant, T is the temperature, F is Faraday’s constant and Z is the valence of the charge carrier.

If we choose the holding potential at -90 mV and apply a symmetric pulsed oscillating waveform with an amplitude of 60mV. The membrane potential will be alternated from -30mV to -150 mV. Based on calculations in table 1, extrusion of three Na ions during the negative half cycle of the oscillating electric field requires 630 meV of energy, which is 180 meV more than at the membrane resting potential. Also, it is worth to mention that single ATP molecule hydrolysis energy is around 550 meV [64], less than the requirement of 630 meV. Therefore, the Na extrusion will be hindered during the negative half-pulse. On the contrary, energy required to extrude 3 Na ions during positive half cycle reduces to 270 meV, much lower than that consumes at membrane resting potential and the ATP hydrolysis free energy. Thereby, Na extrusion will be favored during the positive half cycle of the oscillating electric field. Similarly, based on the intra- and extracellular K ion concentrations of 115 and 5 mM, respectively, the K equilibrium potential of -90 mV can be obtained. Based on calculations in table 1, energy required for 2 K ions intrusion within positive half cycle is 120 meV, which becomes -120 meV during negative half cycle. The negative sign suggests that K ions could actually gain 120 meV energy from negative half cycle. Thus, K ions intrusion favors negative half cycle of the oscillating electric field. The more detailed theory of SM technique has been presented along with the computer simulation [65, 66].

Based on the calculations and analysis above, by applying well designed oscillating electric field, working paces of the Na/K pumps can be synchronized. More specifically, all of the Na extrusions and K intrusions will be synchronized into positive half cycle and negative half cycle of the oscillating electric field, respectively. The schematic picture of Synchronization Modulation is shown below.

In practical, the Synchronization Modulation technique consists of two steps: synchronization
Table 1: Energy Profile of \( Na^+ \) and \( K^+ \) translocations

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<th>2 ( K^+ ) intrusion energy (meV)</th>
<th>3 ( Na^+ ) extrusion energy (meV)</th>
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<tr>
<td>Positive half cycle</td>
<td>((90-30)^*2 = 120)</td>
<td>((60+30)^*3 = 270)</td>
</tr>
<tr>
<td>Negative half cycle</td>
<td>((90-150)^*2 = -120)</td>
<td>((60+150)^*3 = 630)</td>
</tr>
<tr>
<td>Resting potential</td>
<td>((90-90)^*2 = 0)</td>
<td>((60+90)^*3 = 450)</td>
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Figure 2: Schematic diagram of synchronization modulation
and modulation. In the synchronization step, a designed oscillating electric field is applied to synchronize the individual pump molecule to run at the same pace, so that the Na\(^+\) ions translocation from individual pump are entrapped into the positive half cycles, while all the K\(^+\) transporters are entrapped in the negative half-cycle as discussed above. The measured Na/K pump currents in response to the SM electric field have been shown with characteristics as following [47]. Initially, the pumps run at random paces with different pumping rates at random phases. The positive half-pulse elicited net outward pump currents, and the negative half-pulse elicited very little current. As more oscillating pulses applied, the negative half pulses gradually elicited distinguishable inward currents which were alternated with the outward components and the magnitude ratio of the outward to inward pump currents was about 3:2, reflecting the stoichiometric number of the Na/K pump [53], [83]. Once reaching synchronization, the field frequency can be adjusted again to a higher value. By this way, the pump molecules can be gradually modulated to higher turnover rates [67].

1.5 Current techniques in synchronizing the Na/K pumps

Results show that original synchronization modulation technique could efficiently synchronize sodium extrusion into positive half cycle and potassium intrusion into negative half cycle of the electric field. Once the pumps are synchronized and working in the same phase with respect to one another, the frequency is adjusted in stepwise increments (modulation) which speeds up the pumps turnover rate. This technique has been applied on multiple cells and tissues and results show that it could efficiently hyperpolarized the membrane potential as well as the transepithelial potential of the kidney renal tubular [68, 69, 70, 71]. But this what we called the first generation SM technique can only synchronize the pumps into each half cycles of oscillating electric field. In other words, we are unable to determine the detailed location of each pump current in their own half cycle [72]. As a result, the pump currents are uniformly distributed and there is no or very small transient current being induced [51].

Indeed, in order to synchronize the individual Na/K pump, many studies have been reported. For example, by shooting laser beam to NPE-caged ATP, people are able to release the ATP molecules simultaneously. As a result, the Na/K pumps will bind ATP to their N domain at the same time, synchronizing their function. It has been reported that large transient pump currents
were obtained using this method [92]. In addition, by applying designed voltage waveforms and analyzing the transient relaxation currents, people were able to distinguish three components of Na ions being released to the extracellular solution [51] and later mechanism of two K ions uptake was also presented[52]. The key point of this technique is that most of the pump molecules are restricted at E2 state by depletion of either Na ions or K ions from the solutions. Next, suddenly change the polarity of the cell membrane by giving a designed voltage, those restricted pump molecules would be activated simultaneously. As a result, large amount of pump molecules will work under the same pace (synchronization) and induced a large transient pump current, from which people dissected detailed components for Na and K binding or releasing information.

However, most of the experiments were conducted on partially functional pump molecules, where the well-known energy provider or driving force, ATP hydrolysis, was interrupted. So any conformational changes related to ATP hydrolysis would be more or less affected. On the other hand, it is well known that phosphorylation and de-phosphorylation are tightly related to conformational changes of Na/K pump such as occlusion and de-occlusion, etc. Hereby, without ATP consumption, conformational changes should be restrained. Moreover, in the partially functional pumps, dynamic correlations between E1 and E2 states are overlooked. For instance, a manner that energy from ATP hydrolysis being transferred from E1 state to E2 state for K intrusion should be exist since ATP hydrolysis occurred on cytoplasmic side of the membrane while K intrusion happened on the other side. This long distance correlation mechanism is impossible to be revealed in the partially worked pump molecule. Thus, it is questionable that whether information obtained under partially functional pumps could reveal the mechanism of natural Na/K pump or not. These discussions lead to the second question: How could we synchronize Na/K pumps into individual steps under physiological condition? To address this issue, a second generation SM technique is presented and developed in this dissection. The investigations based on this new version SM are organized as following:

- In Chapter 2, we presented the mechanism of modified synchronization modulation or what we called second generation synchronization modulation technique. The characteristics of the synchronized pump current (mainly synchronization part), voltage dependency of the synchronized pump currents, ouabain inhibition effect, synchronized currents under Na/Na exchange mode as well as computer simulation were also included.
• In Chapter 3, we investigated the single channel configuration that revealed by second generation SM. We found that when the concentration gradient of K reduced and meanwhile the applied voltage was large enough, the activation and relaxation current obtained from K/K exchange mode of Na,K-ATPase was no longer symmetric. Based on these results, we proposed that instead of two structural access channels in the transmembrane domain, there is only. While several negatively charged amino acid located in the middle of the ion pathway form an energy trap which seems divide this channel into two segments.

• In Chapter 4, we re-studied the slow down effect of D$_2$O on Na/K pumps utilizing second generation SM as a platform. The key point of this study is to compare the magnitudes of synchronized pump current at different frequencies in D$_2$O and H$_2$O. Results showed that the maximum synchronized current was obtained when the frequency of SM was set at 50Hz in H$_2$O. Whereas, the frequency reduced to about 25Hz in D$_2$O, which suggested that D$_2$O slowed down the turnover rate of Na/K pump.

• In Chapter 5, we analyzed the synchronized and modulated pump current (mainly modulation part). We found that by gradually modulating the frequency of SM electric field upward in a stepwise fashion, the transient pump currents increased correspondingly.

• In Chapter 6, we tested the capability of second generation SM in hyperpolarizing the cell membrane potential. We showed that the SM technique could consistently hyperpolarize the membrane potential by 3-4 mV in a short time under physiological condition. Additionally, we increased the extracellular potassium concentration which artificially mimicked the hyperkalemia condition. Noticeably, the hyperpolarization of membrane potential induced by SM was more potent with magnitude about 6-7 mV. Thees results unleash the potentials of applications of modified SM on certain pathological situations such as intensive-exercise induced hyperkalemia.
Chapter 2 Transient Na/K pump currents induced by SM

2.1 Introduction

Transient Na/K pump current or pre-steady-state pump current has been studied for many years. There are usually two different methods to obtain it. One used caged-ATP, in which ATP is released from a non-hydrolyzable cage by an intense ultraviolet laser beam [73, 74]. Based on the reaction sequence of Na translocation,

\[ \text{Na}_3\text{E}_1 \rightleftharpoons \text{Na}_3\text{E}_1-\text{ATP} \rightleftharpoons (\text{Na}_3)\text{E}_1-P \rightleftharpoons P - E2(\text{Na}_3) \rightleftharpoons P - E2 \]

it will generate a high concentration of \( \text{Na}_3\text{E}_1 \) state in the absence of ATP. Then a rapid release of ATP from NPE-cage would result with a right shift of equilibrium to \( \text{Na}_3\text{E}_1-\text{ATP} \) and the following steps. To a certain extent, the Na/K pumps are synchronized to the same pace and generate a transient pump current. By means of this method, synchronized pump currents are obtained with time constant in 100 ms range. Another method is to apply sudden voltage jumps to Na/K pumps that either under Na/Na mode or K/K mode, which people also called partial reactions of Na/K pumps [75, 76]. In the absence of either Na or K ions, the Na/K pumps will be concentrated in certain states (P-E2 for instance) of the pumping cycle. A sudden membrane polarity change by voltage jumps would also shift the equilibriums to the following states simultaneously, synchronizing the pace of Na/K pumps. The time constant of the transient pump current is much shorter than that with ‘caged-ATP’ method discussed above, with a value of several milliseconds. In both techniques, Na/K pump molecules are initially in a steady state and then fueled by a sudden change of either ATP substance or a voltage jump. As a result, the pace of Na/K pumps are somewhat synchronized, inducing transient pump current.

However, the rate constants calculated from transient currents varied significantly even with the same technique and experimental conditions on similar cell types. For example, Fendler et al, reported a rate constant of 20 s\(^{-1}\) on purified Na+ ,K+-ATPase-containing membrane fragments adsorbed to a lipid bilayer membrane using the caged-ATP technique [77, 78]. Whereas, under a similar condition, Apell et al concluded a rate of at least 200 s\(^{-1}\) [79]. Moreover, using voltage
jump technique on heart cell, Nakao et al announced a rate constant less than $200 \text{ s}^{-1}$ \cite{76}, while Hilgemann et al measured as $600 \text{ s}^{-1}$ \cite{75}. Time constants are obtained from macroscopic current recording which is summation of each single pump currents. So, distribution of single pump current or quality of synchronization technique may potentially affect the results, which could explain differences of rate constants of the same protein. Obviously, when all of the pumps are perfectly synchronized to the same pace, the total pump current will reflect the properties of each pump function accurately. Thus, finding a more efficient and stable synchronization technique becomes crucial for dynamic study of Na/K pump.

In this study, we investigated the characteristics of pump currents induced by the second generation Synchronization Modulation technique. Results showed that transient pump currents were induced at the beginning of positive and negative half cycles. The ratio between pump-mediated charges in the positive and negative half cycle was less than but close to 3:2, stoichiometric number of Na/K pump. The transient currents were highly sensitive to ouabain and the inhibition was in a time-dependent manner. By fitting transient currents with mono-exponential equation, we observed that the time constant of each pulse reduced while amplitude increased along the SM trace, which indicated that the synchronization modulation was a dynamic process. In addition, we extended our investigation to the effect of SM technique on the pumps that under Na/Na exchange mode. Based on our results, Na ions intrusion and extrusion can be distinguished and the ratio became 1:1, stoichiometric number under Na/Na mode. In conclusion, the results demonstrate our hypothesis that by modified SM, sodium ions extrusion and potassium ions intrusion can be synchronized into the very beginning of positive and negative half cycles, respectively, thus inducing transient pump currents. More importantly, by modified SM, we are capable of synchronizing the pump functions under physiological condition, which unleashed its potential in studying the mechanisms of Na/K pump.

2.2 Methods and materials

2.2.1 Skeletal muscle fiber preparation

The animals are anesthetized and euthanized following the protocol approved by the Institutional Animal Care and Use Committee (IACUC). Single muscle fiber is separated and chosen using the procedure elaborated before \cite{101}. Briefly, Semitendinous muscle fibers are obtained from American Bullfrog and then transferred to a Petri dish filled with a high potassium concentration relaxing
solution. Relaxing solution, just as its name implies, will relax the muscle fiber by depolarizing the membrane potential to prevent its contraction during experiment procedures. A single muscle fiber with 50–100 um diameter and 3-5 mm length is hand-dissected from its surrounding connect tissue and transferred to a double vaseline gap chamber. There are three pools of the chamber, two end pools sandwiched with a central pool. The details of this chamber can be found in [80]. The isolated muscle fiber is mounted in the notches of the two partitions filled with thin vaseline and clamped by two Delrin clips on both sides. Then under the microscope, gently moving those two clips and place a tension on the fibers to stretch the sarcomere to a length of 3–3.5 um which prevent the cell from contracting during the experiment. Thin vaseline will be used to fill the two notches to the same height of the partitions. Last but not least, end pools will be covered by two glass slips. Solutions inside the end pools will be replaced by internal solution and external solution for the central pool. Three agar bridges connect the three pools to small ponds filled with 3 M KCl.

![Diagram of the double vaseline gap technique](image)

**Figure 3:** Double vaseline gap technique
2.2.2 Templet subtraction method

**Figure 4:** Templet subtraction method. (A) Applied synchronization modulation pulse; (B) The last pulse elicited transmembrane currents; (C) Transmembrane currents elicited by the templet pulse; (D) Transient pump current which was obtained by subtracting current in Panel B from that in Panel C.

To study the pump-mediated current with high time resolution, the experimental system as well as the muscle fiber itself must remain perfectly stable. Any small perturbation of the electrical parameters of the system will result in non-negligible errors. Traditionally, Na/K pump current is recognized as ouabain or other cardiac glycosides sensitive current. To ensure mostly inhibition of Na/K pumps, there is always a waiting time (usually minutes), during which some electrical parameters of the system may change. For example, changes of equivalent of the 'Frankenhaeuser-Hodgkin space' surrounding the muscle fiber which are known occur over time will dictate the series
resistance [81]. So, to avoid or minimize any uncertainty, we proposed a new subtracting method called templet subtraction. Idea of this method comes from p/4 method used to subtract the linear capacitance current in studying ion channels. Briefly, SM-induced Na/K pump currents are obtained by subtracting each oscillating pulse from a pre-generated templet pulse who has the same amplitude and frequency. The advantages of this method are that on one hand, the linear membrane conductance current is mostly eliminated; on the other hand, it reflects the instantaneous effect of the SM oscillating electric field on Na/K pump without any contamination.

### 2.2.3 Composition of solutions

**Relaxing solution (in mM):**
120 Potassium Glutamate, 5 K₂PIPES, 1 MgSO₄, 0.1 K₂EGTA;

**Ringer solution (in mM):**
120 NaCl, 2.5 KCl, 2.15 Na₂HPO₄, 0.85 NaH₂PO₄.H₂O, 1.8 CaCl₂;

**Internal Solution (in mM):**
58 K-Glutamate, 6.8 MgSO₄.7H₂O, 5 MOPS, 20 EGTA, 10 CsOH, 3 Na₂-Creatine phosphate, 5 5-ATP-Na₂. Final [K]ᵢ 140mM, [Na]ᵢ 16Mm, PH=7.3. Stored at -20 C degree. For Na/Na exchange mode, K-Glutamate was replaced by Tetramethylammonium chloride (TMA-Cl);

**External Solution (in mM):**
3.5 3,4-Diaminopyridine (3,4-DAP), 86 NaCl, 10 CsCl, 4 KCl, 2.15 Na₂HPO₄, 0.85 NaH₂PO₄.H₂O, 1.8 CaCl₂, 1.5 BaCl₂.2H₂O, 0.001 Tetrodotoxin (TTX). Final [K]ₒ 4mM, [Na]ₒ 90mM, PH=7.1. For Na/Na exchange mode, KCl was replaced by NaCl.

### 2.2.4 Data analysis

Data is collected with Dagon T200 TEVC. Data is analyzed using pClamp10 (Molecular Devices) and a Java program by J. Mast. Significant differences were determined with Students t test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, n.s. P<0.05.
2.3 Results

2.3.1 Second generation SM induces transient pump currents

Different from the first generation SM, we added an overshoot at the end of each half cycle of the oscillating electric field (see Fig.5). The templet pulse was shown as pulse a. The mechanism has been discussed in the method section. To test if there was massive leakage induced by SM electric field, a post templet pulse was introduced as pulse c. For example, if current induced by pulse c was comparable with that by pulse a, there was no or negligible leakage and vice versa. Results showed that the transient current was highly ouabain sensitive as shown in Panel B and D. It has been well accepted that ouabain is a specific Na,K-ATPase inhibitor, which indicates that the transient currents obtained here are Na/K pump currents. In the meantime, there was no significant membrane leakage induced by SM in both conditions, which can be manifested by small current in Panel C and E. Moreover, the peak currents of positive half cycle and negative half cycle were around 30nA and -22nA, respectively (Panel F). The ratio between them was close 3:2, which was also consistent with currents obtained by original SM technique. It is well known that for each pumping cycle, Na/K pumps exported 3 Na ions and imported 2 K ions, matching the ratio above. So, we proposed that by second generation SM technique, we were able to synchronize most of the Na ions extrusion and K ions intrusion in the beginning of positive and negative half cycles, respectively.

In addition, the whole SM trace elicited pump currents after templet subtraction were presented in Fig 6. Initially, the pumps run at random paces with different pumping rates at random phases. As a result, the positive half-pulse elicited net outward pump currents, and the negative half-pulse elicited very little current (first a few pulses). However, as the membrane potential continued to oscillate; the elicited pump currents began to exhibit the following characteristics: (1) The negative half-pulses gradually generated distinguishable inward pump currents which were alternated with the outward components; (2) the magnitude of the outward pump current and inward current had a ratio about 3:2; (3) The transient current saturated after about 20 oscillating pulses; (4) The transient currents were totally abolished in the presence of 500 µM ouabain, which confirmed that the transient currents obtained were Na/K pump currents.

One argument was that other than synchronizing Na/K pumps, oscillating electric field may cause leakage accumulation as well. Accordingly, we extended our study to the inhibition of ouabain
Figure 5: Transient pump current induced by modified SM technique. (A) Applied synchronization modulation pulses; (B and D) Currents induced by pulse b subtracted from templet pulse a in the absence and in the presence of ouabain, respectively. (C and E) Currents induced by pulse c subtracted from templet pulse a in the absence and in the presence of ouabain, respectively. F. The peak currents of positive and negative half cycle with and without ouabain as indicated. G. The charges obtained by integrating the first 200us of transient currents in Panel B. (n=15)
Figure 6: Whole trace of SM induced pump current (Upper) Whole trace of pump current generated by template subtraction method; (Lower) Pump current generated in the presence of 500 uM ouabain.

on the transient currents in different time line (Fig.7). Our logic is that if there is leakage accumulation induced by SM, it would not be affected by ouabain in time dependent manner. Accordingly, transient currents were obtained every 3 minutes after ouabain addition. Results showed that the amplitude of transient current was inversely proportional to the time after ouabain and fully inhibition occurred at around 12 minutes which was consistent with previous study. Several conclusions can be drawn from this experiment. Firstly, membrane leakage accumulation current induced by oscillating electric field was trivial. Because if not, the amplitude of transient currents would be independent of ouabain or even became directly proportional with time. Secondly, the pump molecules that have not been inhibited would remain synchronized under SM, which can be manifested from the lower panel of Fig. 7. Clearly, the ratio of positive charge and negative charge remained unaltered in spite of time.
Figure 7: Time-dependent manner of ouabain inhibition (Upper panel) Current generated in the absence of ouabain and with Ouabain addition after 3 minutes, 6 minutes, 9 minutes and 12 minutes; (Lower panel) Pump mediated charge as a function of time after ouabain addition
2.3.2 Characteristics of transient pump currents

To obtain more detailed information, the total membrane current, the 4th pulse and the last pulse induced transient currents were superimposed and enlarged (Fig.8). It was noticeable that there was a phase shift between total membrane current (black, mainly capacitance current) and the transient currents (red and green), which suggested that they did not share same time course. This was another evidence that the transient currents are not capacitance current.

Figure 8: Superimposition of total membrane current (black), the 4th transient current (red), and the last transient current (green). The total membrane current was scaled 140 times smaller. Solid lines represent mono-exponential fits of those three pulses.

Under physiological condition, where Na ions extrusion and K ions intrusion were randomly distributed, Na/K pump currents were outward only and in steady state [43]. In experiments with caged-ATP [73], transient currents were obtained but with relatively large time constants. This is
because diffusion of ATP molecules from the cages to the binding site is rate limited. While, in Na/Na [51] or K/K mode [52], where the Na/K pump functions were fully synchronized, transient pump currents with smaller time constants were recorded. So, it is reasonable to use time constant from mono-exponential fit $I = I_{max} \ast e^{-t/\tau} + C$ to consistently monitor the synchronization status along the SM pulses.

Figure 9: Time constants of positive transient current, negative transient current as well as total membrane current from mono-exponential fits.

Results showed that for positive half cycle on Fig.8, time constants were 34s, 73 s and 38 s for total membrane current (black), 4th transient current (red) and last transient current (green), respectively and for negative half cycle, the corresponding time constants were 34s, 51s and 36s. Clearly, both positive and negative time constants of the last pulse were smaller than the 4th pulse. Moreover, the time constant of each pulse was plotted on Fig.9. Obviously, the more pulses applied,
the smaller the time constant. Several conclusions can be drawn: firstly, SM induced transient current (black and red) possesses distinguishable time constant from membrane current (green and blue), which means that it is not residue of membrane capacitance current. Secondly, the time constant of Na transient current (+) is always longer than K transient current (-) until they aligned with membrane current at the end of the trace. This can be explained by different mechanism of Na ions and K ions translocations. It has been demonstrated that releasing three Na ions to the extracellular side is slower than releasing two K ions, which may result with more difficulty to synchronize Na ions extrusion than K ions intrusion. Last but not least, it is obvious that time constant of both positive and negative transient current decreases with more pulse application until aligned with membrane current, which indicates that synchronization status is a dynamic process. The more pulse applied, the better the synchronization until reaching saturation.

2.3.3 Effect of overshoot pulses on the synchronized transient pump currents

![Figure 10: Energy barrier with different magnitudes](image)

The first generation SM technique, in which no overshoot pulses are applied, can only synchronize Na/K pumps into each corresponding half cycle. As a result, individual Na extrusion or K
intrusion is still uniformly distributed and no transient pump current is induced. On the contrary, the second generation SM presented here with overshoot pulses induces large transient pump current. Accordingly, our results suggested that the overshoot pulses could significantly affect the synchronization of Na/K pump molecules. To obtain more detailed information, we extended our investigation of overshoot pulses effect on synchronization from two aspects: magnitude and duration. Firstly, we varied the magnitude of overshoots pulses from 0 % of the activation pulse to 100 % as shown in Fig.10. Results showed that when overshoot pulse was the same as activation pulse (cyan), very little transient pump current was induced, which was consistent with the results from first generation SM [66]. When the magnitude of overshoot pulses increased to a higher level, larger transient pump currents were induced until saturated at about 100 % (black). The results here confirm that the magnitude of overshoot pulses are crucial for generating transient pump currents. The mechanism will be presented in the discussion section.

![Figure 11: Energy barrier with different durations](image)

Next, we adjusted the duration of overshoot pulses from 0.5ms to 2ms and observed its effect on synchronized pump current (Fig.11). Results showed that the longer the duration, the bigger the
transient pump current. However, the difference between each trace was slight. Thus, the effect of duration of overshoot pulses on synchronization of Na/K pumps was less significant than that of magnitude shown above.

In conclusion, we obtained transient pump current by second generation SM under physiological condition. The transient current in the positive and negative half cycles which represent Na extrusion and K intrusion had a ratio close to 3:2, the stoichiometric number of Na/K pump. In the presence of ouabain, the transient currents were mostly eliminated in a time dependent manner. Moreover, the synchronization was a dynamic process which could be manifested by gradually reduced time constant of individual pulse. This observation indicated that with more pulses applied, the better synchronization of the Na/K pumps was obtained. Lastly, we showed that the magnitude of the overshoot pulses significantly affected the synchronized pump current while the effect of duration of overshoot pulses was trivial.

2.3.4 Computer simulation results

It is well known that the macroscopic pump current is the summation of single pump current. Accordingly, we propose that while synchronized, most of the pumps are transporting Na or K ions at the start of the SM pulses, resulting in transient macroscopic current. While, under physiological condition, most of the Na/K pumps are in random phases and uniformly distributed, which would result in outward-only and steady-state macroscopic current. If so, the summation of single pump currents with random phases should be similar as macroscopic current under physiological condition.

According to the results and analysis above, we synchronized most of the Na/K pumps into the same phase, which indicated that the single pump current can be obtained by (macroscopic transient pump current)/(number of the pumps synchronized). The total number of Na/K pumps in our experiments is about $10^4$ based on the previous study [82]. So, we divided the last five synchronized pump currents by 10,000 to estimate the single pump currents shown in Fig.6 A. Simulation results were obtained by summation of N traces in panel A but with random phases. Results showed that when N were relative small numbers (10 or 100), the macroscopic current contained both positive and negative components. However, when N went up to 1000, the macroscopic current becomes outward-only and even smoother when N=10000. Moreover, experimental results of macroscopic pump current which was recognized as ouabain-sensitive current was obtained as well (Fig.6 E, red). Obviously, the simulation result (black) and experimental result (red) were in good agreement with
To sum up, by computer simulation, we obtained macroscopic pump current by summation of single pump current with random phases. The result was comparable with pump current from experiments under physiological condition. These results provide another evidence that the individual pump molecules are synchronized into the same pace.

2.3.5 Synchronization of pumps under Na/Na exchange mode

To further investigate effect of SM technique on Na/K pumps, experiments were conducted in the absence of extracellular potassium, which would force Na/K pump to run under Na/Na exchange mode. Firstly, to measure pump current under Na/Na mode, 40mV (same as the activation voltage of SM) voltage step with 100 ms duration was applied both under control and in the presence of Ouabain. Results showed that the ouabain-sensitive current was barely identified, which was consistent with previous data [83]. It can be explained by that under physiological condition, the Na/K pumps exported 3 Na ions and imported 2 K ions per cycle, resulting in outward pump current.
On the contrary, under Na/Na exchange mode, the pumps translocate 3 Na ions in both directions, becoming electroneutral. Next, SM electric field was applied as shown on Fig. 13. Interestingly, transient pump currents in both positive and negative half cycles were obtained. Moreover, instead of 3:2 under Na/K mode as shown previously, the positive and negative peak current had a ratio around 1:1, the stoichiometric number under Na/Na mode. The results demonstrated that the SM technique can separate the ions extrusion and intrusion even under Na/Na exchange mode.

2.4 Discussion

In the present study, we modified the Synchronization Modulation technique by adding an overshoot pulse at the end of each half cycle. We proposed that the high energy level of the overshoots will prevent corresponding ions from being translocated to the other side until the membrane
polarity change. As a result, all Na ions extrusion would happen at the very beginning of the positive half cycle and K intrusion would be forced into the beginning of negative half cycle. Our hypothesis was demonstrated by results that transient currents were induced in the beginning of each half cycles. Moreover, the transient currents were highly ouabain sensitive in a time dependent manner, which confirmed that they were Na/K pump currents. In addition, computer simulation by summation of synchronized pump currents with random phases resulted in comparable currents with the experimental results. Lastly, with modified SM technique, we distinguished the sodium outward translocation from the inward under Na/Na exchange mode.

2.4.1 Different mechanisms of first and second generation SM techniques

Figure 14: Mechanisms of first and second generation SM techniques

For both first and second SM techniques, oscillating electric field with 50 Hz frequency were used to train the enzymes working under the same phase. Results in the previous studies showed that synchronized pump current was uniformly distributed using first generation SM technique. While, synchronized pump current under 2nd generation technique had a transient or pre-steady-state part. Mechanism difference was illustrated in the following.
The first generation SM technique would restrict most of the Na transporters into the positive half cycle and K into the negative half cycle and mechanism has been elucidated previously based on energy consumption difference. However, as no restriction in applied pulses, the transmembrane ions movement could occur anytime in the corresponding half cycle as shown in upper Panel of Figure 14. As a result, Na and K translocation current are uniformly distributed in the positive and negative half cycle. In other words, we are not able to determine the detailed location of each pump current. Whereas, when an energy trap pulse is added, the high energy level of the overshoot in the negative half cycle will prevent Na ions from being translocated to the extracellular showed in the lower panel of Figure 14. In other words, the ones that are ahead of the applied field, leading the phase, will be slowed down and the ones that are behind, lagging the phase, will be speeded up until all the molecules are in phase with each other. The positive half cycles are vice versa. As a result, all Na extrusion would happen at the very beginning of the positive half cycle and K intrusion is forced into the beginning of the negative half cycle, which would induce a transient pump current in each half cycle.

2.4.2 Importance of synchronized transient pump current

Several studies were able to distinguish three components of Na ions being released to the extracellular solution [50] and later two K ions [52]. The key point of this technique was that most of the pump molecules were restricted at E2 state by depletion of either sodium ions or potassium ions in the solutions. Then by suddenly changing the polarity of the cell membrane, those restricted pump molecules were activated simultaneously, inducing transient currents.

However, all of the experiments above were conducted on dialyzed pump molecules and more specifically, internally dialyzed. Under this condition, ATP hydrolysis, the energy provider or driving force, was interrupted. So any conformational change related to ATP hydrolysis would more or less be affected. It is well known that phosphorylation and de-phosphorylation are tightly related to conformational changes of Na/K pump such as occlusion and de-occlusion. So, it is reasonable to speculate as to whether or not information obtained from dialyzed mode could reveal mechanism of natural Na/K pump. Moreover, in the dialyzed pump, dynamic correlations between E1 and E2 states were overlooked. For instance, there must be a way that energy released internally is transferred from E1 state to E2 state for K intrusion because ATP hydrolysis occur on cytoplasmic side of the membrane while K intrusion happened on the other side.

In the present study, experiments were conducted under physiological conditions instead of being
dialyzed. Thus, the Na/K pump dynamic cycle was complete, including ATP hydrolysis, protein conformation changes, ions transmembrane movement, etc. From the synchronized pump current, some important information can be obtained. For instance, the transient current in the positive half cycle which represents Na extrusion and that in the negative half cycle which represents K intrusion always has a ratio close to 3:2, which shows another way to determine the stoichiometric number of Na/K pump. It has been shown previously that the second generation SM pulses contains two peaks: activation pulse and overshoot pulse (Fig.4). There are also two transient currents that are induced in the total membrane current. However, the subtracted pump current only exhibits single transient current locates at the very beginning of each half cycle. Accordingly, there should be only one channel or one electrogenic step inside the transmembrane domain of Na/K pump under physiological condition. The detailed information can be obtained in chapter 3.
Chapter 3  Single channel configuration in Na/K pump

3.1  Introduction

The question whether there is a single channel or two access channels in Na/K pump has been debated and investigated for decades. Studies of the partially dialyzed pump molecules have shown that a stimulation-triggered forward pump current is always followed by a backward current with the similar magnitude and time-course. These charge-movement-like pump currents indicate that the pump channel is obstructed deeply inside the membrane which separates the pump channel into two segments (access-channels).

On the contrary, Studies of Polytoxin-treated Na/K pumps showed a pathway from the extracellular to intracellular solution [84]. Cysteine-scanning mutagenesis studies of the -helices in transmembrane domain with MTSET or MTSES demonstrated that the amino acids in alpha-helices affecting the channel conductance are mainly located at the ends of channel [85, 86, 87]. Moreover, the synchronized transient currents discussed in chapter 2 also indicated that there was only one electrogenic step inside the transmembrane domain of Na/K pumps. These studies implied that Na/K pumps have a single channel configuration with orifice at ends of the channel.

To further address this question, experiments were conducted in the absence of Na ions which equivalently force pump running K/K mode [52]. It has been demonstrated that Na/K pumps under this mode will be restricted into steps shown as red box in Fig.15. In the previous studies, investigators analyzed relaxation pump current induced by a series of stimulation pulses, from where they dissected detailed ions movement information. This methodology was employed in this study along with some modifications including: Firstly, higher extracellular K concentrations were used varied from 8mM up to 40 mM, which would reduce the concentration gradient for K ions across the cell membrane. Secondly, instead of applying both positive and negative pulses, we only applied a series of negative stimulations in a wide range of magnitudes. Because we mainly focusing on the forward pumping cycle which would inwardly drive K ions movement.

Results showed that with lower extracellular K concentration that was comparable with the previous study, the relaxation pump currents were similar with activation pump current in both
magnitude and time constant. The results were consistent with data presented before [52]. We named this pump current as charge-movement-like pump current since it possessed characteristics of charge movement. Whereas, when extracellular K concentration was set at 40 mM which was five times higher than the original concentration, the activation current and relaxation current were no longer symmetric if the stimulation pulses were high enough. Specifically, we successfully observed the forward-only pump currents in responding to an electric stimulation without the backward-current component, which indicated that ions had been translocated to the other side of plasma cell membrane and could not come back even the polarity change. We named this unidirectional pump current as transmembrane pump current.

3.2 Materials and solutions

The animals are anesthetized and euthanized following the protocol approved by the Institutional Animal Care and Use Committee (IACUC). Single muscle fiber is separated and chosen using the procedure discussed in Chapter 2. The experiments were conducted on frog skeletal muscle fibers using the double Vaseline-gap voltage clamp technique. The pump molecules were internally
dialyzed by eliminating both the internal and external Na ions. Recipes of the internal and external solutions are as following.

Relaxing solution (in mM):
120 Potassium Glutamate, 5 K<sub>2</sub>PIPES, 1 MgSO<sub>4</sub>, 0.1 K<sub>2</sub>EGTA;

Ringer solution (in mM):
120 NaCl, 2.5 KCl, 2.15 Na<sub>2</sub>HPO<sub>4</sub>, 0.85 NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 1.8 CaCl<sub>2</sub>;

Na-Free Internal Solution:
L-Glutamic acid potassium salt monohydrate (K-glutamate) 58mM;
MgSO<sub>4</sub> 6.8mM;
3-(N-Morpholino) propanesulfonic, 4-Morpholinepropanesulfonic acid (MOPS) 5mM;
Ethylene glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA) 20mM;
Dibasic potassium phosphate (K<sub>2</sub>HP<sub>4</sub>) 4mM;
Cesium hydroxide hydrate (CsOH) 10mM;
Adenosine 5-triphosphate dipotassium salt hydrate (5-ATP-K<sub>2</sub>) 5mM;
Adjust the final pH to 7.30 via KOH at room temperature; store in the freezer at -20 C.

Na-free External Solution:
3,4-Diaminopyridine(3,4-DAP) 3.5mM;
Tetramethylammonium chloride (TMA-Cl) 85.35mM;
CsCl 10mM;
KCl from 4mM to 36mM as indicated;
Dibasic potassium phosphate (K<sub>2</sub>HPO<sub>4</sub>) 1.5mM;
Potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) 1mM;
CaCl<sub>2</sub> 1.8mM;
BaCl<sub>2</sub> 1.5mM;
Tetrodotoxin (TTX) 1M;
Ouabain 500M as indicated;
Adjust final pH to 7.10 via HCl at room temperature.
3.3 Results

In this study, in order to demonstrate whether there is a single channel or two channels in the Na/K pump, K/K exchange mode is re-studied using series of gradually decreased negative pulses. The Na/K pump currents are identified as ouabain-sensitive current and the detailed information can be obtained from Fig. 16. It is known that the inhibition of ouabain on Na/K pumps is relatively slow usually in minutes, during which some electrical parameters of the system may change. Any changes may result in considerable errors and contaminations in the pump currents. Therefore, to monitor the stability of system, there is always a time control trace for both with and without ouabain groups. The duration between each time control trace is the same as the waiting time for ouabain inhibition. For example, results from the subtraction of pulse 1 and 2 as well as pulse 3 and 4 are almost zero in Fig. 16, which suggests that the system is stable. However, subtraction between in the absence and presence of ouabain results with a large transient current for both activation and relaxation parts, which indicated that the transient currents were related to Na/K pump.

When extracellular K concentration was set as 8mM, which was comparable with previous study, the magnitudes of pump currents showed a voltage dependent manner (Fig.17). Also, the activation current and relaxation current were almost symmetric and independent of pulses applied. Those features were similar as charge-movement current and consistent with data presented before.

Nevertheless, when extracellular K concentration was 40mM, 5 times higher than the original concentration, the similarity of the forward and backward transient pump currents remained only for certain voltage. When applied pulses were less than -120 mV, the forward and backward transient pump currents remained comparable (zone 1 of Figure 18) which was similar as at lower K concentration. The on-charges remains similar as the off-charges, as shown in the lower panel. As the stimulation pulses increasing, the fast component of the backward transient current became smaller than the forward activation current as shown in zone 2 of Figure 18. In like manner, the backward charge was also less than the forward, which indicated that activation pulses induced fast K inward charge movement was more than fast relaxation outward charge. While noticeably, the slow component remains unaltered in zone 2. If the magnitude of stimulation pulses further increased, the slow components of the backward pump currents started to reduce. In response to a -200 mV stimulation pulse hyperpolarizing the membrane to -260 mV, both fast and slow components of the backward pump currents fully vanished as shown in zone 3. This implies that
all the K ions driven by the stimulation pulse flowing into the channel were no longer flowing back. In other words, at higher extracellular K concentration or lower concentration gradient, a high stimulation pulse can drive the external K ions to overcome both the ionic concentration gradient and the obstacle in the channel to cross the cell membrane. The unidirectional single transient pump current indicates a single electrogenic step is involved in the ion-transport across the cell membrane, or single channel configuration.

We also measured the pump currents generated by the negative stimulation pulses at different extracellular K concentrations. The ratio of the off-charge over the on-charge as a function of the magnitude of stimulation pulses at different extracellular K concentrations were concluded in Fig.19. In response to lower stimulation pulses, the ratio remains at about 1 regardless of the extracellular K concentration, which is the same as shown in zone 1 of Figure 18. The pump currents are the two-directional charge-movement currents. Whatever the K ions flowing into the channel in responding to the stimulation pulse will fully flow back once the stimulation is over.

**Figure 16:** Identification of Na/K pump currents
When the magnitude of stimulation pulse increases, the ratio becomes less than one indicating some of the K ions flowing into the channel no longer flow back, as shown in zone 2 in Figure 18. The higher the concentration of the extracellular K ions, or the lower the ionic concentration gradient, the less the ratio is, or the easier for the K ions moving through the channel into the intracellular solution. At about 40 mM extracellular K ions, in response to a -200 mV stimulation pulse, the ratio becomes almost zero, see zone 3 of Figure 18. The pump currents are no longer the two-directional charge-movement currents but uni-directional transmembrane currents, suggesting that K ions passing through the channel across the cell membrane to the intracellular side.

Our results presented above suggest that instead of two structural access channels in the transmembrane domain, there is only one channel. While several negatively charged amino acid located in the middle of the ion pathway would form an energy trap which seems to divide this channel into two segments. In order to confirm the effects of collimator and energy-well due to the negatively charged amino acids deeply inside the pump channel, we redid the above experiments in the extremely low external pH of 4.6. The extracellular K concentration was remained at 40 mM,
Figure 18: Na/K pump currents when $[K]_o=40\text{mM}$

and again, the negative stimulation pulses from -20 mV up to -200 mV were applied to the cell membrane. The measured pump currents were shown in Figure 20. Interestingly, in response to the same magnitude of stimulation pulses, the transient pump currents were significantly reduced but the plateau currents increased. These results are similar as data obtained previously from other labs [88, 89]. Most impressively, regardless of the increments in the stimulation pulse, the transient backward pump currents responding to the falling phase only slightly decreased but never disappeared. These results demonstrate that most of the flowing-in K ions driven by the stimulation pulse cannot pass through the channel across the cell membrane but returning to the external solution once the stimulation is over.

Comparing pH values of 4.6 and 7.1 at physiological condition, concentration of proton increased almost by three orders. $H^+$ is smaller than either Na or K which can pass through the orifice into the channel and interact with the negatively charged amino acids. Due to neutralization of the negatively charged amino acids, there will be less or no energy-well to attract the flowing-in K ions. The only driving force for the moving ions is the applied stimulation pulse. The lower speed of
the flowing-in K ions result in the lower magnitude of the transient pump currents and the higher plateau. In addition, because the collimator function is reduced. The flowing-in K ions cannot be well aligned in the lumen of pump channel. To sum up, under physiological condition, ions translocated across the membrane along the channel under collimation, which ensure the highest efficiency of energy use. If the extracellular PH is low enough for extra proton binding with the negatively charged amino acid as known as protonation, the collation system would be interrupted and the cation ions no longer would go through the pathway even with high energy. Instead, most of the ions would be stuck in the channel which consequently presents as increase of the steady-state plateau current. Therefore, regardless of the magnitude of stimulation pulse, most of the flowing-in K ions will flow back once the stimulation is over.

Figure 19: Ratio of relaxation and activation charges at different $[K]_o$ concentrations
3.4 Discussions

In this study, we demonstrated that there is only one structural channel in the transmembrane domain of Na/K pumps by electrophysiological method. We proposed that several negative charged amino acids located in the middle of transmembrane domain will form an energy trap, obstructing K ions translocation. Two situations are discussed: low [K]o and high [K]o. At low extracellular K concentration (8mM), energy from the activation pulse is not enough to overcome the energy trap. Inevitably, it will cause accumulation of K ions inside the energy trap and when membrane potential comes back to holding potential, the binding K ions will be released into the extracellular solution driven by the ionic concentration gradient. As a result, the activation current and relaxation current will show a symmetric feature.

When external K concentration is set to be higher (40mM), the energy from activation pulse is capable of driving K ions escaping from the energy trap and being translocated into the intracellular side when the applied voltage is high enough. As a result, there will be less K ions come back to extracellular when membrane potential drops to holding potential, which presents an asymmetrical characteristic for the activation and relaxation currents. Specifically, when applied pulse is extremely high (-200 mV), the pump current becomes unidirectional with no backward current, which indicates that all of K ions have been translocated to the cytoplasmic side. The proposed
Figure 21: Proposed mechanisms of symmetric and asymmetric pump currents

mechanisms are concluded in Fig. 21.

To further confirm our hypothesis, we protonated the negatively charged amino acids by low PH extracellular solution. Interestingly, the asymmetrical feature of activation and relaxation charges disappeared even the applied energy was high enough. This result suggest that the negatively charged amino acids in the TM domain are crucial for ions translocation in Na/K pump.
Chapter 4 Slow down effect of D₂O on Na, K-ATPase

4.1 Introduction

Deuterium isotope effects on membrane proteins including ion channels and transporters has been investigating for many years. For instance, when studying the proton channels in rat alveolar epithelium, people find that the time constant of H⁺ current activation is about three times slower in D₂O than in H₂O [90]. Moreover, results from the study of the nerve fiber behavior under heavy water suggested that D₂O substitution prolongs the action potential, which is resulted from a reduction of the magnitude and slowing of the time course of the ionic permeability changes [91]. Lastly, Daniel A Alicata, etc. reported that when D₂O is used as the solvent, gating of Na+ channel is slowed [92, 93]; specifically the last stage in gating. From the results of D₂O effect on sodium channel, they conclude that D₂O-sensitive primary activation and D₂O-insensitive tail current deactivation involve separate pathways [92].

Inhibitory effect of D₂O on Na/K pumps has been reported by different groups. For example, K. Ahmed, etc. present that substitution of D₂O for H₂O in the Na, K-ATPase reaction is inhibitory in a concentration dependent manner [94]. The experiments are conducted on microsomal membrane fraction from rat brain by measurement the changes of radioactive ¹⁴C-ATP and ¹⁴C-AMP. They also find that D₂O reduces the K sensitivity of the efflux but increases the affinity for K. As an explanation, they suggest that D₂O shifts the Na/K pump equilibrium from E₁ to E₂. Additionally, D. Landowne, etc. reported that D₂O replacement reduces the Na efflux of squid axons by about a third and meanwhile confirms the results from K. Ahmed, etc. that Na efflux reduces but K binding affinity increases [95]. To explain these results, they hypothesize that the enzyme stays at E₂ state or what they called K-bound state longer with D₂O than H₂O. Again, the results are presented based on the differences of radioactive ²²Na in H₂O and D₂O.

In this study, effect of D₂O on Na/K pump was re-investigated. Instead of using radioactive methods discussed above, we employed a new designed technique named Synchronization Modulation (SM). Briefly, well-designed oscillating pulses were used to separate the sodium ions extrusion from the potassium ions intrusion based on their energy profiles. The mechanism of this technique
has been reported previously [68]. Recently, we modified the original SM technique by adding an overshoot at the end of each half cycle and successfully obtained transient pump currents. We have demonstrated that the synchronized macroscopic transient current is the summation of each individual pump currents. In other words, the more the Na/K pumps are synchronized, the larger the transient current will be obtained.

Our results showed that using H$_2$O as solvent, the maximum transient pump currents were obtained when the frequency of the SM electric field was 50 Hz. It indicated that most if not all of the pumps were running at 50Hz in this scenario. Whereas, when H$_2$O was substituted by D$_2$O, the optimized frequency of oscillating electric field was no longer 50Hz, but reduced to about 25Hz. This results suggested that in the presence of D$_2$O, more pumps were working at around 25Hz. Based on the recently finding that TM5-TM6 and their hairpin loop move during conformational changes, we propose the slow down effect of D$_2$O on Na/K pumps is due to the higher viscosity of D$_2$O which will hinder the essential movements.

4.2 Methods and Materials

4.2.1 Muscle fiber preparation

The animals were anesthetized and euthanized following the protocol approved by the Institutional Animal Care and Use Committee (IACUC). Single muscle fiber was separated and chosen using the procedure elaborated before. Twitch skeletal muscles, semitendinosus and ilio, were hand dissected from American Bullfrog and placed in relaxing solution as in prior work [96, 97, 98]. The experiments are conducted in a double-vaseline-gap chamber [80].

4.2.2 Composition of solutions

Relaxing solution (in mM):
120 Potassium Glutamate, 5 K$_2$PIPES, 1 MgSO$_4$, 0.1 K$_2$EGTA;

Ringer solution (in mM):
120 NaCl, 2.5 KCl, 2.15 Na$_2$HPO$_4$, 0.85 NaH$_2$PO$_4$.H$_2$O, 1.8 CaCl$_2$;

Internal Solution (in mM):
58 K-Glutamate, 6.8 MgSO$_4$.7H$_2$O, 5 MOPS, 20 EGTA, 10 CsOH, 3 Na$_2$-Creatine phosphate, 5 5-ATP-Na$_2$. Final [K]$_i$ 130mM, [Na]$_i$ 16Mm, PH=7.3. Stored at -20 C. For Na/Na exchange mode, K-Glutamate was replaced by Tetramethylammonium chloride (TMA-Cl);
External Solution (in mM):
3.5 3,4-Diaminopyridine (3,4-DAP), 86 NaCl, 10 CsCl, 4 KCl, 2.15 Na$_2$HPO$_4$, 0.85 NaH$_2$PO$_4$.H$_2$O, 1.8 CaCl$_2$, 1.5 BaCl$_2$.2H$_2$O, 0.001 Tetrodotoxin (TTX). Final $[K]_o$ 4mM, $[Na]_o$ 90mM, PH=7.1. For Na/Na exchange mode, KCl was replaced by NaCl. In the experimental group, H$_2$O is replaced by D$_2$O as solvent. D$_2$O was purchased from SIGMA with isotopic purity of 99.8 atom % D.

4.2.3 Applied SM pulses

The applied SM pulses is shown in Fig.22. Briefly, Na/K pump currents are obtained by subtracting each pulse of SM from a pre-generated templet pulse who has exactly the same amplitude and frequency. The detailed mechanism of this technique has been discussed in Chapter 2. The advantages of this method are that on one hand, the membrane conductance current and other linear leakage current are mostly eliminated; on the other hand, it reflects the instantaneous accumulation effect of the SM on Na/K pump without any contamination.

![Figure 22: Applied SM pulses](image)

4.2.4 Data analysis

Data was collected with Dagon TEVC 200 device. Data was analyzed using pClamp10 (Molecular Devices), GraphPad Prism (GraphPad Software) and a Java program. Significant differences were determined with Students t-test. In all cases, data represent with mean and SEM.

4.3 Results

4.3.1 50 Hz SM electric field induced Na/K pump currents

Synchronization Modulation is developed based on studies of oscillating electric fields activation on Na, K-ATPase [54, 99]. Briefly, well designed oscillating electric field are used to separate
the sodium ions extrusion and potassium ions intrusion based on their different energy profiles. More recently, we modified the waveform of the original waveforms of SM and obtained transient pump current at the start of each half cycle. We have demonstrated that the higher the peak of the transient current, the more Na/K pumps are synchronized and vice versa. One of the crucial points of this technique is that the frequency of oscillating electric field that being used has to match the turnover rate of the Na/K pumps. If the frequencies of electric fields are too high or too low, compare with the pumping rate, the Na/K pumps will not be fully synchronized [66].

According, oscillating electric field with frequency of 50Hz, matching the turnover rate of natural Na/K pump, was used first as shown in Fig.23, upper panel. The experiments were conducted using H₂O as solvent. Results showed that the transient currents were increasing along the SM trace and reaching saturation at about 30th pulse (Fig.23, middle panel), which was consistent with the previous results. It suggests that the synchronization is a dynamic process, in which the more pulses are applied, the more pump molecules are synchronized until reaching a steady state. Moreover, the peak value of last positive current, representing sodium ions extrusion, was about 35nA. While, the value became -25 nA for the negative current, representing potassium intrusion. The ratio between them is around 1.4, close to the stoichiometric number of Na/K pump which is 3:2. In addition, the transient currents were almost eliminated in the presence of 500uM ouabain, which indicating
that they were highly ouabain-sensitive. Statistical results from 15 different experiments were plotted in Fig.24. The averaged positive peak current was around 36nA and averaged negative peak current was about -26nA (black dots). Also, the statistical results showed that ouabain significantly suppressed the transient currents.

Next, we substituted the solvent of experiments from H₂O to D₂O. The experimental procedures and SM electric field protocols were exactly the same as before. However, results showed that transient pump currents of both positive and negative half cycles were suppressed in the presence of D₂O (Fig.25). The results were confirmed by statistical analysis of 15 individual experiments and averaged positive peak current and negative peak current were about 26nA and -19nA, respectively (Fig.26). Again, these transient currents were totally abolished in the presence of ouabain, which indicated they were Na/K pump currents. The comparison of currents obtained in H₂O and D₂O was shown in Fig.27. Statistically, transient pump current reduced by 30 percent when using D₂O as solvent.

**Figure 24:** Statistical analysis when f=50Hz and solvent=H₂O
There are two potential explanations for this 30 percent suppression of transient currents. First, D$_2$O may inhibit the Na/K pumps like ouabain does. As a result, the number of working Na/K pumps will be reduced and therefore suppress the pump current. Second, D$_2$O slows down the Na/K pumps. If so, the frequency of SM electric field of 50Hz would be too high to synchronize all of the Na/K pumps and thus the transient pump currents are lessened. To address this issue, SM electric field with lower frequency is used. It is necessary to point out that all other parameters of the electric field are the same including: magnitude, number of pulses, waveforms, etc. except for the frequency. The purpose of this experiment is that if the first explanation that D$_2$O inhibits Na/K pump function is true, adjusting the frequency of SM electric field should not alter the magnitude of pump currents. While if the second explanation that D$_2$O slows down the Na/K pump fits, SM with lower frequencies which match the turnover rate of pumps in this situation, could improve the synchronization and induce larger pump currents.

Interestingly, our results showed that when using H$_2$O as solvent, the pump current obtained was significantly attenuated (Fig.28) at 25Hz. The averaged positive and negative peak current
Figure 26: Statistical analysis when f=50Hz and solvent=D$_2$O

Figure 27: Comparison of pump currents in H$_2$O and D$_2$O when f=50Hz
Figure 28: Synchronized pump currents using H$_2$O and D$_2$O as solvent at 25 Hz

were about 23nA and -17nA, respectively. On the contrary, in D$_2$O, the pump currents were remarkably increased with averaged positive peak of 33nA and negative of -23nA. The detailed statistical information can be obtained in Fig.29. Results from this experiment suggests that D$_2$O slows down the turnover rate of Na/K pumps, which could explain the 30 percent suppression of synchronized pump current in D$_2$O at 50Hz.

4.3.3 Pump currents in both H$_2$O and D$_2$O as a function of frequency

To further investigate the slow down effect of D$_2$O on Na/K pump, we applied SM electric field with 4 different frequencies, 25Hz, 50Hz, 70Hz and 100Hz, in both H$_2$O and D$_2$O solvents. The last synchronized pump currents from these 4 modes were concluded in Fig.30. Also, the pumps mediated charges obtained by integrating the transient part of each currents were presented in Fig.31. When using H$_2$O as solvent, the maximum pump current was recorded at 50Hz. For frequencies both higher and lower than 50Hz, the elicited pump currents were suppressed in a bell-shape pattern, which suggested that the further the frequency away from 50Hz, the smaller the pump current was obtained. Whereas, when the solvent was D$_2$O, the maximum pump current was
Figure 29: Comparison of pump currents in H$_2$O and D$_2$O when f=25Hz

recorded at 25Hz. Similarly, the pump current gradually reduced when frequencies went higher. It has been reported that Na/K pumps can be optimally synchronized only when their turnover rates relatively matches the frequencies of SM electric fields [66, 100]. Thus the frequencies at which the largest pump currents were obtained also represented the turnover rates of Na/K pumps at that specific situation. That is to say that in H$_2$O, the turnover rate of most Na/K pumps should be round 50Hz and in D$_2$O, it reduces to about 25Hz.

Turnover rate of each pump molecule dependents on many factors, such as membrane potential, temperature, ion concentration gradients and surrounding environment [101]. In our experiments, the membrane potential remained consistent for all of the experiments. This will rule out the effect from membrane potential. Similarly, all of our experiments were conducted in room temperature that was accurately controlled. Indeed, people found that when D$_2$O is used as the solvent, gating of sodium channel was slowed [93], which would potentially affect the sodium concentration gradient. While in all of our experiments, TTX was used to block the Na channels, which eliminate the possibility that D$_2$O affects the ion concentration gradients. Therefore, the only factor that reduced the turnover rate of Na/K pumps is the surrounding environment change caused by D$_2$O substitution.
Figure 30: Pump currents as a function of frequency of SM electric field
4.4 Discussion

In the present study, the D$_2$O inhibitory effect on Na/K pump is re-investigated with modified Synchronization Modulation technique. We have demonstrated previously that the synchronized transient current is the summation of individual pump current and thus, the more pumps synchronized, the larger the transient current. Results show that using H$_2$O as solvent, the maximum transient current is obtained when the frequency of oscillating electric field is 50Hz. However, when H$_2$O is replaced by D$_2$O as solvent, the turnover rate of Na/K pumps reduces to 25Hz. The result is consistent with the previous conclusion that D$_2$O slows down the Na, K-ATPase.

4.4.1 Potential mechanism of D$_2$O slow down effect on Na/K pump

Previously, several observations of D$_2$O effect on Na/K pump have been reported: D$_2$O reduces the sodium efflux but increases the binding affinity of potassium; D$_2$O inhibit the formation of E-P

Figure 31: Pump current and pump-mediated charge as a function of frequency
(phosphoenzyme) and this inhibition has a linear relationship with the concentration of \( \text{D}_2\text{O} \), etc. However, information about the mechanism of this effect is insufficient.

More recently, with the development of structure biology and site mutation techniques, more detailed information of Na/K pump can be obtained. Several studies have suggested that the H5-H6 loop in Na/K pump is important in energy transduction. For example, people reported that TM5TM6 hairpin moves outwards in response to enzyme phosphorylation during the E1E2 conformational change [102]. Also, when studying the ouabain binding sites in the Na/K pump, people found that H5-H6 hairpin loop remained embedded in the membrane upon digestion in the presence of ouabain or cations but is released into the soluble fraction in the absence of these ligands. These results indicated that the cations or ouabain interactions restricting the free movement of this domain [103]. Linking ouabain inhibitory mechanism also suggests a possible explanation for \( \text{D}_2\text{O} \) effect on Na/K pump. It is known that the viscosity of \( \text{D}_2\text{O} \) is larger than \( \text{H}_2\text{O} \) by 25 percent because of the stronger hydrophilic bonding in \( \text{D}_2\text{O} \) than \( \text{H}_2\text{O} \) [104]. Thus, \( \text{D}_2\text{O} \) could potentially slacken the protein by directly hindering the movement of the H5-H6 extracellular loop that is required for cation translocations. This explanation also supports the fact that K binding affinity increases in the presence of \( \text{D}_2\text{O} \) because the Na/K pump spends more of its time in the E2 State [105].

### 4.4.2 Advantages of SM in studying the mechanism of Na/K pump

It has been demonstrated that Na extrusions and K intrusions of individual Na/K pump molecule can be synchronized into the beginning of positive half cycle and negative half cycle, respectively, thus transient pump currents are induced. By analyzing the characteristics of the transient pump current, many information about mechanism of Na/K pump can be gathered. For example, in this study, by comparing the differences of synchronized transient pump currents in different solvents, we confirmed that \( \text{D}_2\text{O} \) could reduce the turnover rate of Na/K pump.

It is necessary to point out that all of the experiments were conducted under physiological condition where the whole pumping cycle is complete. Indeed, there are other techniques that are capable of inducing transient pump current. For example, people obtained Na and K transient pump currents from partially functional Na/K pumps, from where they dissected different steps of ions translocations [50, 52]. However, their experiments were conducted under either Na/Na exchange mode or K/K exchange mode, in which ATP hydrolysis, phosphorylation or de-phosphorylation were not considered. Therefore, this technique is limited to studies of ion translocations where
the conformational changes related to ATP hydrolysis are not included. On the contrary, the
Synchronization Technique presented here can be applied under physiological condition, which
unleashes its potential in studying the mechanism of natural Na, K-ATPase.
Chapter 5  Modulation of Na/K pumps by modified SM technique

5.1  Introduction

In chapter 2, it has been demonstrated that individual pump molecules working at random paces can be synchronized by an oscillating electric field if the frequency of that electric field and the turnover rate of pumps are relatively matching. It is reasonable to hypothesize that once synchronized, by adjusting the frequency of electric field by a proper step, the Na/K pumps can be re-synchronized to the new frequency. If so, by gradually increasing the frequency of oscillating electric field in a stepwise manner, the pumping rates can be accelerated and thus enhancing the function of Na/K pump.

In order to prove our hypothesis, we applied the synchronization modulation train (Fig.32) to the cell membrane. The train includes three parts: templet pulses (pre-pulses), 50Hz (synchronization), 50-150Hz (modulation) and their roles are described as following:

Pre-pulses: in this part, templet pulses at each frequency are generated. Templet pulses are used to subtract the capacitance current and other leakage current form the data acquisition pulses at individual frequency and therefore, the mainly component left would be Na/K pump current.

50Hz: in this part, oscillating electric field with frequency of 50Hz is applied to synchronize the Na/K pumps to run at the same pace. Detailed mechanism has been discussed previously [66, 100]. Briefly, sodium ions extrusion and potassium ions intrusion possess different energy profile, which can be calculated easily from Nernst-Planck equation. By analyzing the results, we find that membrane depolarization will facilitate the Na+ export and hinder K+ import, while membrane hyperpolarization does the opposite. Accordingly, oscillating electric field with frequency of 50Hz, matching the natural turnover rate of Na/K pump, is applied. Massive data has shown that Na ions extrusion and K ions intrusion will be synchronized into positive half cycle and negative half cycle, respectively.

50Hz-150Hz: once the pumps are synchronized to the same pace, the frequency of oscillating pulse is adjusted to a higher rate. Then, the frequency will be maintained at this rate for certain pulses to re-synchronize the pump molecules. By duplicating this pattern, the rates of Na/K pumps...
will become higher and higher in a stepwise manner until reaching the designed frequency which is 150Hz.

5.2 Methods and Materials

5.2.1 Muscle fiber preparation

The animals were anesthetized and euthanized following the protocol approved by the Institutional Animal Care and Use Committee (IACUC). Single muscle fiber was separated and chosen using the procedure elaborated before. Twitch skeletal muscles, semitendinosus and ilio, were hand dissected from American Bullfrog. The detailed information can be obtained in chapter 2.

5.2.2 Composition of solutions

Relaxing solution (in mM):
120 Potassium Glutamate, 5 K₂PIPS, 1 MgSO₄, 0.1 K₂EGTA;
Ringer solution (in mM):
120 NaCl, 2.5 KCl, 2.15 Na₂HPO₄, 0.85 NaH₂PO₄.H₂O, 1.8 CaCl₂;
Internal Solution (in mM):
58 K-Glutamate, 6.8 MgSO₄.7H₂O, 5 MOPS, 20 EGTA, 10 CsOH, 3 Na₂-Creatine phosphate, 5 5-ATP-Na₂. Final [K]ᵢ 130mM, [Na]ᵢ 16Mm, PH=7.3. Stored at -20 C. For Na/Na exchange mode, K-Glutamate was replaced by Tetramethylammonium chloride (TMA-Cl);
External Solution (in mM):
3.5 3,4-Diaminopyridine (3,4-DAP), 86 NaCl, 10 CsCl, 4 KCl, 2.15 Na₂HPO₄, 0.85 NaH₂PO₄.H₂O, 1.8 CaCl₂, 1.5 BaCl₂.2H₂O, 0.001 Tetrodotoxin (TTX). Final [K]₀ 4mM, [Na]₀ 90mM, PH=7.1.

Figure 32: Modulation pulses applied
5.3 Results

The full SM train of data acquisition part was shown in Fig.33 upper panel. Clearly, as the frequency went higher, the electric pulses were more condensed. The frequency steps of the applied electric field was shown in Fig.33 lower panel. The frequencies were adjusted in a stepwise manner and in each step, the electric field was maintained for designed period to ensure synchronization of pumps at that frequency. The results of synchronized and modulated Na/K pump currents showed that the magnitudes of pump currents for both positive and negative half cycles were progressively increased along with SM train (Fig.34, upper panel). The pattern of the pump currents increase was in good agreement with the frequency steps (Fig.33, lower panel). In addition, the result obtained in the presence of Ouabain was shown in the lower panel. Clearly, the currents for both positive and negative half cycles were almost eliminated, which confirmed that the currents obtained were Na/K pump currents.

**Figure 33:** Full trace of modulation pulses and the frequency steps

To collect more detailed information, the pump currents at 50Hz, 75Hz, 100Hz, 125Hz and 150 Hz were plotted individually (Fig.35). Glancing at all of the five currents, the inward pump currents are all clearly distinguishable alternating with the outward pump currents. In addition, the ratios of the outward pump currents to the inward current for all five current traces were similar, close to 3:2. The averaged magnitude of pump currents responding to both positive and negative half pulses were calculated and concluded in Table 2 (second and third row, respectively). At 50Hz, the
magnitudes of outward current and inward current were 35.92nA and -24.75nA, which increased to 91.6nA and -64.78nA when the frequency was 150Hz. This a little bit less than three-time increment was in good agreement with the frequency ratio of 150/50. The Na/K pump-mediated charges fluxes were obtained by integrating the areas underneath the outward and inward currents, respectively. The results were listed in the fourth and fifth rows of Table1. Interestingly, the numbers of charges translocated during both half cycles of SM electric field were approximately the same for all five frequencies. The similarity of ionic fluxes suggested that most of the pump molecules had been synchronized through the whole SM traces because if not, there would have been discrepancies between different frequencies. In addition, we can obtain the ratio between number of efflux ions and influx ions for each frequency listed. The results were shown in row 6 which were 1.38, 1.41, 1.52, 1.28 and 1.43 for different frequencies, respectively. All of them were close to 1.5, the stoichiometric ratio of the Na/K pump molecules.

To further investigate the frequency-modulation effects on the pump currents, we superimposed all of the pump current traces in Fig. 36 with respect to the starting point of each current. The positive half pulse and negative half pulse were presented separately. Several conclusions can be drawn from this result:

firstly, the magnitude of pump currents in both half cycles were increasing along with the
increment of frequencies. The details have been discussed above.

Secondly, not only the magnitudes of currents changed, there was also a phase shift shown as the black arrow. It has been demonstrated that almost all of the pumps are synchronized by SM train, which can be manifested by the similarity of ionic fluxes with different frequencies. The only explanation for this phase shift would be that the turnover rate of each pump molecule increased which would accelerate the translocation of ions. This phase shift provided another evidence that the pumping rates was upregulated by SM electric field.

Lastly, the phase shift for positive half cycle, representing Na ions extrusion, was more significant.
Table 2: Comparison of inward and outward pump currents for each frequency

<table>
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<th>50Hz</th>
<th>75Hz</th>
<th>100Hz</th>
<th>125Hz</th>
<th>150Hz</th>
</tr>
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<tr>
<td>Average outward peak current (nA)</td>
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<td>46.81</td>
<td>65.23</td>
<td>77.80</td>
<td>91.62</td>
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<tr>
<td>Average inward peak current (nA)</td>
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<td>-32.64</td>
<td>-43.92</td>
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<td>-64.78</td>
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<tr>
<td>Pump mediated efflux charge (pC)</td>
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<td>15.85</td>
<td>15.63</td>
<td>15.04</td>
<td>14.82</td>
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<tr>
<td>Pump mediated influx charge (pC)</td>
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<td>-11.26</td>
<td>-10.27</td>
<td>-11.80</td>
<td>-10.34</td>
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<td>Charge ratio (+/-)</td>
<td>1.38</td>
<td>1.41</td>
<td>1.52</td>
<td>1.28</td>
<td>1.43</td>
</tr>
</tbody>
</table>

Figure 36: Superimposed pump currents from different frequencies
than that of negative half cycle, representing K ions intrusion. This can be explained by different mechanism of Na ions and K ions translocation. It has been demonstrated that releasing three Na ions to the extracellular side is much slower than releasing two K ions. Also, they proposed that releasing three Na ions has distinct steps while releasing two K ions occurred simultaneously, which may potentially result with more difficulty to synchronize Na ions extrusion than K ions intrusion.

5.4 Discussion

It has been well documented that the stoichiometric number of the Na/ K pump function remains constant over a wide range of membrane potentials. Therefore, modulation of the pumping rate to a higher value results in an increase in the pump currents. This can be seen by comparison of five current traces shown in Fig. 35 and the measurements listed in Table 2. It is necessary to point out that in all of our experiments the pulse magnitude remained at a constant value. The increase in the pump currents solely resulted from the pumping-rate modulation. The result can be explained by a simply calculation. Assuming that all N pump molecules are synchronized to a field with frequency f. During the positive half-cycle, $3*N$ sodium ions will be extruded in a time period of $1/(2f)$. The magnitude of the outward pump currents should be $(3Ne) *2f$, where e is charge of a monovalent ion. When the frequency is gradually modulated to 2f, the duration of half pulse would be reduced to $1/(4f)$. Therefore, the magnitude of the outward pump current becomes $(3Ne) * 4f$, which is double that of the low-frequency value. Similar explanation also works for the inward pump currents which represents the K intrusion.

In this study, we demonstrated that after synchronization of Na/K pumps, by gradually modulating the frequency of SM to higher value, the turnover rate of Na/K pumps can be accelerated to the corresponding level. This upregulation of Na/K pump function has many biological significance and potential applications. For example, as discussed in chapter 1, multiple diseases are related to either loss-of-function mutations of Na/K pump or reduction of ouabain binding sites. The contents of Na/K pumps may not be altered by SM technique, however, the function of the remained pumps will be significantly enhanced. As a result, the integral function of Na/K pump may be rescued. The detailed information can be obtained in chapter 6, where we investigated the SM effect on skeletal muscle fiber.
Chapter 6   Membrane hyperpolarization induced by modified SM

6.1 Introduction

The regulation of Na/K pump on skeletal muscle contractility has been well documented and concluded in multiple excellent reviews [18, 106]. In general, the skeletal muscle excitation is elicited by a rapid influx of Na through Na channel, followed by a similar efflux of K across sarcolemmal and t-tubular membranes. Thus, during intense work, the interstitial K+ are accumulated and temporarily reaches a relative high level. For example, studies with micro-dialysis probes gave K values in the range 10-13 mM in the interstitial space of working human muscle [19]. Also, a more precise measurement in rat Extensor Digitorum Longus (EDL) muscle suggested that the average extracellular concentration of K would increase from 4 to 10 mM in around 2 s [107]. Moreover, K concentration in arterial blood could reach values above 8 mM after intense exercise to fatigue [108]. On the other hand, there is a highly significant correlation between the rate of increase in extracellular K and the reduction of excitability, contractile force and contractile endurance [109, 110]

Fortunately, there are large amount of Na/K pumps located in the sarcolemma and t tubules of skeletal muscle, whose responsibility is reestablishing the transmembrane Na and K gradients. Multiply studies have demonstrated that even if the contractility of muscle is inhibited by high extracellular potassium, stimulation of Na/K pumps with hormones , such as insulin and amylin, leads to contractile force recovery and cell membrane repolarization [23]. Furthermore, it is well-documented that inhibition of Na/K pumps with cardiac glycosides, such as ouabain, would induce significant reduction in twitch force in single skeletal muscle fibers [111]. Taking together, these studies demonstrated that Na/K pumps are crucial in the maintenance of skeletal muscle excitability and contractility by restoring the Na and K concentration gradients. Any downregulation of Na/K pump could interfere skeletal muscle performance including contractile force, endurance and recovery from fatigue.

However, in certain diseases, the ouabain binding sites are severely less than normal situation. For example, studies suggested that the contents of ouabain binding sites were 3-6 folds lower in
skeletal muscle samples from patients with myotonic muscular dystrophy than control group [33]. The result was confirmed by another report that there were 30 percent fewer ouabain binding sites in cultured cells from myotonic dystrophy patients than control group [32]. As a result, muscles from patients with muscular dystrophy show significant membrane depolarization, which may contribute to impairment of muscle contraction and physical disability [34]. These observations necessitate an efficient way to upregulate the function of Na/K pumps in skeletal muscle.

It has been demonstrated in chapter 2 that the Na ions extrusion and K ions intrusion can be synchronized in the beginning of each half cycles of the second generation SM electric field, inducing transient pump currents. Also, in Chapter 5, we confirmed that by gradually modulating the frequency of applied electric fields, the turnover rate of Na/K can be accelerated to the designed frequency. In this study, we extended our investigations to see if the second generation SM technique could hyperpolarize the cell membrane potential. Our results showed that there were approximately 3-4 mV hyperpolarization of membrane potential induced by SM. The hyperpolarization was highly ouabain sensitive, which indicated that our SM electric filed mainly targeted on Na/K pumps. Moreover, we found that applications of electric fields with random frequencies, constant frequencies or backward-SM had no effect on MP. These results suggested that the turnover rate of Na/K pumps can be accelerated only by following the synchronization and modulation pattern and omitting any steps in the SM trace would result in null effect. Moreover, the longer the SM traces, the more potent the hyperpolarization was. In conclusion, the Synchronization Modulation technique can efficiently repolarize the membrane potential, which unleashes its potential in improving certain pathological conditions, such as exercise-induced hyperkalaemia.

6.2 Methods and materials

6.2.1 Composition of solutions

Relaxing solution (in mM):
120 Potassium Glutamate, 5 K₂PIPES, 1 MgSO₄, 0.1 K₂EGTA;
Ringer solution (in mM):
120 NaCl, 2.5 KCl, 2.15 Na₂HPO₄, 0.85 NaH₂PO₄·H₂O, 1.8 CaCl₂;
Internal Solution (in mM):
58 K-Glutamate, 6.8 MgSO₄·7H₂O, 5 MOPS, 20 EGTA, 10 CsOH, 3 Na₂-Creatine phosphate, 5 5-ATP-Na₂. Final [K]ᵢ 140mM, [Na]ᵢ 16Mm, PH=7.3. Stored at -20 C. For Na/Na exchange mode,
K-Glutamate was replaced by Tetramethylammonium chloride (TMA-Cl);

External Solution (in mM):
3.5 3,4-Diaminopyridine (3,4-DAP), 86 NaCl, 10 CsCl, 4 KCl, 2.15 Na₂HPO₄, 0.85 NaH₂PO₄.H₂O,
1.8 CaCl₂, 1.5 BaCl₂.2H₂O, 0.001 Tetrodotoxin (TTX). Final [K]₀ 4mM, [Na]₀ 90mM, PH=7.1.
For Na/Na exchange mode, KCl was replaced by NaCl.

6.2.2 Skeletal muscle fiber preparation

The animals are anesthetized and euthanized following the protocol approved by the Institutional
Animal Care and Use Committee (IACUC). Single muscle fiber is separated and chosen using the
procedure elaborated before. Briefly, Semitendinous muscle fibers are obtained from American
Bullfrog and then transferred to a Petri dish filled with a high potassium concentration relaxing
solution. Relaxing solution, just as its name implies, will relax the muscle fiber by depolarizing
the membrane potential to prevent its contraction during experiment procedures. A single muscle
fiber with 50–100 um diameter and 3–5 mm length is hand-dissected from its surrounding connect
tissue and transferred to a double vaseline gap chamber.

There are three pools of the chamber, two end pools sandwiched with a central pool. The
details of this chamber has been discussed in Chapter 2. The isolated muscle fiber is mounted in
the notches of the two partitions filled with thin vaseline and clamped by two Delrin clips on both
sides. Then under the microscope, gently moving those two clips and place a tension on the fibers
to stretch the sarcomere to a length of 3–3.5 um which prevent the cell from contracting during the
experiment. Thin vaseline will be used to fill the two notches to the same height of the partitions.
Last but not least, end pools will be covered by two glass slips. Solutions inside the end pools
will be replaced by internal solution and external solution for the central pool. Three agar bridges
connect the three pools to small ponds filled with 3 M KCl.

6.2.3 Synchronization Modulation electric field

Synchronization Modulation pulses are generated using JaVa program. The stimulation field con-
Sisted of three consecutive pulse-trains: synchronization, modulation and high frequency mode.
In details, each pulse contained two parts: activation and overshoot. The electric field had an
oscillating square waveform with an initial synchronization frequency of 50 Hz, which is assumed
to be close to the natural physiological turnover rate of the Na, K-ATPase pump molecules. Then
immediately, pulse frequency is gradually modulated to 150 Hz in a stepwise fashion. The step of
frequency increase was 3% - 5% for every 100 pulses. In the last part, the pulse frequency is fixed at 150 Hz for 2000 and 8000 pulses as indicated. The total time for synchronization and modulation are 50 and 80 seconds. All the pulses have the same magnitude and waveform without any time gap. The field strength of activation pulse is adjusted from 50-70 mV as indicated followed by a doubled overshoot pulse.

6.2.4 Data analysis

Data is collected with Dagon T200 TEVC. Data is analyzed using pClamp10 (Molecular Devices) and a Java program by J. Mast. Significant differences were determined with Students t test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, n.s. P<0.05.

6.3 Results

6.3.1 Modified SM induces membrane potential hyperpolarization

The applied SM trace is shown in Fig.37. Briefly, there are three segments in a trace. Synchronization: in this segment, 50 Hz oscillating electric pulses are applied to synchronize the Na/K pump molecules. Modulation: in this segment, the frequency of applied pulse is gradually modulated to 150 Hz in aiming to accelerate the pumping rate of the synchronized Na/K pumps in the previous step. Maintenance: in this segment, the frequency of electric field is maintained at 150 Hz, which aims to keep Na/K pumps working at high rate.

Firstly, we tested the capability of SM in hyperpolarizing the membrane potential. Results showed that SM could hyperpolarize the membrane potential by about 3mV and the membrane potential gradually returned to origin upon removal of electric field (Fig.38). While on the contrary, in the presence of ouabain, application of same electric field slightly depolarized the membrane.
potential, which indicated that the SM induced membrane potential hyperpolarization was highly related to Na/K pumps.

It has been proposed that external electric field would increase cell membrane permeability by inducing microscopic pores or pore-like structures in the plasma membrane \[112, 113\]. Thus, the applied SM electric field may alter dielectric strength of the hydrophobic interactions of the membrane, inducing slight membrane leak. While, even though SM may slightly alter the membrane permeability, it will not significantly affect concentration gradients across cell membrane. Because SM induced membrane potential hyperpolarization could last several minutes upon electric field removal, which is impossible if massive leak existed on the membrane.

Moreover, to investigate the traits of SM effect, we varied the length of SM traces from 2000 pulses to 8000 pulses, with total time from 50 seconds to 80 seconds. Interestingly, results showed that the longer the SM trace, the more potent the hyperpolarization (Fig.39) was and meanwhile, the longer it would last. It has been well demonstrated that Na/K pumps can be activated by absorbing energy from oscillating electric fields \[58, 68, 114\]. Accordingly, the results presented above can be explained by the following hypothesis. With more SM pulses applied, the time that Na/K pumps work at high rate (150Hz) was prolonged. As a result, the K+ concentration gradient established would be steeper and consequently more hyperpolarization was observed.

To sum up, we successfully achieved membrane hyperpolarization with Synchronization Modulation technique. In addition, we found that SM with longer duration could induce more potent membrane potential hyperpolarization. While, longer duration may potentially cause more electroporation on the cell membrane. So, parameters of SM electric field should always be optimized,
Figure 39: Membrane potential hyperpolarization with different length of SM traces
embracing the highest efficiency of SM and meanwhile diminishing the damage to cell membrane.

6.3.2 Right frequency pattern is required for membrane hyperpolarization

Next, we would like to further confirm that the membrane potential hyperpolarization is mainly due to the pumping rate acceleration entrained by the SM electric field. For this purpose, we firstly applied electric field with the same magnitude but random frequency to the muscle (Fig.40). The total length of the trace was about 80 seconds, which was the same as the previous SM trace. Results showed oscillating pulses with random frequencies slightly depolarize the membrane.

Then, we applied electric field with constant frequency of 50 Hz (Fig.41), which was comparable with physiological turnover rate of Na/K pump, but without modulation mode. The field induced membrane potential change was trivial. Statistical results showed that 50Hz-only electric field
**Figure 41:** Electric fields with constant frequency could not hyperpolarize the membrane

**Figure 42:** Backward modulation could not hyperpolarize the membrane potential
induced membrane potential change is negligible regardless of the field application.

One argument could be that 50Hz may be too low to accelerate the pump function. So we further applied electric field in the constant frequency mode of 150 Hz, which was the same as the end frequency of the SM electric field. Similarly, no hyperpolarization of the membrane potential change was observed. We also applied synchronization with backward modulation to the muscle (Fig. 42). The waveform, magnitude, and initial frequency remained the same as regular SM pulse, but in the modulation stage, the frequency gradually reduced to 20Hz. Again, the membrane potential remained unaltered.

In conclusion, we proved that membrane potential hyperpolarization is mainly due to the pumping rate acceleration entrained by the SM electric field. In order to achieve membrane potential hyperpolarization, the Na/K pumps have to be synchronized to the same pace first (Synchronization), then by gradually increasing the frequency of electric field (Modulation), the turnover rate would be accelerated. As a result, more K ions would be intruded in a given time, hyperpolarizing the membrane potential. Omitting any steps in the SM traces or disrupting the pattern of frequencies will result with null effect, which can be demonstrated by that electric fields with random frequencies, constant frequencies, or backward-SM are not capable of hyperpolarizing the membrane potential (Fig.43).
6.3.3 SM effect under hyperkalemic condition

There are good evidences that Na/K pumps are tightly related to exercise-induced hyperkalemia. For example, Clausen, et al showed that Na/K pump stimulation improves contractility in isolated muscles of mice with hyperkalemic periodic paralysis [115]. Moreover, downregulation of Na/K pump contents such as congestive heart failure would result in more pronounced hyperkalemia [116]. Also, in McArdle disease, where energy supply from glycogenolysis was restricted, ouabain binding sites to muscle biopsies showed a significant reduction [117], which resulted more pronounced exercise-induced hyperkalemia. Accordingly, we extended our investigation to see if SM could rescue membrane potential under high [K]o situation, which mimic the Hyperkalemia in pathology.

Figure 44: Membrane hyperpolarization induced by SM under high [K]o condition
Physiologically, [K]o concentration remains between 3.5-5 mM [63]. While, multiple studies have shown that after intensive exercises or long period electrical stimulation, extracellular K concentration could increase up to 10 mM [19]. Herein, we incubated isolated skeletal muscle fiber in high [K]o solution contains 10 mM potassium.

Intriguingly, our results showed that the SM induced membrane potential hyperpolarization in high extracellular potassium condition was more potent than under physiological condition, with magnitude averaged around 6-7 mV (Fig.44). The effect disappeared when applying electric field with random frequencies. Under physiological condition, the averaged membrane resting potential was -57 mV, which became -49 mV when [K]o was set to 10mM. The averaged difference was around 8 mV, which was consistent with prior study [118]. Also, we compared the SM induced membrane potential hyperpolarization in both conditions. Clearly, before SM application, the membrane potentials were largely separated. Whereas, SM hyperpolarized the membrane potential more under high [K]o condition, restoring the concentration gradient back to the level that was comparable with physiological condition.

The detailed mechanistic understanding needs further investigation. Potential explanation could be that under physiological condition, where K concentration is well established, the turnover rate of Na/K pump is slower than the designed frequency (50Hz). On the contrary, Na/K pumps run faster with depolarized membrane potential, which can be manifested from sigmoid shape of the I-V curve. As a result, the number of synchronized pumps under [K]o condition is larger than physiological condition.

6.4 Discussion

6.4.1 SM electric fields mainly target on Na/K pumps

When an oscillating electric field applied to cell membrane, in addition to Na/K pump molecular, other membrane proteins such as voltage-gated Na channel or rectifier K channels may be affected as well. The voltage-gated sodium channels, as studied previously, could be totally inhibited by specific blocker TTX. In terms of K channels, addition of 3, 4 DAP and Cs would efficiently inhibit voltage dependent K channels [119] and inwardly rectifier K channels [120], respectively. Most importantly, the SM induced membrane potential hyperpolarization was highly ouabain sensitive (Fig.1), which indicates that the effect is mainly from activation of Na/K pumps.

Indeed, there were studies showed that electrical stimulation would increase the contents of
Na/K pump in skeletal muscles. While in these studies, people used 12V electrical stimulation pulse to generate action potential which excited the muscle and induced contraction. As discussed above, continuous contraction would inevitably increase the internal Na concentration or Na binding affinity [121], activating Na/K pumps activity, which indicates that the effect on Na/K pumps is passive and indirect. While in the present study, we applied SM electric field with maximum amplitude of 140 mV, which is about two order smaller than previous studies. Moreover, previously data from our lab showed that the electric field is directly applied on Na/K pump molecules, which can be manifested by folds increase of Na/K pump current [47].

6.4.2 Potential pathological applications of SM

There are good evidences that Na/K pumps are tightly related to exercise-induced hyperkalemia. For example, Clausen, et al. showed that Na/K pump stimulation improves contractility in isolated muscles of mice with hyperkalemic periodic paralysis [115]. Moreover, downregulation of Na/K pump contents such as congestive heart failure would result in more pronounced hyperkalemia. Also, in McArdle disease, where energy supply from glycogenolysis was restricted, ouabain binding sites to muscle biopsies showed a significant reduction [117], which resulted more pronounced exercise-induced hyperkalemia.

Moreover, it has been reported that multiply cardiac muscle diseases or disorders are highly related to reduction of Na/K pump contents. For example, the contents of ouabain binding sites in the vastus lateralis from congestive heart failure patients reduced by 25-37 percent compared with control group [158]. In addition, muscles obtained from patients with muscular dystrophy showed 3-6 folds reduction of Na/K pumps [122]. Also, in patients with McArdle disease, where energy supply from glycogenolysis is restricted, the Na/K pumps contents reduced by 27% [123]. By applying SM technique, even though the contents of Na/K pumps may not change, the function of remained pumps would be significantly enhanced. As a result, muscle contractivity will be significantly improved.
In this dissertation, we studied the second generation Synchronization Modulation electric field to synchronize the Na/K pumps function into individual steps. We have demonstrated that by applying this new version SM technique, the Na ions extrusion and K ions intrusion will be synchronized at the beginning of the positive half cycle and negative half cycle, respectively. As a result, transient pump currents or so called pre-steady state pump currents are obtained. Some important information about the mechanism of Na/K pumps can be revealed from the synchronized pump current. Among them, the single-channel configuration of and D2O slow down effect on the Na/K pumps are discussed in this dissertation. Moreover, it has been confirmed that by gradually modulating the frequency of SM electric field to higher value, the turnover rate of the Na/K pumps can be potentiated to the designed level. Lastly, we find that the second generation SM can significantly hyperpolarize the cell membrane potential by activating the function of Na/K pumps.

The novel SM technique we developed have many advantages. First of all, compared to traditional SM technique that only synchronize Na/K pumps into half cycles of the electric field, the second generation SM has the capability to synchronize the pump function into individual steps. Thus, the synchronized macroscopic current shares the same characteristics with the single pump current, which indicates that more detailed information about the mechanism of Na/K pump can be revealed. Second, different from the transient currents that obtained in partially functional Na/K pumps, our experiments are conducted under physiological condition, in which the complete cycle of the Post-Albert model is included such as ATP hydrolysis, phosphorylation and de-phosphorylation, etc. The dynamic correlations between E1 and E2 states are not overlooked as well. Third, for the caged-ATP method, even though the whole cycle of Na/K pump function is included, the rate of the obtained transient current is limited by the diffusion speed of the released ATP molecules to their binding sites (A domain). Our second generation SM technique, on the contrary, does not has such restrictions, which can be manifested by that the transient currents we obtained are much narrower than that from caged-ATP experiments. Finally, by applying modified SM, the Na/K pump function is significantly enhanced, which unleashes its broader applications.
in diseases involving Na/K pumps deficit.

For example, at the end of this dissertation, we tested one of the applications of this technique: hyperpolarizing the cell membrane potential. Our results showed that the membrane potential can be hyperpolarized by about 3 mV. Also, in some extreme situations such as high extracellular K concentration which is also called hyperkalemia, our data showed that the hyperpolarization of membrane potential was more potent with an average about 6-7 mV (not shown here). These data indicate that by applying SM, the homeostasis of sodium and potassium can be quickly established. In addition, we have applied SM technique to rat kidney and our preliminary results show that the TEPD of kidney can be hyperpolarized by about 5-6 mV. The TEPD of the kidney tubular reflects the ion (mainly Na) concentration gradients across the transepithelial cells. The larger the TEPD, the higher the Na concentration gradient. Thus, our results suggest that the concentration gradient of Na ions are significantly enhanced by SM, which could provide more energy for other secondary transporters such as Na/Glucose symporter. As a result, the kidney reabsorption function will be improved.

In the future, we will keep exploring other mechanisms that can be revealed by the second generation SM technique, such as energy transduction pathways in the Na/K pump. In the meantime, we will investigate more potential applications of SM technique. For example, we will continue the study of SM effect on kidney reabsorption function under some pathological conditions such as ischemia.
References


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Appendix A: Na/K pump inhibitor-Ouabain

Ouabain is a cardiac glycoside and in lower doses, can be used medically to treat hypotension and some arrhythmias. It acts by inhibiting the Na/K-ATPase, also known as the sodium-potassium ion pump. The binding site of the cardiac steroid on the Na/K pump has been more difficult to define. Comparison of ouabain-sensitive and -resistant isoforms of the Na/K pump and subsequent mutagenesis work initially pointed to the outer part of the first hairpin (the transmembrane segments 1 and 2 and the short extracellular loop between them), but extensive mutagenesis work disclosed the important contribution of the fifth, sixth, and seventh transmembrane segments [124], and recent work demonstrated that the third hairpin (transmembrane segments 5 and 6) provides the structure of the binding site itself [125]. People found that H5-H6 hairpin loop remains embedded in the membrane upon digestion in the presence of ouabain or cations but is released into the soluble fraction in the absence of these ligands indicative of the cations or ouabain interactions restricting the free movement of this domain [126].

Figure A1: Chemical structure of Na/K pump blocker ouabain
Appendix B: Double Vaseline Gap chamber and TEVC-200

The TEV-200 is a high performance general purpose two electrode clamp. It’s an ideal assay clamp for studying receptors and channels.

There are three pools of the chamber, two end pools sandwiched with a central pool. The isolated muscle fiber is mounted in the notches of the two partitions filled with thin vaseline and clamped by two Delrin clips on both sides. Then under the microscope, gently moving those two clips and place a tension on the fibers to stretch the sarcomere to a length of 3-3.5 um which prevent the cell from contracting during the experiment. Thin vaseline will be used to fill the two notches to the same height of the partitions. Last but not least, end pools will be covered by two glass slips. Solutions inside the end pools will be replaced by internal solution and external solution for the central pool. To ensure the replacement is complete, the procedure has to be repeated by at least 3 times for each solution. Three agar bridges connect the three pools to small ponds filled with 3 M KCl. Ag/AgCl pallets are used to connect the small ponds with channel 1 and channel 2 on the Two-electrode voltage clamp instrument.
**Figure A2:** Two electrode voltage clamp instrument (Figure from Dagan Corporation website)

**Figure A3:** Double vaseline gap chamber
Appendix C: Capacitance of single muscle fiber

Figure A4: Illustration of Capacitance measurement

A small voltage-clamp step is delivered of size 10mV (top panel) and long enough for the resulting current to come to steady state. The clamp current required to bring this about is measured (bottom panel). The area under the transient current is then integrated to calculate the transient charge delivered during the voltage-clamp step (shaded area, denoted Q). The transient charge is then divided by the size of the voltage clamp step to yield an estimate of the cell capacitance $C = Q/v$ [127, 128]. Using this method, our result is around 6.82nF. (n=15)
About the author

Pengfei Liang originally comes from Shandong, China. He graduated from University of Shanghai for Science and Technology in China with an undergraduate degree in Physics in 2012. He received an M.S. in Applied Physics and Medical Physics from University of South Florida in 2016, where he continued the Ph.D. program in Biophysics.