J Bioenerg Biomembr (2008) 40:347–357
DOI 10.1007/s10863-008-9150-1

Synchronization of Na/K pump molecules by an oscillating electric field

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Received: 2 August 2007 / Accepted: 28 June 2008 / Published online: 2 August 2008

Abstract Synchronization of the Na/K pump molecules in a cell membrane was studied in frog skeletal muscle fibers using double Vaseline-gap voltage-clamp techniques. We found that the pumping rate of naturally random-paced pump molecules can be artificially synchronized by a pulsed, symmetric, oscillating membrane potential with a frequency comparable to the physiological turnover rate. The synchronized pump currents show separated outward and inward components, where the magnitude of the outward component is about three times the randomly-paced pump currents, and the magnitude-ratio of the outward to inward pump currents is close to 3:2, which reflects the stoichiometric ratio of the pump molecules. Once synchronized, the pumping rate is restricted to the field frequency, and the pump currents are mainly dependent on the field frequency, but not the field strength. In contrast to previous work, which by restraining the pumps at a presteady state succeeded in triggering the steps of the pump cycle only individually and between interruptions, here we synchronize the pumps running continuously and in a normal running mode.

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

Introduction

The concept of synchronization has been previously used to explain physiological phenomena in biological systems, such as the contraction and relaxation of heart muscle cells and the generation of epilepsy, which generally represent a simultaneous stimulation of the cells resulting in channels opening at the same time.

Unlike ion channels which mainly have two states, open or closed, many electrogenic pumps, such as the Na/K pumps, are often envisioned as a loop consisting of many steps (Albers 1967; Post et al. 1972; Apell and Karlish 2001; Hilgemann 1994). Most voltage-gated ion channels are in the closed state at the membrane resting potential and switch to the open state after responding to a stimulation potential. The Na/K pumps keep running at all physiological membrane potentials, as the pump's equilibrium potential is far more negative than the membrane resting potential (Gadsby et al. 1985; Gadsby and Nakao 1989; Rakowski et al. 1997). Pumping rate is often used to describe the speed of the pumping loop.

Gadsby and Nakao (Gadsby and Nakao 1989; Rakowski et al. 1997) estimated the turnover rate of around 50 Hz in membrane potential depolarization. Based on thermodynamic principles, the turnover rates of pump molecules should follow some kind of statistical distribution. The estimated turnover rate is most likely an averaged value for all of the pumps in the study. We now use another term, turnover phase, which seldom appears in literature, to represent the pace of stepping in the loop, in relation to other pump molecules. Due to their structural independence, it is reasonable to assume that the pumps' turnover phases are randomly distributed.

Microscopically, current generated by each Na/K pump should include two alternately appearing components: outward Na and inward K pump currents. However, the inward K pump current cannot be distinguished from the outward Na current in routine measurements due to random paces. In all experimental measured pump currents only a

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net outward current can be observed. Based on these complications, it is much more difficult to synchronize the pump molecules than the ion channels. Elegant work has been done to simultaneously trigger a specific step in an interrupted loop (Apell and Bersch 1987; Bamberg et al. 1993; Sokolov et al. 1998; Holmgren et al. 2000; Forbush 1987). In these studies, the pump loop was purposely interrupted by various chemicals in order to force the pump molecules to stay at the same specific pumping state right before the ion-transports. Then, either an optical signal or an electrical stimulation was used to trigger the corresponding ion-transport, simultaneously, across all the pump molecules in order to measure the transient pump currents. These can be considered as the synchronization of a specific transient pump current in an interrupted mode. Synchronization of the Na/K pump molecules in a physiological running mode has been seldomly reported.

In the previous study, we showed synchronization of the Na/K pump molecules in a physiological running mode by using a train of DC pulses (Chen and Zhang 2006). In this paper, we further study synchronization of the pump molecules by an AC electric field. We employed voltage-clamp techniques to alternate the membrane potential of skeletal muscle fibers and monitor the corresponding changes in the pump currents. We found that the pumping loop of the Na/K pump molecules can be synchronized by a well designed oscillating electric field. The synchronized pump molecules clearly showed separated outward and inward components of the pump currents in an alternating pattern. The magnitude of the outward currents was observed to be three times higher than that of the randomly paced pump currents. The magnitude ratio of the outward to inward pump currents was close to 3:2 which is shown to reflect the Na/K pumps' stoichiometric ratio.

Experimental methodology

A double Vaseline gap voltage clamp was used to measure the pump currents in frog skeletal muscle fibers. This technique was developed by Hille and Campbell (1976) and has been used in many labs including ours to study charge movement currents (Adrian et al. 1976; Kovacs et al. 1983: Irving et al. 1987; Hui and Chen 1992; Chen 2004; Chen and Wu 2002). Single skeletal muscle fibers were hand dissected under a microscope from twitch muscles, *semitendonosis* of *Rana pippiens* frogs, and mounted into a custom-made chamber. The procedure for dissecting and mounting cut fibers in a double Vaseline-gap chamber has been described previously (Chen and Zhang 2006; Chen 2004). The fibers were electrically and ionically separated by two Vaseline partitions into three segments: end pool one, central pool, and end pool two. The dimensions of the partitions and the central pool were 100 μm and 300 μm, respectively. The fiber segments at the two end pools were treated with 0.01% saponin for 2 min 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. In addition to the studies of charge movement currents, we recently used this technique to measure the Na/K pump currents in the skeletal muscle fibers and to study their voltage dependence (Chen and Zhang 2006; Chen and Wu 2002).

The compositions of internal and external solutions follow the recipes used in other labs and ours in the study of Na/K pump currents. We also followed Gadsby's work (Gadsby et al. 1985) and adjusted the concentrations of Na and K in the external and internal solutions in order to increase the pump currents. The solution compositions were as follows:

> Internal solution (mM): Na-glutamate, 40; K-glutamate, 22.5; $MgSO_4$, 6.8; Cs_2–EGTA, 20; Cs_2–PIPES, 5; $Tris_2$–Creatinephosphate, 5; and Na_2–ATP, 5.5.
> External solution (mM): TEA–Cl, 22.5; $CsCl_2$, 20; NaCl, 50; 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.

TTX and 3, 4-DAP were used to block Na and K channel currents. All experiments were performed at room temperature, 24 °C. Previous study showed that TEA had some effects on the Na/K pumps. We found that in skeletal muscle fibers 22.5 mM TEA–Cl did not significantly reduce the pump currents (Chen and Zhang 2006; Chen and Wu 2002).

The Na/K pump currents have been widely studied in cardiac cells (Gadsby et al. 1985; Gadsby and Nakao 1989; Nakao and Gadsby 1989; Glitsch 2001) and oocytes (Schweigert et al. 1988). Very few studies have been reported studying the pump currents in skeletal muscle fibers. That is probably because of the fiber's large size which is not suitable for the microelectrode patch clamp. On the other hand, the double Vaseline-gap voltage clamp has been well developed to successfully study the intramembrane charge movement currents in skeletal muscle fiber for decades (Hille and Campbell 1976; Adrian et al. 1976; Kovacs et al. 1983; Irving et al. 1987; Hui and Chen 1992). The order of magnitude of the charge-movement currents is comparable to, or even smaller than, that of the Na/K pump currents. In addition, due to the small series resistance in the clamp pathway, less than 1 kΩ compared to megaohms resistance in the microelectrode, we are able to transiently change the membrane potential such as in an

alternating field, which is an advantage over the microelectrode patch clamp technique. However, it is impossible to get a gigohm seal, and therefore, the leakage current is relative large. The p/4 method has been widely used to remove the leakage currents in the study of intramembrane charge movement currents.

We measured the Na/K pump currents in skeletal muscle fibers using the double Vaseline-gap voltage clamp techniques and the p/4 method to remove the leakage currents. In all experiments, the membrane potential was held at −90 mV, the membrane resting potential of skeletal muscle fibers. The membrane potential was first hyperpolarized to −110 mV followed by four negative p/4 pulses whose waveforms are identical to, but the magnitudes are one fourth of, the following corresponding stimulation pulses. The currents generated by the hyperpolarization p/4 pulses are mainly the membrane leakage current and the leakage current through the Vaseline seals. Those leakage currents are added up and then subtracted from the currents elicited by the following full strength stimulation pulse.

In this study, we employed several stimulation pulse-trains. In all of these trains there were two parts to the pulses, the first parts are the p/4 pulses, and the second parts are pulse-trains. The pulse-train consists of a number of synchronization pre-pulses followed by four data acquisition pulses. Only the currents elicited by the data-acquisition pulses were recorded and resolved into pump currents. All of the pulses in each train were identical except when explicitly pointed out in figures. All of these pulses are symmetrical, having the same magnitude, alternating the membrane potential from −30 to −150 mV. The pulse-duration for each stimulation train is marked separately. The name of the train is defined by the number of synchronization pre-pulses. Train T0 is a control only having a few data-acquisition pulses without synchronization pre-pulses. Synchronization train T100 has 100 oscillating pre-pulses followed by the data-acquisition pulses.

The protocol of the experiments was as follows: the control train, T0, was always applied first to the cell membrane five times over, then, the synchronization pulse-train, T100, was applied five times over. The time intervals between the train T100 applications were always 30 s. Our experimental results showed that 30 s is more than enough for the synchronized pump molecules to return to a random pace. The external solution was then changed to the external solution with ouabain, a specific inhibitor of the Na/K pumps. Then, the same procedure was reapplied to the cells.

In data analysis, after digitally eliminating the leakage currents, the currents in the presence of ouabain were subtracted from those in the absence of ouabain, yielding the ouabain-sensitive currents, or the Na/K pump currents. The final pump currents were averaged from five repeated stimulations.

Experimental results

Figure 1 shows the ouabain-sensitive currents, or the Na/K pump currents elicited by a single 30 ms DC pulse depolarizing the membrane potential to −30 mV. The pump currents show only an outward current. The transient currents in response to the rising and falling phases of the pulse which are also shown in later figures were due to imperfect matching in p/4 subtraction. This result is consistent with those obtained from other labs using the microelectrode patch clamp techniques (Gadsby et al. 1985; Gadsby and Nakao 1989; Nakao and Gadsby 1989; Glitsch 2001; Schweigert et al. 1988).

Figure 2 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 is very similar to those obtained from other preparations, such as cardiac cells (Gadsby et al. 1985, Gadsby and Nakao 1989, Nakao and Gadsby 1989; Glitsch 2001), nerve cells (Rakowski et al. 1989), and Xenopus oocytes (Schweigert et al. 1988).

The only difference is that the pump current obtained using a microelectrode is the absolute value of the pump currents. Here, because of using p/4 pulse subtraction, the currents we measured were the pump currents relative to that at the membrane potential of −110 mV. Therefore, in this *I–V* curve, the pump current at the membrane potential of −110 mV is zero.

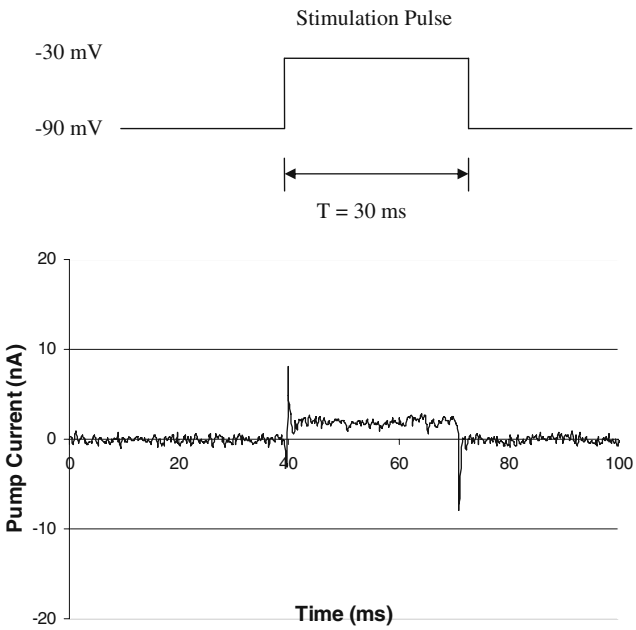

Fig. 1 *Upper panel*: a single stimulation pulse used to elicit the pump currents. *Lower panel*: The ouabain-sensitive currents or the Na/K pump currents. The transient charge- and discharge-currents responding to the rising and falling phases of the pulse are due to imperfect matching during p/4 subtraction. Similar results are shown in the following figures

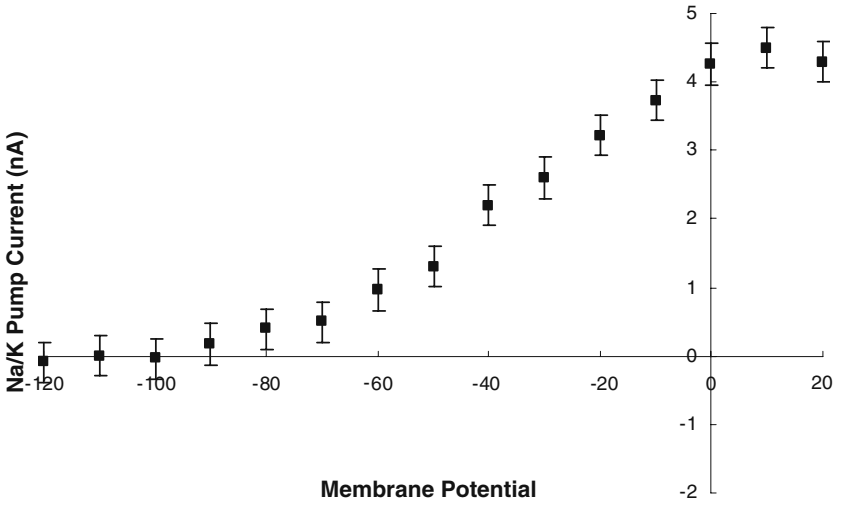

Fig. 2 Na/K pump currents as a function of the membrane potential. Seven experiments were conducted. The *bars* represent the standard deviation. Because we used the p/4 method, the pump current presented here is the relative pump current with respect to that at the membrane holding potential of −110 mV. Therefore, the pump current at −110 mV is zero

The curve exhibits a sigmoidal shape and the slope gradually increases as the membrane potential is depolarized. As 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 decreases. In contrast, the slope becomes very shallow in the hyperpolarization potential range and the generated pump currents are very small.

We first compared the effects on the Na/K pump currents by the stimulation trains, T0 without, and T100 with, 100 pre-pulses. The middle panel of Fig. 3 shows the pump currents elicited by T0. The pump currents are mainly generated by the positive half-pulses while the negative half-pulses generate very little currents. That is because the pump's *I–V* curve has a very shallow slope at the hyperpolarization region (Gadsby et al. 1985; Gadsby and Nakao 1989; Nakao and Gadsby 1989; Glitsch 2001; Schweigert et al. 1988). The magnitude of the pump currents in response to the positive half-pulse was estimated by averaging the last 20 points of the currents. For this fiber, the current magnitude is 1.5 nA.

The lower panel of Fig. 3 shows the pump current elicited by the synchronization pulse-train, T100, with 100 pre-pulses. The pump current became significantly different from that shown in the middle panel. First, the pump current responding to the positive half-pulse was significantly increased. The magnitude was estimated as 4.3 nA, which is about a three-fold increase from that (1.5 nA) elicited by train T0.

Secondly, in contrast to the mainly positive half-pulse of train-T0-elicited pump currents, the negative half-pulses in train T100 are clearly seen to also generate distinguishable pump currents. The negative half-pulse induced pump current is estimated as 2.5 nA, Interestingly, the magnitude ratio of the pump currents induced by the positive and negative half-pulses (4.3:2.5) is a little higher than 3:2. More than ten experiments have been conducted. The resultant magnitude ratios for all the experiments were a little higher than, but close to 3:2, in a range from 3.3:2 to 3.7:2. The ratio of 3:2 is the stoichiometric ratio of the Na/K pumps.

Figure 4 provides the detailed changes of the pump currents in response to the synchronization pre-pulses within the pulse-train T100 recorded from another fiber. The half-pulse duration was 10 ms. The pump currents shown in the upper panel were elicited by the first 20 synchronization pulses, while those shown in the lower panel were elicited by the last 20 pulses. Initially, the pump currents could mainly be generated by the positive half-pulses and the negative half-pulses elicited very little current. As the membrane potential oscillated, the negative half-pulses generated larger and larger pump currents, and so did the positive half-pulses. Finally, when reaching a steady-state, both currents became saturated, showing a magnitude ratio of 3:2, reflecting the stoichiometric number of the Na/K pumps.

Our working hypothesis is that a continuous oscillation in membrane potential may be able to synchronize the Na/K pump molecules. Once synchronized, all the pump molecules may extrude Na ions in the time period corresponding to the positive half-pulse, and then, pump in K ions in the period during the negative half-pulse. Consequently, the pump currents elicited by the positive half-pulses mainly reflect the outward Na currents, while those generated by the negative half-pulses reveal the inward K currents. The magnitude ratio of the outward currents over inward currents should be coincident with the pump's stoichiometric number, 3:2. Our results showed that the ratio is higher than, but close to, 3:2 which indicates that the majority, but not all, of the pumps are synchronized. In addition, considering the tiny pump currents generated by the negative half-pulse in T0, the ratio may

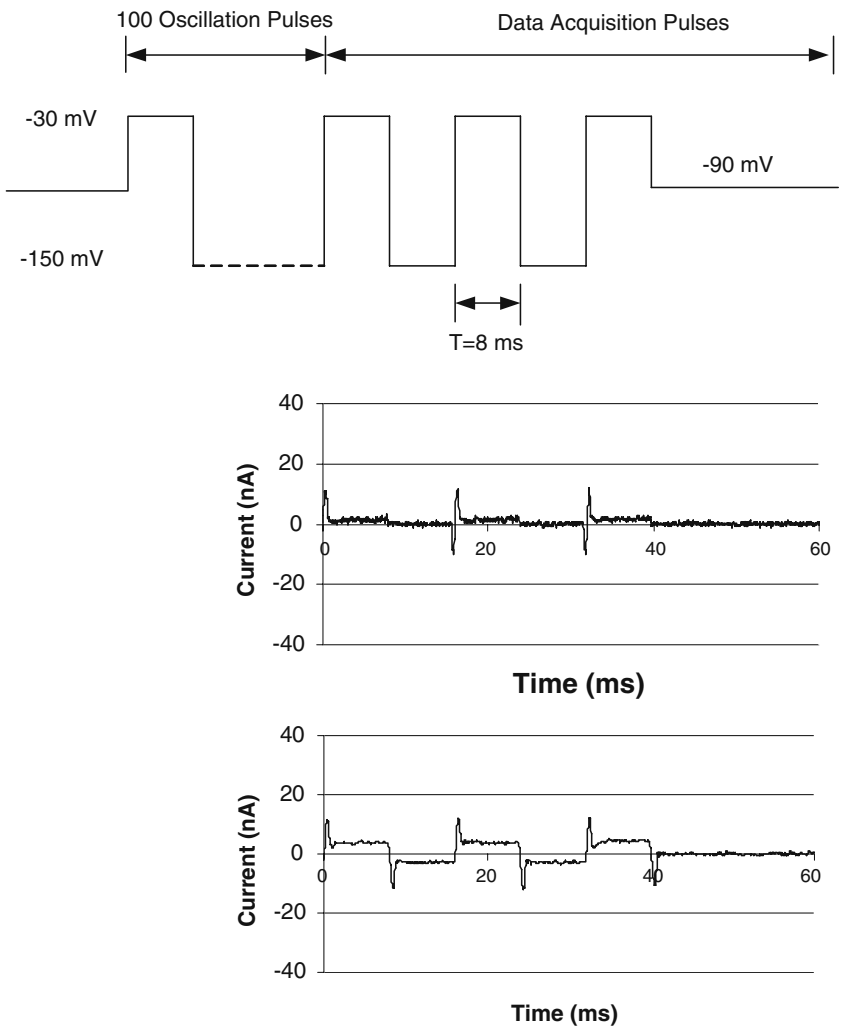

Fig. 3 *Upper panel*: the synchronization pulse-train, T100. There were 100 symmetric oscillating pulses prior to three data acquisition pulses. All of the pulses were the same alternating the membrane potential from −150 to −30 mV. *Middle panel*: pump current elicited by the control train, T0, which is the same as train T100 shown in the upper panel except without the 100 oscillating pre-pulses. The pump current is mainly elicited by the positive half-pulses, while the negative half-pulses generate very little currents. *Lower panel*: Pump currents elicited by the synchronization pulse train, T100, showing alternating outward and inward currents corresponding to the positive and negative half-pulses, respectively. The transient currents corresponding to the polarity change of the pulses are artificial due to imperfect matching of the current traces in subtraction. The lower trace shows less noise than the upper trance. That is because of the much lower data acquisition rate for train T100 then T0, and the lower trace was an average of five traces. The fiber diameters used in this study were in a range from 40 to 60 μm

have an inaccuracy. Because the pump currents generated by the negative half-pulses in train T0 were very small, only about 3% of those generated by train T100, the induced inaccuracy should be very small.

In contrast, for the randomly paced Na/K pumps, the recorded pump currents in response to either a positive or a negative half-pulse are a summation of the outward and inward pump currents from individual pumps. As a result, there can be only a small net outward current.

As the next step, we would like to confirm our working hypothesis of synchronization of the pump molecules. The experimental design was based on the following ideas: let's assume that the pump molecules were synchronized to the pre-pulse oscillation so that all the pumps run at the same pace in response to the oscillating pulses. When the oscillating membrane potential is in the negative half-pulse, all the pumps are forced to pump in K ions. If the oscillation stops and the membrane potential stay at the negative value, the field no longer forces the pumps to the next step extruding Na ions. The pumps lose their synchronization quickly and become randomly paced starting at the time when the field supposes to change its polarity. In other words, the time before the onset of desynchronization is just as long as the half period of the oscillation that just ceased.

We conducted experiments using a modified synchronization pulse-train, which is shown in the upper panel in Fig. 5. All of the stimulation pulses and data acquisition pulses remain the same as the pulse-train, T100, except the membrane potential ends at the negative half-pulse, −150 mV. The half-pulse duration is 6 ms. The elicited pump currents are shown in the lower panel of Fig. 5. Before the membrane potential was ended at −150 mV, the pump currents had shown the separated inward and outward pump currents, and roughly a 3:2 magnitude ratio. When the membrane potential was ended at the negative half-pulse of −150 mV, the inward pump currents remained the same magnitude as that elicited by the previous negative half-pulses. Interestingly, about 6 ms after cessation of the oscillation (pointed out with an arrow), which is the half-pulse duration, the inward pump current started an exponential-like decay. This decay in the inward pump

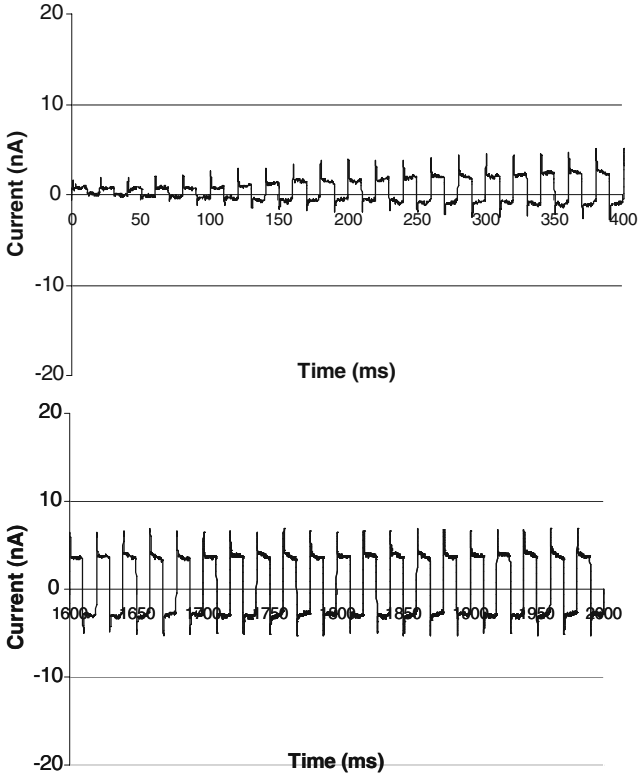

Fig. 4 The Na/K pump currents elicited by the synchronization pre-pulses in the pulse-train, T100, which was the same as that used in the above figure except that the half-pulse duration was 10 ms. *Upper panel:* the pump currents recorded from the first 20 pulses. The currents elicited by the first a few pulses mainly show outward components in response to the positive half-pulses while the inward components in response to the negative half-pulses are very small. *Lower panel:* the pump currents elicited by the last 20 synchronization pre-pulses. When it reaches a steady-state, the magnitude ratio of the outward over inward pump currents is close to 3:2, the stoichiometric ratio of the Na/K pumps

current signifies the pump molecules return to a random pumping pace. The maintenance of the inward pump current for exactly another half-cycle further suggests that the pump molecules had been synchronized before the ending of the membrane potential oscillation.

In addition to a proof of synchronization, the exponential-like decay represents the kinetics of de-synchronization. It took many cycle oscillations in the membrane potential to synchronize the pump molecules; it took only tens of milliseconds to return to the random pumping pace.

Figure 5 clearly demonstrates that the time period in maintenance of the inward pump current is 6 ms after the membrane potential changed to the negative value, which is exactly the half-pulse duration, or the half-cycle of the pumping loop. The question we asked ourselves was whether this coincidence is accidental or causal. If this is due to synchronization of the pump molecules, the time to keep the inward current before the exponential decay should be exactly the half-pulse duration of the synchronization pre-pulses, or the half-cycle of the synchronized pumping loop.

Therefore, we repeated the experiments using another modified synchronization pulse-train, as shown in upper panel of Fig. 6. All of the pulses are the same as those shown in Fig. 5 except that the half-pulse duration was increased to 12 ms. Again, the oscillating membrane potential was terminated at the value of negative half-pulse, −150 mV. The elicited pump currents are shown in the lower panel. After ending the oscillation, the inward pump currents were kept for 12 ms before exponential decay. This 12 ms was, again, exactly the duration of the oscillating pre-pulse. Both Figs. 5 and 6 consistently show that after the membrane potential is ended at the negative half-pulse value, the inward pump currents remained for another half-pulse-duration before decreasing to zero. These results provide strong evidence that the pump molecules had been synchronized by the oscillating pulses.

We further conducted another group of experiments to verify the synchronization of the pump molecules. Let's assume that the pump molecules are synchronized by the oscillating pre-pulses so that the pumps' turnover rates are restricted to the pulse-frequency. If that is the case, when only increasing the pulse-magnitude but remaining at the oscillating frequency, there should be no change at all in the inward pump currents regardless of the magnitude change. This occurs because the stoichiometric numbers of the Na/K pumps remain constants in a wide range of the membrane potentials. Therefore, the magnitude of the pump currents mainly depends on the pumping rate.

The modified synchronization pulse-train is shown in the upper panel of Fig. 7, which is the same as the original train except for the data acquisition pulses. All of the pulses including the oscillating pre-pulses and the four data-acquisition pulses have the same waveform and half-pulse duration of 10 ms. As usual, the oscillating pre-pulses and the first two data acquisition pulses alternated the membrane potential from −150 to −30 mV. The magnitude of the following two data-acquisition pulses was increased by 20 mV to alternate the membrane potential from −170 to −10 mV. The measured pump currents are presented in the lower panels. Interestingly, an increment in the pulse-magnitude was not met with a noticeable increase in the inward pump current at all. In contrast, the outward pump current clearly showed some increase.

This result can be explained as follows: based on Boltzmann theory, turnover rates of individual pump molecules should follow some kinds of distribution. An oscillating electric field with a fixed frequency cannot to synchronize all of the pumps. Our theoretical analysis and computer simulation have consistently showed that an oscillating electric field can mainly synchronize the pump molecules whose pumping rates are comparable to the

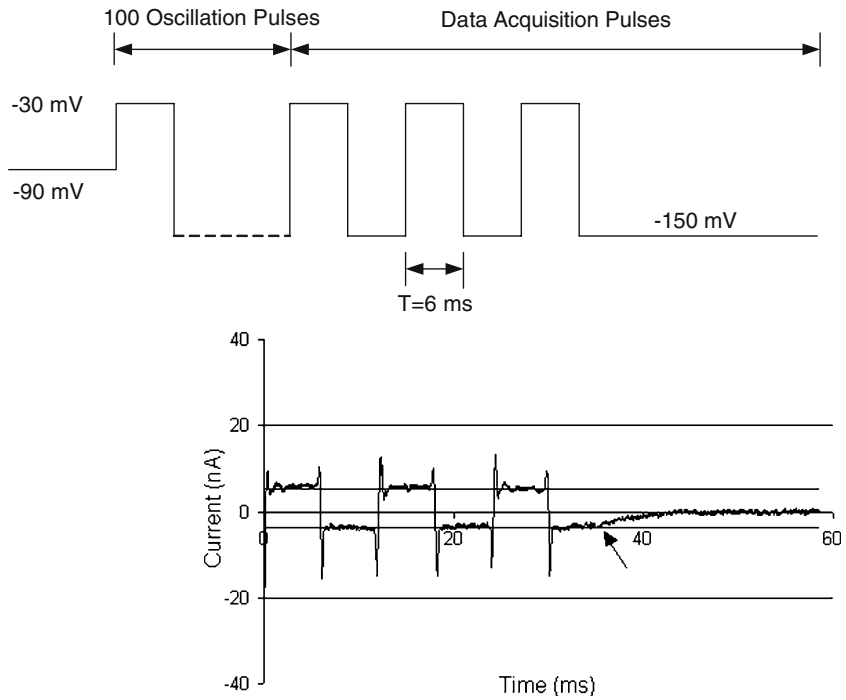

Fig. 5 *Upper panel*: a modified synchronization pulse-train T100. The oscillating membrane potential was terminated at −150 mV, the value of the negative half-pulse. The half-pulse duration was 6 ms. *Lower panel*: the elicited pump currents. The inward pump currents remained for 6 ms, the duration of the pre-pulses, and then, exponentially decayed to zero starting at the time pointed by an *arrow*

field-frequency (Chen 2008; Chen and Huang 2008). Even though we purposely selected the field frequency of 50 Hz comparable to the pumps' natural turnover rates, there must be some pumps still random paced. The pump currents we measured were a sum of both synchronized and unsynchronized pump currents.

In reference to the pump currents responding to the negative half-pulses, the inward currents were mainly contributed to by the synchronized pump molecules. It can be seen in the middle panel of Fig. 3 that the randomly paced pumps generated very little inward pump currents. This is understandable because a negative pulse of −150 mV can elicit very little pump current for the randomly paced pumps. The pump currents elicited by the negative half-pulse have to be mainly the synchronized inward pump currents. The same inward pump current

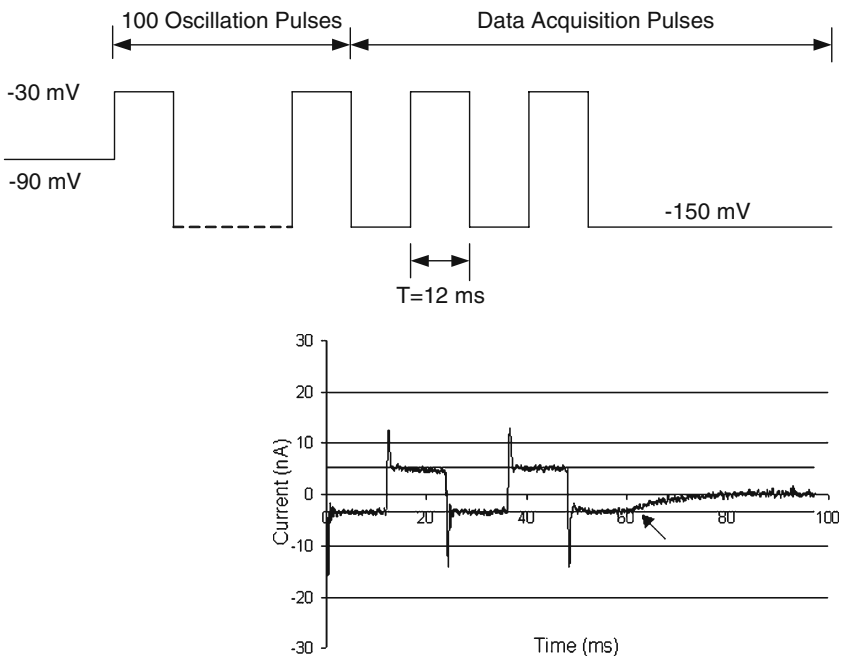

Fig. 6 *Upper panel*: another modified synchronization pulse-train T100 with half-pulse duration of 12 ms. Again, The oscillating membrane potential was terminated at −150 mV, the value of the negative half-pulse. *Lower panel*: the inward pump currents remained for another half-pulse duration prior to an exponential decay

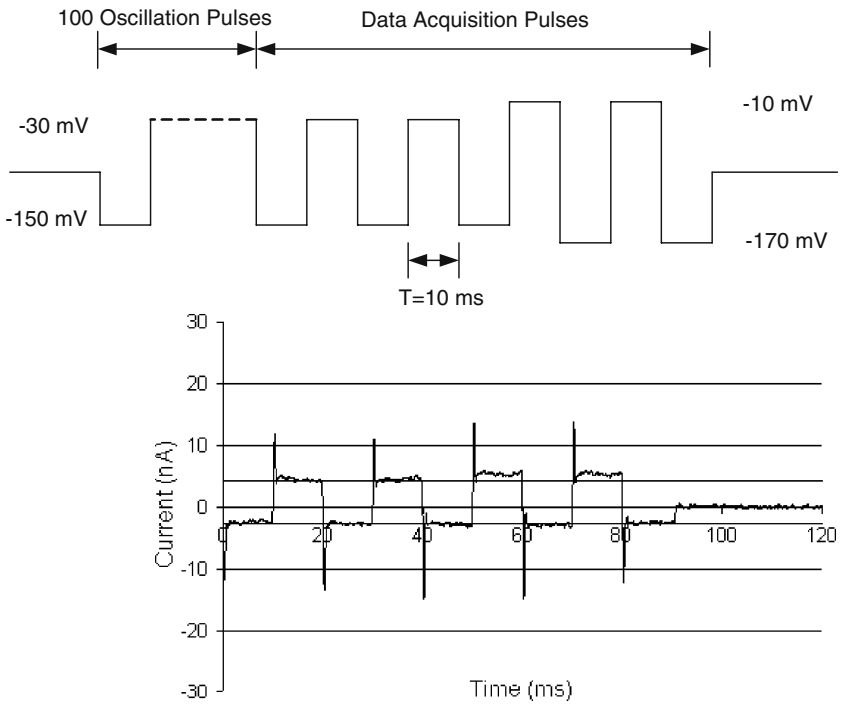

Fig. 7 The synchronized pump currents depend only on the synchronization frequency. *Upper panel*: a modified synchronization pulse-train T100. The first two data acquisition pulses were the same as the oscillating pre-pulses. The second two data acquisition pulses have an increased magnitude but remain the same half-pulse duration. *Lower panel*: the elicited pump currents. The outward pump currents showed some increment, but the inward currents had very little change in response to the increase in the pulse magnitude

regardless of the increase in pulse magnitude indicates that the synchronized pump currents are independent of the pulse magnitude if the field frequency remains the same.

In contrast, the outward pump currents show a noticeable increase. That is because the outward pump currents are contributed to by both synchronized and unsynchronized pump currents. Even though the synchronized pump currents might remain the same, the unsynchronized pump currents were certainly increased because of their voltage-dependence (Fig. 2). Therefore, the total outward pump currents were increased. The results shown in Fig. 7 that the increase in the magnitude of the second two data-acquisition pulses only increased the outward pump currents, but had no, or very little, effect on the inward pump currents indicate that the pump molecules had been synchronized by the oscillating membrane potential.

Discussion

Potential mechanisms involved in synchronization of the pump molecules

We studied an oscillating electric field-induced change in the Na/K pump currents. The Na/K pump currents or the ouabain-sensitive currents were identified by subtracting the currents in the presence from those in the absence of ouabain, a specific inhibitor of the Na/K pumps, with ion channel currents maximally blocked. The pump currents were fully abolished by ouabain. After a number of oscillations in the membrane potential, the pump currents gradually changed from unidirectional outward currents to the separated outward and inward pump currents. When the pump currents reached a steady-state, the ratio of the outward to inward pump currents showed 3:2, reflecting the stoichiometric number of the Na/K pumps. The pump currents were then no longer sensitive to the membrane potential as long as the field frequency remained the same. All these features were eliminated in tens of millisecond after the membrane potential oscillation was stopped.

Based on these results, our working hypothesis is that the pump currents were synchronized by the oscillating electric field. The experimental magnitude ratio is a little larger than, but close to, 3:2, the stoichiometric ratio. The discrepancy arises because not all pumps are synchronized. The unsynchronized pumps always contribute more to the positive current than the negative currents. In addition, the inward pump currents may be underestimated by the p/4 method.

In terms of time-courses of synchronization and desynchronization, it takes many oscillating cycles to synchronize the pumps, but once the field is removed the synchronized pumps quickly become randomly paced in tens of ms. The significant difference in the time-courses can be explained as follows: pump synchronization is a process in which individual pumps are forced to run at the same pace. Entropy of the system is reduced, which is not a natural process. The significant effort requires external energy. The electric field provides the needed energy alternatively influencing the voltage-dependent steps in

the two transport limbs. Because the voltage-dependent steps are not the rate-limiting step in the pumping loop, it may take many oscillating cycles to force the individual pumps finally to run at the same pace as the field oscillation. In contrast, desynchronization is a natural process increasing the entropy of the system, which happens spontaneously without any external effort.

Theoretical studies of the potential mechanisms involved in synchronization of the carrier-mediated ion-exchangers and computational simulation has been reported separately (Chen 2008; Chen and Huang 2008). Here, we only briefly describe the potential concepts involved in Na/K pump synchronization.

Synchronization of pumping loop to an oscillating electric field can be explained in terms of energy. The electric field at two half-cycles causes the energy barrier to be significantly different for the two transports. For example, for skeletal muscle fibers, the Nerstian equilibrium potential of Na and K are 60 and −90 mV, respectively, intracellular with respect to the extracellular fluid (Robertson and Astumian 1991). During the negative half-pulse of −150 mV, extrusion of three Na ions requires 3×60=180 meV to overcome the ionic concentration gradient and 3×150=450 meV to overcome the electric potential. To complement the transport, energy of at least 180+450=630 meV is needed. Similarly, during the positive half-pulse of −30 mV, the energy barrier for pumping in two K ions over the electrochemical potential is 2×(90−30)=120 meV. Therefore, when the Na-extrusion falls into the negative half-pulse and K-influx into the positive half-pulse, the two hindering half-pulses, the energy needed to overcome the electrochemical potential is 630+120=750 meV. Considering the protein's conformational change, more energy may be needed. However, hydrolysis energy from a single ATP is only about 550 meV (Weiss 1996; Domaszemicz and Apell 1999). Clearly, the pumping loop is inhibited. In contrast, the electric field at a positive half-pulse reduces the energy barrier for the Na-extrusion to 3×(60+30)=270 meV, which is smaller than the ATP hydrolysis energy of 550 meV. Therefore, Na-extrusion favors the positive half-cycle.

In the pumping loop, the ATP binding and hydrolysis are located in the Na extrusion limb. The majority of the ATP hydrolysis energy is consumed by the Na-extrusion and proteins' conformational change. The K-pumping in step is mainly determined by the membrane potential. The electric field at the negative half-pulse reduces the K-influx energy barrier to a negative value of 2×(90−150)=−120 meV. Due to the energy barrier of +120 mV in the positive half-pulse the K-transport favors the negative half-pulses. If only one transport falls into the hindering half-pulse, the pumping loop continues to run but the time-course of the hindered transport will be significantly increased.

Therefore, initially, the individual pumps may run at different pumping rates and random phases. When they reach a steady state, the pumps will extrude Na ions during the positive half-pulse and then pump in K ions during the negative half-pulse. In other words, the pumping loops will eventually match the pace of the field's oscillation.

Pump currents within the corresponding half-pulses

One argument is why the synchronized pump currents show relatively equal distribution within each half-pulse. In contrast, in the voltage-jump experiments on interrupted pumping loops, the pump currents showed initial maximum with exponential decay having a time-course less than 1 ms. In these studies, all the pumps were triggered simultaneously from a pre-setup state to generate pump currents from a specific step in one ion-transport (Apell and Bersch 1987; Bamberg et al. 1993; Sokolov et al. 1998; Holmgren et al. 2000; Forbush 1987).

In this study the pumps are in a physiological running mode. The pumping loop for each ion-transport consists of a sequence of reaction steps of ion binding, occlusion, protein conformational change, deocclusion, and ion unbinding (Apell and Karlish 2001; Hilgemann1994; Domaszemicz and Apell 1999; Apell 2003; Jorgensen and Pedersen 2001). The electrogenic steps are mainly related to ion binding and unbinding. Based on these findings, a single pump current can be expressed as two outward current-pulses followed by two inward current-pulses. The outward currents represent Na ions transport out the cell and the two current-pulses correspond to the Na-binding and unbinding steps, respectively. Similarly, the two inward current-pulses stand for the binding and unbinding steps of K ions. Under different membrane potentials, durations of these current-pulses will be changed significantly.

When synchronized, both binding and unbinding Na current-pulses fall into the field's positive half-pulse and both binding and unbinding K current-pulses into the negative half-pulse. Even though the binding current-pulse may be stuck to the rising-phase of the half-pulses, the unbinding current will certainly not.

In addition, in the voltage-jump experiments, the pump currents from all the pumps were triggered simultaneously. In physiological running mode, all the pump molecules initially run at random pumping phase. The Na-outward and K-inward currents from individual pumps may be trapped into the positive and negative half-pulses, respectively, but they may remain in different phases.

Moreover, the individual pumps may not be running at the same pumping rate. Environment fluctuation such as the channel current-induced local changes in ionic concentration and temperature may affect the pumps nearby. In addition, due to the series resistance in the current injection

pathway, the membrane potential for individual pumps may not be the same, which will result in different pump currents. Because the stoichiometric numbers of the Na/K pumps remain constants for a wide range of membrane potentials (Rakowski et al. 1989; DeWeer et al. 1988), different pump currents mean different pumping rates, or different durations of the binding and unbinding current bars, and their time-interval. This will further smooth the distribution of the pump currents within the corresponding half-pulses. In addition, we do not expect that all the pump molecules can be synchronized by a single oscillating electric field, especially for those turnover rates far away from the field frequency. Those unsynchronized pump currents make the total currents more evenly distributed in the half-pulses.

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

Tsong and Astumian studied absorption and conversion of electric field energy by membrane bound ATPases (Tsong and Astumian 1986; Robertson and Astumian 1991; Markin et al. 1992) and elegantly elaborated a process of energy transduction between an oscillating field and the enzyme. The underlying mechanisms involved in these previous studies have some fundamental differences from those that are involved in this paper, including the assumptions, approaches, and the targets in the protein's structures.

In those studies, a fundamental assumption is the existence of an optimal frequency window(s) for the pump molecules. When the frequency of an applied electric field matches the optimal frequency window(s), the electrical energy is absorbed by the pumps, and the pump functions are activated. In contrast, this study is based on the experimental results that the pump's turnover rates are adjustable without the assumption of optimal frequency window(s). We show that under the influence of a special designed oscillating electric field, different turnover rates of individual pumps can be entrained to match the field oscillating frequency. Synchronization only means that all the pumps are running at the same pace as the oscillating field, which does not indicate any activation of the pump functions. Figure 7 shows that increasing the magnitude of the synchronization field can not increase the pump functions at all.

Secondly, we have further shown that once synchronized, the pump molecules can be modulated to higher and higher pumping rate (Chen et al. 2007) if one gradually increases the field's synchronization frequency. The applied oscillating electric field has to be specially designed with a changing frequency dynamically. This approach gradually entrains the pump molecules in order to activate their pump functions, which is different from the optimal frequency window model where the oscillating electric field has fixed frequency(s) falling in the window(s).

Thirdly, the previous studies implied that the optimal frequency window(s) is in the range of kilo and mega-Hz, which may not reflect the functions of the entire pump molecule because the pump's turnover rate is only at about 50 Hz. Therefore, the target of the optimal frequency approach may focus on some specific domain(s) of the pump molecule or some step(s) or sub-step(s) in the pumping loop. In contrast, the synchronization frequency we used is about 50 Hz, comparable to the pumps' natural turnover rates, and their turnover rates can be modulated by gradually changing the synchronization frequency (Chen and Huang 2008). All of this indicates that this approach is likely focused on the entire protein.

Acknowledgment The authors wish to thank Drs. T. Y. Tsong and L. Horn for valuable comments and suggestions. We also own thanks to Dr. D. Robson for his instructive discussion and reading the paper. This study is partially supported by the research grants from National Institute of Health (NIH), 2R01 NIGM50785 and National Science Foundation (NSF) PHY 0515787.

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