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ORIGINAL PAPER

Entrainment of Na/K pumps by a synchronization modulation electric field

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Abstract We studied entrainment of the catalytic cycle of the Na/K pumps by an imposed external AC electric field. Our results show that a well designed dichotomous oscillating electric field with a frequency close to the pumps' natural turnover rate can synchronize the pump molecules. Characteristics of the synchronized pumps include: (1) outward pump currents responding to Na-extrusion and inward pump currents responding to K-pumping in are separated; (2) magnitude of the outward pump currents can be up to three times higher than that of the randomly paced pump currents; (3) magnitude ratio of the outward over inward pump currents reveals the 3:2 stoichiometry of the pumps. We, further, gradually increased the field oscillating frequency in a stepwise pattern and kept pump synchronization in each step. We found that the pumps' turnover rate could be modulated up as the field frequency increased. Consequently, the pump currents significantly increased by many fold. In summary, these results show that the catalytic cycle of Na/K pumps can be synchronized and modulated by a well designed oscillating electric field resulting in activation of the pump functions.

Keywords Na/K pump · Synchronization · Modulation · Membrane proteins · Electric field

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Introduction

Many molecular pumps in cell membranes function as "housekeepers" to maintain, at the expenditure of energy, ionic concentration gradients and an electrochemical potential difference across the cell membrane. The ionic concentration gradient is critical to many cell functions, including controlling cell volume, generating electrical signals, and providing energy for other active transporters. A stable membrane resting potential is vital to many cells, especially to the excitable cells. Many diseases or physiological emergencies involve dysfunctions of the pump molecules either due to the lack of ATP to fuel the pumps or due to a reduced density of pump molecules in the cell membrane. A fascinating question is whether we can artificially or physically control these pump functions.

Pioneering work by Teissie and Tsong (1980) in study of Rb accumulation in red blood cells showed that a weak oscillating electric field could activate the Na/K ATPase in erythrocytes. Blank and Soo (1989) have reported that an alternating current can either stimulate or inhibit ATP hydrolysis activity of the pumps, depending on the concentration ratio of Na and K ions. Recently, they also studied the effects of an AC magnetic field on the pump functions (Blank and Soo 2001, 2005) Based on the experimental results in measurement of ion movement by radioactive tracers (Serpersu and Tsong 1983; Liu et al. 1990), several theoretical models have been built to explore the possible mechanisms involved in electrical activation of the pump molecules, such as the resonance frequency model (Tsong and Astumian 1986, 1987; Markin et al. 1992; Robertson and Astumian 1991), Brownian ratchet model (Astumian 1997; Tsong 2002), and adiabatic pump model (Astumian 2003).

Previously, we developed a technique of synchronization-modulation to electrically activate the functions of the Na/K pumps. Theoretically, we showed that by synchronization modulation of the pumps' turnover rates, the pump functions can be significantly activated (Chen 2006; Chen, submitted, in review). Experimentally, we have applied the synchronization modulation electric field to frog skeletal muscle fibers (Chen and Dando 2006, 2007) and mammalian cardiomyocytes (Chen and Dando, submitted), and shown that the field-induced pump activation can effectively reinstate, and hyperpolarize, the membrane resting potential. In terms of underlying mechanisms involved in this technique, we have demonstrated the synchronization of the Na/K pumps by both DC and oscillating pulses (Chen and Zhang 2006; Chen and Zhang, submitted). In this paper, we further present the results of our studies in modulation of the pumps' turnover rates and activation of their functions.

In this study, we continue to use frog skeletal muscle fibers and monitor the pump currents by using a double Vaseline-gap voltage clamp technique. This technique has been used to study intramembrane charge movement currents in skeletal muscle fibers for decades (Hille and Campbell 1976, Kovacs et al. 1983; Irving et al. 1987, Hui and Chen 1992) and recently used in our lab to study the Na/K pump currents (Chen and Zhang 2006, Chen and Zhang, submitted, and Chen and Wu 2002). In terms of the concern of the technique in measurement of pump currents, we will first compare the results with those obtained using the microelectrode patch-clamp on other cells. Then, we will show that by keeping the pump synchronization and gradually increasing the field's oscillating frequency, the pump molecules can be modulated to a significantly higher pumping rate.

Methodology

The double Vaseline–gap voltage clamp technique was developed by Hille and Campbell (Kovacs et al. 1983), and has been used in many labs including ours to study charge movement currents and Na/K pump currents. Briefly, single skeletal muscle fibers were hand dissected from twitch muscles, *semitendonosis* of *Rana pippins* frogs, and mounted into a custom-made chamber. The fibers were electrically and ionically separated by two Vaseline partitions into three segments: End pool 1, Central pool, and End pool 2. The dimensions of the partition and the central pool are 100 μm and 300 μm, respectively. The fiber segments at the two end pools were treated with 0.01% saponin for two minutes and washed out. A voltage clamp (Dagan TEV 200) was connected to the three pools through six Ag/AgCl pellets in order to clamp the membrane potential and to monitor the transmembrane currents.

The compositions of internal and external solutions follow the recipes used in ours and other labs in study of Na/K pump currents. We also followed Gadsby's work (Gadsby and Nakao 1989) and adjusted the concentrations of Na and K in the external and internal solutions in order to increase the pump currents. The solution compositions are the following:

Internal solution (mM): Na-glutamate, 40; K-glutamate, 22.5; $MgSO_4$, 6.8; Cs_2-EGTA, 20; Cs_2-PIPES, 5; $Tris_2$–Cretinephosphate, 5; and Na_2-ATP, 5.5.

External solution (mM): TEA-Cl, 27.5; NaCl, 75; KCl, 5.4; Na_2HPO_4, 2.15; NaH_2PO_4, 0.85; $CaCl_2$, 1.8; $RbCl_2$, 1.5; $BaCl_2$, 1.5; and 3.4-DAP, 3.5 and 1 μm TTX.

External solution with ouabain: the same composition as above but with 1 mM ouabain.

In all of our experiments, we blocked most of channel currents. To focus on the field effects on the pump molecules, all of the experiments were performed at room temperature, 24 °C.

Due to the less than 1 kΩ series resistance in the clamp pathway comparing to megaohms in the microelectrode, the double Vaseline-gap voltage clamp technique allows us to transiently change the membrane potential or alternate the potential polarity, which is superior to the microelectrode patch clamp. However, it is impossible to get a gigaohm seal resistance, and therefore, the leakage current is relative large. The p/4 method has been widely used to remove the linear leakage currents.

Figure 1 shows the protocol of the p/4 method. Before the application of a stimulation pulse to the cell membrane, the membrane potential is purposely set to a hyperpolarization potential of −110 mV followed by a group of four sub-pulses which have the same waveform as, but only one fourth of the magnitude of, the following stimulation pulse. The polarity of the sub-pulse may be the same as, as shown in Fig. 1, or opposite to, the following stimulation pulse. Because of the low magnitude, especially with a negative polarity, the currents elicited by the p/4 sub-pulses are mainly the linear (capacitance and leakage) currents with little residual pump currents. In all experiments, the membrane potential was held at −90 mV, the membrane resting potential of skeletal muscle fibers. The currents generated by the p/4 sub-pulses were added up and then subtracted when we wanted to focus on non-linear effects at full pulse strength.

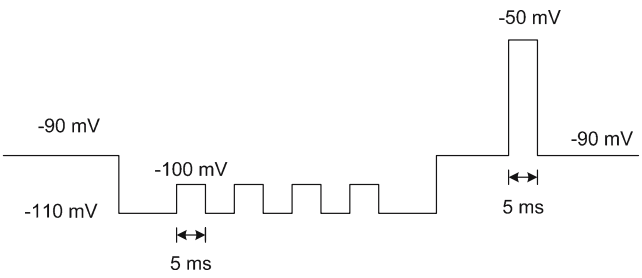

Fig. 1 Protocol of p/4 pulses to subtract the linear currents

Stimulation protocol

In this study, all the stimulation pulse-trains consist of symmetric oscillating rectangular pulses having the same magnitude alternating the membrane potential from −30 to −150 mV. In each train, the first part is the p/4 sub-pulses, followed by the stimulation pulses which consists of a number (*N*) of oscillating pre-pulses, and three data-acquisition-pulses. The control pulse-train, T0, consists of only three data-acquisition-pulses without pre-oscillating pulses (*N*=0). The upper panel in Fig. 4 shows the control pulse-train.

The synchronization pulse-train, T100, consists of 100 pre-oscillating pulses preceding the three data-acquisition-pulses, which is shown in the upper panel of Fig. 5.

The synchronization-modulation pulse-train, TM100, consists of five blocks with gradually reduced half-pulse duration. Each block has 100 pre-oscillating pulses followed by three data-acquisition-pulses. The half-pulse duration is initially 15 ms in the first block and then gradually reduced to 10, 6, 4 and 3 ms, respectively. The equivalent oscillating frequencies for the five blocks are increased from 33 to 166 Hz in a stepwise fashion. There was no time-gap between any two blocks. The synchronization modulation pulse-train, TM100, is shown in Fig. 6.

The stimulation protocol was as follows: The control pulse-train, T0, was first applied to the cell membrane five times. The time intervals between the train applications, here and thereafter, were always three minutes. When studying synchronization, the synchronization pulse-train, T100, was then delivered to the cell membrane five times. Our previous studies showed that at our experimental conditions, 100 cycles of oscillation in the membrane potential can synchronize the pump molecules and that three minutes is enough for the pump molecules to return to their initial random paces. When studying the modulation, the synchronization-modulation pulse-train TM100 was applied to the cell membrane five times. Then, the external solution was changed to that with ouabain, a specific inhibitor of the Na/K pump currents. After that, the same procedure was reapplied to the cells.

In data analysis, the linear transmembrane currents elicited by p/4 sub-pulses were first added and then subtracted from the currents induced by the following data-acquisition-pulses. The resulting non-linear transmembrane currents in the presence of ouabain were subtracted from those in the absence of ouabain. The resultant currents are defined as the ouabain-sensitive currents, or the Na/K pump current.

The resulting pump currents from five repeated stimulations were averaged to increase the signal/noise ratio. For synchronization-modulation studies, the pump currents from the three data-acquisition-pulses in each group were further averaged. The results are shown in the corresponding figures.

Experimental results

I-V curve of the Na/K pumps

Figure 2 shows the ouabain-sensitive currents, or the Na/K pump currents elicited by a single 30 ms pulse depolarizing the membrane potential to −30 mV. The pump currents show only an outward current. This result is consistent with those obtained form other labs using the microelectrode patch clamp techniques (Gadsby and Nakao 1989; Nakao and Gadsby 1989; Rakowski et al. 1991; and Rakowski et al. 1989a).

Figure 3 shows the pump current as a function of the membrane potentials, or the steady-state I-V curve of the Na/K pumps. The curve exhibits a sigmoidal shape with a shallow slope gradually increasing as the membrane potential is depolarized. At the membrane potential gets close to 0 mV, the pump currents are saturated showing a plateau of the curve. When the membrane potential is further depolarized, the pump current is even shown to fall, showing a negative slope. The curve is very similar to those obtained from other preparations, such as cardiac cells (Gadsby and Nakao 1989; Nakao and Gadsby 1989), neuron cells (Rakowski et al. 1989a), and Xenopus oocytes (Rakowski et al. 1991) using the microelectrode patch clamp technique.

The only difference from those results obtained using the microelectrode is that the absolute values of the pump currents

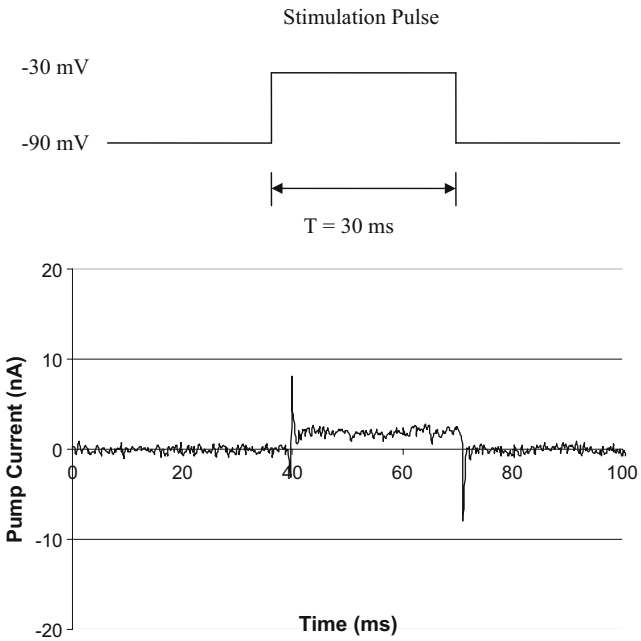

Fig. 2 *Upper panel* A single stimulation pulse used to elicit the pump currents. *Lower panel* The ouabain-sensitive currents, or the Na/K pump currents. The transient charge and discharge currents responding to the rising and falling phases of the pulse are due to un-perfect matching during p/4 subtraction. Similar results are shown in the following figures

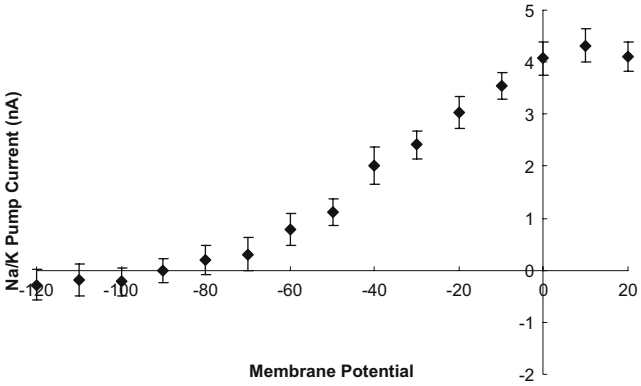

Fig. 3 Na/K pump currents as a function of the membrane potential. Seven experiments were conducted. The *bars* represent the standard deviation. Because we used the p/4 method, the pump current presented here is the relative pump current with respect to that at the membrane holding potential of −90 mV

were presented in those I-V curves, while the pump currents presented in this I–V curve were the values relative to those at the membrane holding potential of −90 mV. This is because of the subtraction of the p/4 pulse-induced currents. Therefore, in this I–V curve, the pump current at the membrane holding potential is zero. In fact, this I–V curve, when transposed upwards for the value of the pump current at −90 mV, will be consistent with those obtained by the microelectrode.

Synchronization

The lower panel of Fig. 4 shows the Na/K pump currents elicited by the control pulse-train, T0, without any oscillating pre-pulses. The pump currents are mainly outward currents responding to the positive half-pulses, while the negative half-pulse elicited very little pump currents, which can be explained by the asymmetry of the I–V curve with respect to the membrane resting potential. The pump currents were calculated by averaging the last 20 points of the currents. For this fiber, it is 2.1 nA.

Figure 5 shows the pump currents elicited by the last three data-acquisition pulses in the synchronization pulse-train, T100. Interestingly, the result is dramatically different from those shown in Fig. 4 even though the data-acquisition-pulses were identical in both trains.

First, the magnitude of the outward pump current responding to the positive half-pulse was significantly increased. The value was calculated as 5.1 nA, which is less than, but close to, three times increase from the outward pump currents (2.1 nA) elicited by Train T0.

Secondly, the pump currents elicited by the control train are mainly the outward pump currents corresponding to the positive half-pulses, as shown in Fig. 4. In contrast, the pump currents induced by the data-acquisition-pulses in the synchronization pulse-train, T100, shows distinguishable outward and inward currents occurring alternatively corresponding to the positive and negative half-pulses, respectively. In other words, the original outward pump currents have been separated into two components: the inward and outward currents. If comparing the magnitudes of the pump currents induced by the negative half-pulses in both trains, the difference is more significant. In response to the control pulse-train, the negative half-pulses elicited negligible inward pump currents comparing to the outward currents, while in response to the synchronization pulse-train, the negative half-pulse generated noticeable inward pump currents (2.9 nA) comparable to the outward currents. Finally, the magnitude ratio of the outward to the inward pump currents is 5.1:2.9, which is close to the stoichiometric ratio of 3:2.

We conducted more than ten experiments using the same control and synchronization pulse-trains. All of these results consistently showed the train T100 generated a close to, but little less than, threefold outward pump currents. A distinguishable inward pump current was always observed in response to the negative half-pulses. Moreover, the magnitude ratio of the outward over inward pump components is always close to, but a little higher than, 3:2. It is necessary to point out that the data-acquisition-pulses were identical in both trains T0 and T100. The only difference is that there existed a

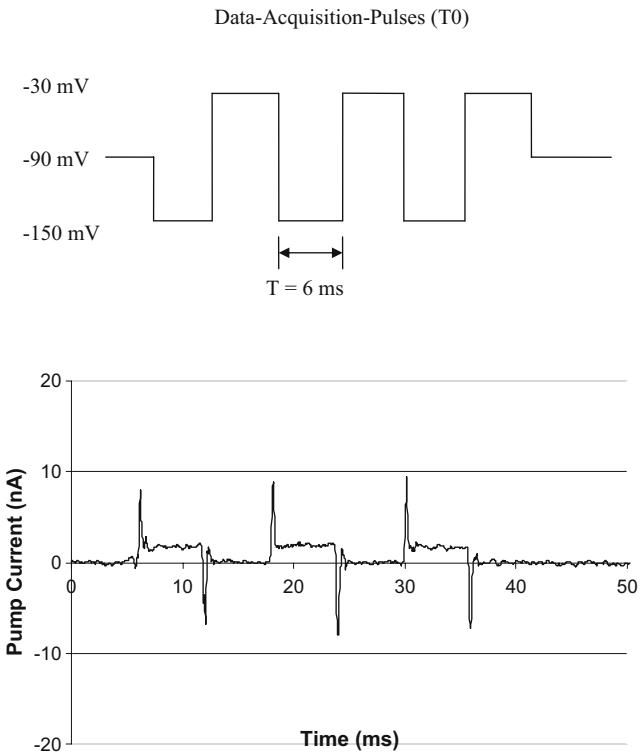

Fig. 4 *Upper panel* The control pulse-train, T0, consists of three data-acquisition-pulses without any pre-oscillation. *Lower panel* Pump currents elicited by Train T0. It is mainly outward pump currents only responding to the positive half pulses. The pump currents induced by the negative half-pulse is very small

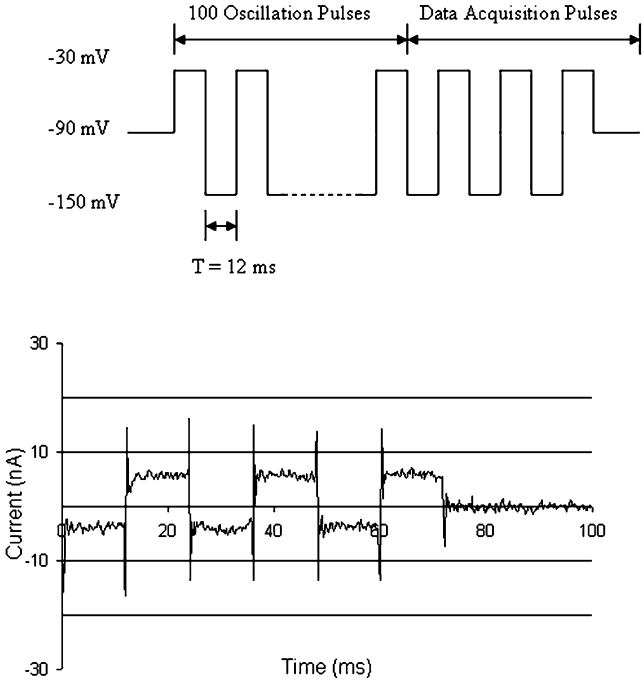

Fig. 5 *Upper panel* The synchronization pulse-train, T100, consists of 100 oscillating pre-pulses followed by three data-acquisition-pulses. *Lower panel* Pump currents elicited by the data-acquisition-pulses in Train T100. Inward component of the pump current is distinguishable from the outward pump current. The ratio of the outward to the inward pump current is close to 3:2

hundred pre-oscillating pulses preceding the data-acquisition-pulses. Clearly, the pre-oscillation in the membrane potential played a significant role in changing the pump currents.

Our hypothesis is that the pre-oscillating pulses which alternate the membrane potential entrain the pump molecules so that the pumps work at the same pace; or the pumps are synchronized. The justification and the mechanisms involved in the pump synchronization will be discussed in the discussion and conclusion section.

Modulation

Let us assume our hypothesis is right that individual pump molecules working at random paces can be synchronized by an oscillating electric field. It is reasonable to imagine that once synchronized, when we increase the field-frequency by a small step, the electric field should be able to re-synchronize the pumps to the new frequency if the frequency-step is small enough and the field is applied for long enough. If that is the case, by gradually increasing the field frequency and keeping the pump molecules synchronized, the pumping rates can be accelerated in a stepwise pattern. In order to prove this hypothesis, we applied the synchronization-modulation Train TM100 (Fig. 6) to the cell membrane. The train consists of five blocks with the half-pulse duration gradually reduced in a stepwise pattern from 15 to 3 ms. The equivalent oscillating frequency of the first block is 33 Hz which is comparable to the Na/K pumps' turnover rate at physiological condition.

With the same method, the pump currents elicited by the data-acquisition-pulses in each block were obtained and averaged for five stimulations. Finally, the pump currents corresponding to the three data-acquisition-pulses in each block were further averaged. The final results for the five blocks are shown in Fig. 7.

Glancing at all of the five current traces, 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 are similar, close to 3:2. However, the magnitude of the pump currents was progressively increased when the half-pulse duration was reduced. The magnitudes of the pump currents responding to both the positive and negative half-pulses were calculated and are listed in the second and third rows in Table 1, respectively. In the first block where the half-pulse duration was 15 ms, the magnitude of the outward pump current was only 2.9 nA. In the last block, the outward pump currents increased to 13.1 nA, a little less than a five-time increment from the first block. Interestingly, the half-pulse duration of 3 ms is also one fifth of that in the first block.

Similar results can be observed by comparing the inward pump currents. The value of the inward pump currents induced in the first block was initially about 2.02 nA and finally reached a value of 9.12 nA, a little less than a five time increase.

The ionic fluxes or the number of ions carried by the pump molecules during the positive and negative half-pulses are represented by the areas underneath the outward and inward pump currents, respectively. This can be obtained by integration with respect to time of the current trace.

The results are listed in Table 1. The fourth row represents the total number of charges extruded from the cell during the positive half-pulse. Similarly, the total numbers of charges pumped into the cell during the

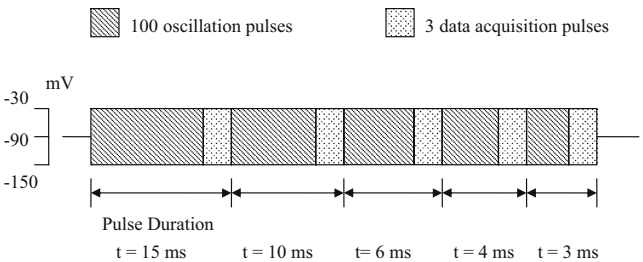

Fig. 6 The synchronization modulation pulse-train (TM100) consisting of five groups with gradually reduced half-pulse durations. Dark color represents 100 oscillation pre-pulses and the lighter color represents three data-acquisition-pulses. All of the pulses have the same magnitude alternating the membrane potential from −30 to −150 mV

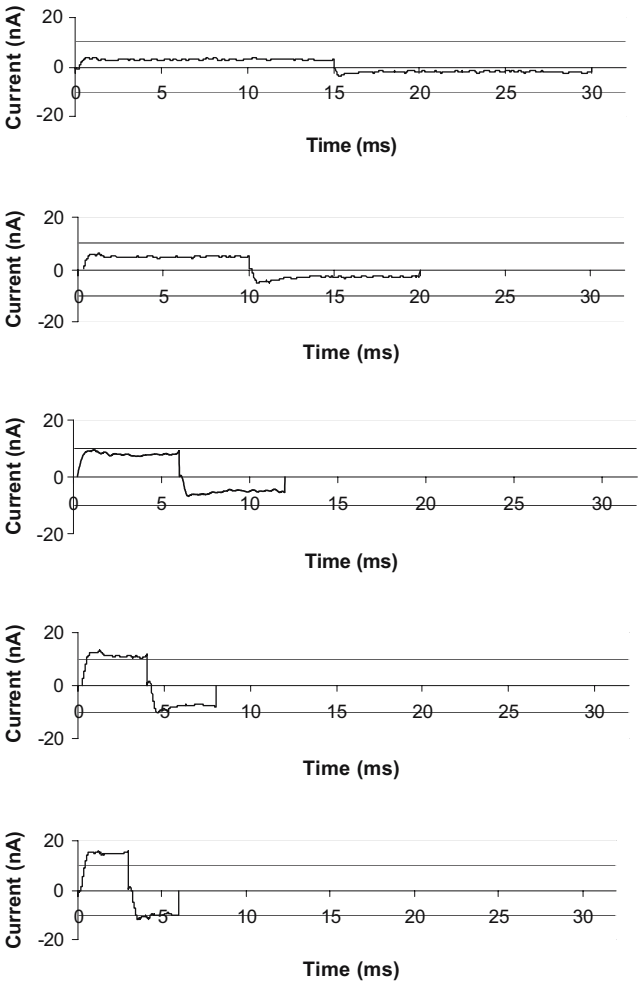

Fig. 7 Comparison of the pump molecules elicited by the data-acquisition-pulses in five consecutive groups of the synchronization-modulation pulse-train (TM100). The pump current traces shown from top to bottom panels correspond to the half-pulse durations of 15, 10, 6, 4 and 3 ms, respectively

negative half-pulses are listed in the fifth row. The numbers of charges moved during both half-pulses are approximately the same for all groups. This similarity is due to the fixed number of pump molecules in the cell membrane and the fixed stoichiometry of the pumps. In fact, this similarity, regardless of pulse duration, is a good sign indicating that most of the pump molecules had been synchronized in each block.

In addition, we can obtain the ratio of the number of charges extruded over that of those pumped-in, in each cycle, by dividing the number in the fourth row over those in the fifth row in the same column. The results show 1.44, 1.35, 1.59, 1.32 and 1.44, respectively, as marked in the sixth row. They are all close to 1.5, the stoichiometric ratio (3:2) of the Na/K pump molecules.

To further compare the frequency-modulation effects on the pump currents, we superimposed all of the pump current traces in Fig. 8. The pump currents are aligned by the middle of the currents responding to the transient change in the polarity of the membrane potential. On the left side are the outward currents responding to the positive half-pulse, and on the right side are the inward currents responding to the negative half pulses. It clearly shows that the areas underneath either the outward or inward pump currents remain the same regardless of the pulse durations. Both the outward and inward pump currents are continuously increased when the synchronization frequency was gradually increased.

Discussions

We first showed that the Na/K pump currents and its I–V curve recorded from skeletal muscle fibers by using the double Vaseline-gap voltage clamp technique are very similar to those measured by the microelectrode patch clamp techniques from other cell preparations. The pump currents are mainly the outward currents. The inward and outward components can not be distinguished.

Synchronization

As the membrane potential is continuously oscillated with a frequency comparable to the pumps' natural turnover rates,

Table 1 Comparison of the magnitudes of the inward and outward pump currents, and the influx and efflux charges responding to two half-pulses in each block of Train TM100

	Oscillation block 1 (15 ms)	Oscillation block 2 (10 ms)	Oscillation block 3 (6 ms)	Oscillation block 4 (4 ms)	Oscillation block 5 (3 ms)
Average outward pump current (nA)	2.90	4.65	7.55	10.2	13.1
Average inward pump current (nA)	−2.02	−3.44	−4.73	−7.75	−9.12
Integral outward pump current (nA ms)	43.6	46.5	45.3	41.0	39.4
Integral inward pump current (nA ms)	−30.3	−34.4	−28.4	−31.0	−27.4
Magnitude ratio	1.44	1.35	1.59	1.32	1.44

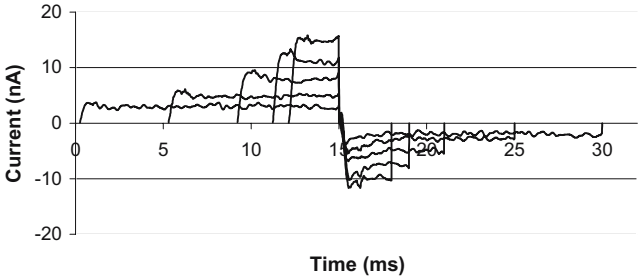

Fig. 8 The pump currents shown in Fig. 7 are superimposed for comparison. The pump currents are aligned by the middle of the currents responding to the polarity change between the two half-pulses. The areas underneath either the outward or inward pump currents remain the same for all five current traces, and the current magnitude gradually increase when the pulse-duration was reduced

the shape and magnitude of the pump currents were changed gradually (data not shown). Finally, the net outward pump current was separated into two components: the outward and inward currents alternatively occurring corresponding to the two half-cycles of the oscillating pulses. The magnitude of the outward pump currents were increased by about three times. The magnitude ratio of the outward over inward components is about 3:2 reflecting the stoichiometric number of the Na/K pump functions (Fig. 5).

Changes in the pump currents can be explained as synchronization of the pump molecules by the oscillating membrane potential. The underlying mechanisms involved in the pump synchronization have been studied previously (Chen and Zhang 2006; Chen and Zhang, submitted). Briefly, because of structural independence, pump molecules may run at individual pumping rates and random pumping phases. When measuring the pump current which is a sum of all the individual pump currents, the outward component representing three Na ions extrusion and the inward component representing two K ions pumping in can not be distinguished. Each pump molecule transports net (3-2) ions out of the cell in one cycle. Therefore, if there are N pump molecules in the study, a total of N net ions are pumped out in each cycle, resulting in an outward pump current (Fig. 4).

From the Post–Albers model of the Na/K pumps (Albers 1967; Post et al. 1972; Rakowski et al. 1997; Lauger 1996; Apell 2003; and Artigas and Gadsby 2003), we can observe the following facts: Firstly, the steps of both Na or K transport are voltage-dependent due to ion movements. Secondly, Na and K transport are in opposite directions; therefore, any membrane potential change will have opposing effects on the two types of ion transport. Thirdly, the two types of ion transport are among the slowest steps in the loop; changing either one will affect the whole net pumping rate. Finally, the two types of ion transport do not happen simultaneously, and instead, are in a sequential pattern. Based on these facts, when an oscillating electric field with a frequency comparable to the pumps' turnover rate is applied to the cell membrane, the field's two half-cycles match the time courses of the two types of ion transport. The oscillating electric field will treat individual pumps differently, based on their turnover rates and phases with respect to those of the field. For the pump whose turnover rate is a little lower than the field frequency, the electric field may facilitate the two types of ion transport, alternatively loop by loop, until the pump's turnover rate matches the field frequency. For those whose turnover rates are a little higher than the field frequency, the electric field may slow them down until reaching the field frequency.

As a result, the pace of the two types of ion transport will be dominated by the two half-cycles of the oscillating electric field, respectively. In other words, the pump molecules are entrained by, or synchronized to, the oscillating electric field. As a result, they extrude three Na ions at the same time and thereafter pump two K ions into the cells. The pump currents are separated into distinguishable outward and inward components (Fig. 5). During the positive half-cycle N pump molecules pump out $3N$ Na ions, which explains the three-times increase from that of the randomly paced pump currents. Then, the pumps bring $2N$ K ions into the cells during the negative half-cycle, resulting in an inward pump current. The magnitude ratio of the outward over inward pump currents, $3N/2N$, represents the stoichiometric ratio 3:2 of the Na/K pump functions (Fig. 5).

Modulation

When the frequency of the oscillating electric field is increased a small amount, the pumping rate will be forced to be synchronized to the new frequency if the step is small enough and the field is applied a sufficient period. Once reaching synchronization, the field frequency can increase again to resynchronize the pumps to the next frequency. Using this method, the pump molecules can be gradually modulated to higher and higher pumping rates. It has been well documented that the stoichiometric number of the Na/K pump function remains constant over a wide range of membrane potentials (Rakowski et al. 1989b). 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. 7 and the measurements listed in Table 1. It is clear that by slowly decreasing the pulse duration of the oscillating electric field and keeping the pump synchronized, the times needed for the two types of ion transport are reduced step by step, and the pumps' turnover rates are gradually accelerated in a stepwise pattern. As a result, the pump current is increased. 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.

Assuming all N pump molecules are synchronized to a field frequency f, during the positive half-cycle, $3N$ Na ions are extruded by the pump molecules in a time period of $1/(2f)$. The magnitude of the outward pump currents is $(3\text{Ne})2f$, where e is charge of a monovalent ion. When the pump molecules are gradually modulated to be synchronized to a frequency of $2f$, the pulse duration reduces to $1/(4\text{f})$. Therefore, the magnitude of the outward pump current becomes $(3\text{Ne})4f$, which is double that of the original value. Similar results can be observed for the inward pump currents.

It is necessary to point out that we only increased the oscillating frequency up to five times which resulted in close to a fivefold increase in the pump currents. Further increase in the field frequency may lead to a progressively higher increase in the pump currents.

Comparison of the underlying mechanisms involved in this technique and those in prior work

An oscillating field has been used to activate functions of membrane proteins for decades. In an excellent review (Tsong 1990), Tsong elaborated the concept of electric field-induced conformational changes in the proteins. In this study, we also used an oscillating electric field to facilitate the pump functions. However, the underlying mechanisms involved in this study have some fundamental differences from those in the previous studies, including the assumptions, targets of the protein's structures, and the results.

In those studies, a fundamental assumption is existence of intrinsic or characteristic frequency(s) of the pump molecules. When the frequency of the applied electric field matches the pumps' intrinsic characteristic frequency(s) or the electric field resonates or is in turn with the pump's characteristics frequency, the pump function can be activated. In contrast, in this study, we do not assume the existence of an intrinsic characteristic frequency of the pump molecules. Instead, the study is based on experimental results that the turnover rates of the pumps are adjustable.

Secondly, in those studies, the characteristic frequency was implied in a range of kilo to megahertz. Therefore, the target of the electric field is most likely not the entire pump molecules, because the pumps' turnover rate (in tens of Hz) is not in that range. Such high frequency electric field may transduce signal to some specific domain(s) of the proteins, functioning on a particular event of the proteins' conformational change. In contrast, the low frequency electric field which is comparable to the pumps' turnover rate most likely functions on the conformational change of entire protein, facilitating its pumping rate.

Thirdly, indeed, the previous studies also implied synchronization of the pump molecules to the applied oscillating electric field. However, the mechanisms involved in those synchronizations are different from that involved in this study. In the previous studies, based on the assumption of existence of intrinsic or characteristic frequency of pump molecules, an ac electric field only with a frequency specifically matching, or in tune with, this frequency can facilitate the proteins' conformational changes. Here, we consider each individual pump running at different pumping rates and random phases, and an ac electric field is used to change them until working on the same pace as the field. The former is a phenomenon of resonance. As long as reaching resonance, the protein can absorb energy from the oscillating electric field. The latter is not. In fact, in our study pump synchronization to the oscillating electric field does not facilitate the pumping rate. It is only the preparation step for modulation. The underlying mechanism involved in this study is an entrainment process by which the pump molecules are trained to run faster and faster, even through the word "train" may not be proper because proteins do not have memory.

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