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# Membrane Potential Hyperpolarization in Mammalian Cardiac Cells by Synchronization Modulation of Na/K Pumps

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**Abstract** In previously reported work, we developed a new technique, synchronization modulation, to electrically activate Na/K pump molecules. The fundamental mechanism involved in this technique is a dynamic entrainment procedure of the pump molecules, carried out in a stepwise pattern. The entrainment procedure consists of two steps: synchronization and modulation. We theoretically predicted that the pump functions can be activated exponentially as a function of the membrane potential. We have experimentally demonstrated synchronization of the Na/K pump molecules and acceleration of their pumping rates by many fold through use of voltage-clamp techniques, directly monitoring the pump currents. We further applied this technique to intact skeletal muscle fibers from amphibians and found significant effects on the membrane resting potential. Here, we extend our study to intact mammalian cardiomyocytes. We employed a noninvasive confocal microscopic fluorescent imaging technique to monitor electric field–induced changes in ionic concentration gradient and membrane resting potential. Our results further confirm that the well-designed synchronization modulation electric field can effectively accelerate the Na/K pumping rate, increasing the ionic concentration gradient across the cell membrane and hyperpolarizing the membrane resting potential.

**Keywords** Na/K-ATPase · Electric field · Synchronization · Modulation · Cardiomyocyte

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## Introduction

The Na/K-ATPase pump molecule is one of the most prevalent housekeeping proteins found within the cell membrane. It famously extrudes three Na ions out of the cell via the exchange of two K ions and consumption of one ATP in each pumping cycle. The ionic concentration gradients generated by the Na/K pumps are critical to many cellular functions, including membrane potential maintenance, signal generation, energy supply and homeostasis. In many diseases or in a physiological emergency, dysfunction of the Na/K pumps is due to either lack of ATP or the low density of the pump proteins within the cell membrane (Clausen, 1998; Rose & Valdes, 1994). Physical manipulation of the pump molecules has become a central target for therapeutic purposes.

The function of Na/K pumps is sensitive to membrane potential. Membrane potential depolarization has been shown in many cases to activate pump functions. However, due to the pump's sigmoid-shaped *I-V* curve, with a shallow slope and saturation behavior (Apell, 2003; Chen & Wu, 2002; De Weer, Gadsby & Rakowski, 1988; Nakao & Gadsby, 1989; Pedemonte, 1988; Rakowski, Gadsby & DeWeer, 1997), a membrane potential depolarization cannot significantly increase the pump currents. The underlying mechanisms have been theoretically discussed previously (Chen, 2006).

In addition, even though membrane potential depolarization can increase the pump currents, it cannot be used in practice. Membrane potential depolarization can only be realized in labs when an electric field is directly applied to the cell membrane. In a real situation, when intact cells are exposed to a DC external electric field, the field-induced membrane potential on the two hemispheres is always of opposing polarity. If the field depolarizes the membrane

potential on one hemisphere, increasing the pump functions, the field must hyperpolarize the membrane on the other hemisphere, decreasing the pump functions at this half of the cell. As a result, the field-induced effects on the pump molecules cancel each other.

In order to activate pump functions, oscillating electric fields have been considered for years. Pioneering work by Teissie & Tsong (1980) used a megahertz AC electric field to activate the Na/K pump molecules in erythrocytes. AC current has been reported to either stimulate or inhibit ATP hydrolysis activity of the enzymes, depending on the ratio of Na and K ions (Blank & Soo, 1989). Later, a resonance-frequency-window model was developed to predict the possible mechanisms involved in electric activation of the pumps (Markin et al., 1992). Detailed information, such as the locations, widths and numbers of these frequency windows, were not provided. Other models include the brownian motion model (Astumian, 1997; Tsong, 2002) and the adiabatic-pump model (Astumian, 2003). Most of the studies are on theoretical modeling. No experimental results have been reported. Meanwhile, Blank & Soo (1996, 2001) have studied the effects of AC magnetic fields on enzyme functions. They found that the pump functions can be activated by an AC electromagnetic field. The underlying mechanism has been postulated as interaction of the electromagnetic field with electrons (Blank & Soo, 2005). Meanwhile, studies also showed that an acute stimulation, such as excitation stimulation, can activate the function of the Na/K pump molecules (Clausen, 2003). A temporary change in membrane potential due to opening of ion channels or other physiological processes will accelerate the Na/K pump rate in order to restore the membrane resting potential.

We recently developed an entirely new technique to electrically activate the Na/K pumps, the underlying mechanism of which is fundamentally different from the above techniques. We consider that activation of pump function using our technique is a dynamic entrainment procedure of the pump molecules using an oscillating electric field. The procedure consists of two steps: synchronization and modulation. First, we apply an oscillating electric field with a frequency comparable to the pumps' natural turnover rate to synchronize the molecule's pumping paces. Then, by keeping this pump synchronization while gradually increasing the synchronization frequency, the pump molecules can be entrained to higher pumping rates.

In theory, we have predicted that using this technique the pump functions can be exponentially activated as a function of the membrane potential (Chen, 2006; Chen, unpublished results). We further experimentally demonstrated synchronization of the Na/K pump molecules by directly monitoring the pump currents using voltage-clamp techniques (Chen & Zhang, 2006, 2008). Then, we designed a synchronization modulation electric field and were able to increase the pump currents by many fold (Chen & Dando, 2008a). This technique was applied to intact fibers from frog skeletal muscles. The results showed that this technique could effectively maintain and even hyperpolarize the membrane resting potential (Chen & Dando, 2008b).

Cardiomyocytes have a high density of Na/K pump molecules. The pump molecules are directly related to the functions of the cardiac cells. Here, we present the results of our studies in mammalian cardiac cells. We applied the synchronization modulation electric field to isolated intact bovine cadiomyocytes and noninvasively monitored changes in the ionic concentration gradient across the cell membrane by using a confocal spectrofluorescent imaging technique. Our results showed that the well-designed synchronization modulation electric field could effectively control the ionic concentration gradient and the membrane potential.

## Materials and Methods

### Isolation of Cardiomyocytes

The isolation protocol followed those developed in other labs (Kaminski & Wolin, 1994). Slaughterhouse-derived bovine cardiac tissue was obtained on ice immediately after death, from a local source, with all subsequent isolation procedures taking place at 4°C unless otherwise stated. All fat, epicardial and endocardial tissues were removed from the ventricle tissue, which was then finely cut with a scalpel and enzymatically dissected using a collagenase solution obtained from Sigma (Atlanta, GA). The cells were incubated at 37°C with 5% $CO_2$ for periods of 30, 60 and 45 min, with the collagenase solution centrifuged off at 1,500 rpm and replaced with fresh solution. After the final incubation, the centrifuged pellet was washed several times with Krebs *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES, KH) solution, then passed through a 95-μm nylon sieve, recentrifuged and incubated in cell culture medium in several laminin-coated optical culture dishes.

### Solutions

Solutions were used at the following concentrations (in mM), with reagents purchased from Sigma, unless otherwise stated: KH solution, 118 NaCl, 10 HEPES, 4.7 KCl, 1.5 $CaCl_2$, 1.1 $MgSO_4$, 1.2 $KH_2PO_4$, 5.6 glucose (pH 7.4); collagenase solution, same as KH solution with 5% type I collagenase; experimental solution, same as KH with 1 $\mu$M

tetramethylrhodamine ethyl ester (TMRE), 1 $\mu$M tetrodotoxin (TTX) (Invitrogen, Carlsbad, CA); culture medium, Dulbecco's modified Eagle medium (DMEM) with 15% fetal bovine serum (FBS) and 1% pen/strep (pH 7.4, from Invitrogen).

## Selection of Fluorescent Dye

We employed confocal microscopic imagining techniques to monitor the ionic concentration gradient throughout the fiber diameter and across the cell membrane. The dye selected for this study was TMRE (Fig. 1), initially developed by Waggoner, 1979 (Gross & Loew, 1989; Sims et al., 1974). TMRE will always show fluorescence without binding to other molecules. The lipophilicity of the TMRE results in high permeability through the cell membrane, which allows redistribution of the dye molecules across the cell membrane when the membrane potential changes.

TMRE is a positively charge dye, which will be drawn into the cells due to the negative membrane potential. Therefore, the ratio of the equilibrium distribution of the dye molecules across the cell membrane is governed by the Nernst equation (Waggoner, 1979):

$$V_n = \frac{RT}{z_n F} \ln\left(\frac{c_n^o}{c_n^i}\right) \quad (1)$$

TMRE is a so-called slow dye because it takes time for the dye molecules to diffuse across the cell membrane and to redistribute throughout whole cells. We are interested in pump activation–induced changes in the membrane potential. It takes time for the pump molecules to build up the ionic concentration gradient. A slow dye fits our requirements well.

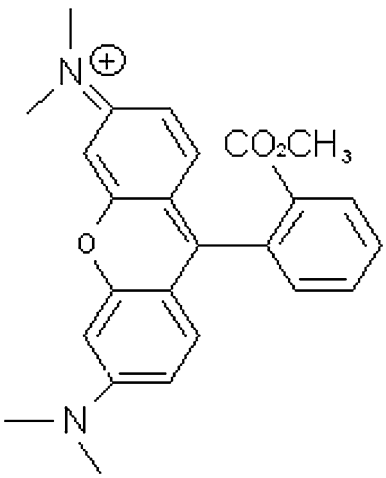


**Fig. 1** Chemical structure of TMRE. The double bond on the upper nitrogen can be thought of as delocalized over the three-ring structure, resonating between the nitrogen bonds, which are covered by hydrophobic methyl groups. This combines with the molecule's ester group to cover the partial positive charge, rendering the dye membrane permeable

Another advantage of TMRE is its high voltage sensitivity. Some fast potential dyes, such as di-4-ANNEPS or di-8-ANNEPS, show approximately only as high as a 10% fluorescent intensity change in response to a 100-m$V$ variation in membrane potential. TMRE shows orders of magnitude higher fluorescence under a similar potential change. Other factors which make this dye an ideal choice for this application are that its spectral properties are independent of environment and that it carries a low rate of phototoxicity (Loew, 1993; Tsien & Waggoner, 1990). Analysis using TMRE is not carried out ratiometrically as the spectral properties of TMRE do not change significantly as a result of factor changes such as pH or, in our case, membrane potential. TMRE was purchased from Invitrogen Corporation (Carlsbad, CA).

## Experimental Procedure

Isolated single cardiomyocytes were transferred to a chamber, which was incubated for a short time at 37°C with 5% $CO_2$, while the laminin acted upon the cells, securing them to the surface of the dish. Subsequently, culture medium was removed from the dishes and replaced with KH solution, and the fiber was mounted on the confocal microscope for background measurement. Background subtraction from both inside the cell and the bathing solution was later calibrated to account for features such as stray light, autofluorescence from the chamber and dark current from the photomultiplier. Next, the solution was changed for that containing the fluorescent dye TMRE. The Na current was removed at this stage using the Na channel blocker TTX (Sigma). The K currents in previous voltage-clamp experiments showed inactivation upon application of a stimulation train such as ours, with a frequency high enough to be considered continuous. Optical culture dishes were examined with transmitted light for viable cardiomyocytes, with subsequent cells placed under a coverslip offering solution depths of less than 100 μm in order to reduce joule heating of the solution. Also, reservoirs of solution were formed outside of the coverslip, to further combat this problem. Ag/AgCl electrodes were placed at each end of the coverslip, 10 mm apart, to provide stimulation via a purpose-built amplifier and a PC running Labview 7.2 (National Instruments, Austin, TX). All of the experiments were performed at room temperature.

Fluorescence images were taken using standard rhodamine optics, employing a green HeNe laser and a fully computer-controlled Olympus IX81 confocal microscope system, with the Fluoview Tiempo analysis package

(Olympus, Tokyo, Japan). Using a x10 dry objective and a confocal aperture of 80 nm, a resolution in the $x$ and $y$ directions of 0.621 μm and a $z$ resolution of 3.09 mm are obtained. Three-dimensional scans were taken every 30 s with the intensity maximum (assumed to eliminate any movement of the cell upon stimulation). The results are extrapolated and subsequently plotted with respect to time.

### Synchronization Modulation Electric Field

The stimulation field consisted of two consecutive pulse trains: synchronization and modulation. The synchronization pulse train was a group of oscillating pulses of 20 Hz. Our previous results showed that an oscillating electric field with a frequency comparable to the pumps' natural turnover rate can synchronize the pump molecules (Chen & Zhang, 2006, 2008). This synchronization pulse train lasted for 10 s, followed by the second pulse train, starting immediately after the first, so as not to lose this synchronization, in which the pulse frequency was raised gradually to 400 Hz in a stepwise pattern. The step of frequency increase was 1% for every 0.1 s. The total time for synchronization and modulation was close to 80 s. All of the pulses had the same magnitude and waveform without any time gap. The field strength was adjusted so that the field-induced membrane potential was about 80 m$V$, peak to peak.

## Results

Figure 2 shows a transmission light microscopic image of bovine cardiomyocytes. After changing to the experimental solution containing the fluorescent dye molecules, the fluorescence intensity inside the cells gradually increased and finally reached a steady state. It took 10–20 min depending on the size of cells to reach this point. Once this steady state was attained, the synchronization modulation electric field was applied to the cells. After 80 s of synchronization modulation, the field frequency remained at 400 Hz until removal of the field. Images were taken every 30 s, scanning from the top to the bottom of the cells. The intracellular fluorescence intensities of individual images were averaged and are plotted as a function of time, shown in Fig. 3. The peaks in the curve represent the slice images that had maximum fluorescence intensities in each scan, which were used to represent the intracellular fluorescence intensity. By this method, any movement of the cell upon stimulation can be eliminated. A period of 60 s before the field application was considered as a control without electric stimulation. At the time marked by the left vertical dotted line in Fig. 3, the

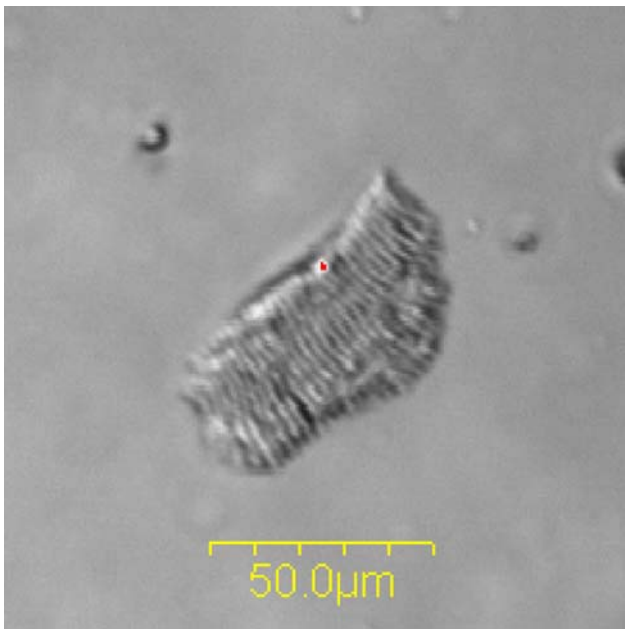


**Fig. 2** A transmission light image of a bovine cardiomyocyte

electric field was applied to the cells until removal, marked by the right vertical line.

Due to the application of the oscillating electric field, after a finite time delay, the intracellular fluorescence intensity gradually and continuously increased, until removal of the field. This result shows that the electric field can increase the number of dye molecules inside the cells. Since TMRE is a positively charged dye, more dye molecules moving into the cells implies a more negative potential inside the cells compared to outside. Therefore, this result indicates that the membrane potential was

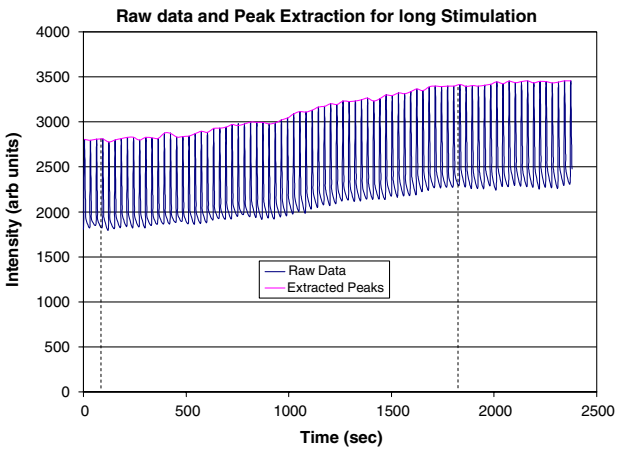


**Fig. 3** Fluorescence intensity from three-dimensional imaging plotted as a function of time. Each data point represents an averaged fluorescence intensity from a sliced image scanned every 30 s in the $z$ direction. There was a 60-s control period before application of the electric field, as marked between the *two vertical dotted lines*

hyperpolarized due to application of the synchronization modulation electric field.

The trace does not show a transient decrease in the fluorescence intensity right after the start of the stimulation as we showed previously in a study of skeletal muscle fibers (Chen & Dando, 2008b). Indeed, the 20-Hz stimulation may open K channels, resulting in a transient reduction in the local K concentration near the cell membrane. However, the fluorescence intensities we measured here were throughout the diameter of each cell, instead of the area in close proximity to the cell membrane. In addition to this, the images were taken every 30 s instead of continuously and, therefore, will not resolve the transient reduction in fluorescence.

The fluorescence intensity initially was initially about 2,790 arbitrary units and in the final situation reached around 3,450 units, showing about a 24% increment. The fluorescence intensity measured in the bathing solution was 900 units. According to equation 1 and considering a background intensity of 780 units, we can estimate the membrane potential before and after the field application to be $-73.5$ and $-80.9$ m$V$, respectively. Application of the synchronization modulation electric field hyperpolarized the membrane potential by 7.4 m$V$, or about 10%.

Eight experiments were conducted. The fluorescence intensities measured in the individual cells were normalized to the corresponding values during the control period and plotted as functions of time. The field-induced increase in the intracellular fluorescence intensity varies in magnitude, but all eight experiments consistently showed increments.

The statistics of the eight traces are shown in Fig. 4. The bars represent the standard deviation. The large deviation is due to variation in cell size on which different membrane potentials were induced by the electric field, in addition to an inherent variation in membrane protein density from fiber to fiber. The average increase in fluorescence intensity after 30 min of field application is about 23%. It is necessary to point out that after field applications, all experiments showed hyperpolarization of the membrane resting potential, which is not simply restoration of the lost membrane potential due to channel opening.

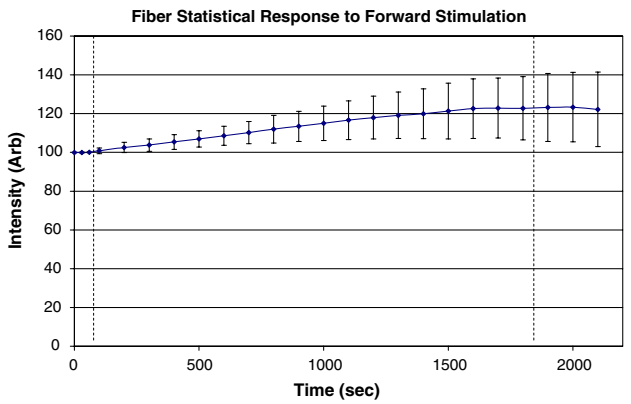


**Fig. 4** Statistics of the field-induced changes in intracellular fluorescence intensities from eight experiments. Each point represents the averaged fluorescence intensity from the slice image having a maximal value. The intensities from each experiment were normalized to the corresponding control value before field application. The results are presented in the following figures using the same method. Bars represent standard deviation

Based on our previous studies, which show that the synchronization modulation electric field can activate the Na/K pump molecules, it would seem reasonable to attribute the membrane potential hyperpolarization to activation of the pump molecules. In order to prove this hypothesis, we repeated the experiments in the presence of 1 mM ouabain in the bathing solution, which specifically inhibited function of the Na/K pump molecules.

Six experiments were conducted. The measured intracellular fluorescence intensities were again normalized to the corresponding control values before field application. Again, the field was applied to the cells during the period between the two vertical lines. For the ouabain-treated cells, no single experiment showed an increase in fluorescence intensity. The statistics of the six traces are shown in Fig. 5. Again, the bars represent the standard deviation.

This decrease in fluorescence intensity may result from two origins. Firstly, as the K channels were not blocked, the resultant opening of these channels may lead to a leakage of K ions and, hence, depolarization of the membrane potential. Secondly, due to the presence of other ionophores or membrane permeabilization, a slow rundown of the ionic concentration gradient is unavoidable when the pumps are inhibited.

This result proves that the synchronization modulation electric field–induced increase in the intracellular fluorescence intensity is ouabain-sensitive and, hence, dependent on the Na/K-ATPase pump molecules. More specifically,

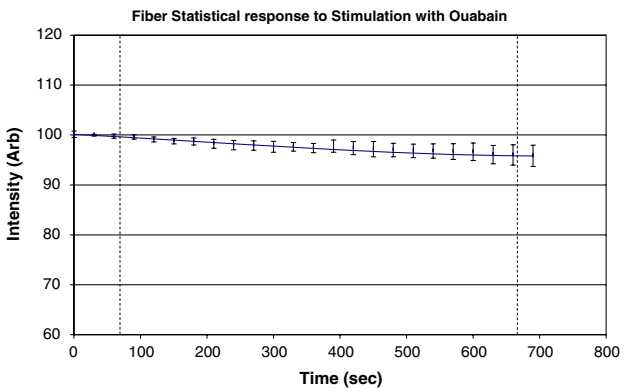


**Fig. 5** Statistical average of the effects of the synchronization modulation electric field on ouabain-treated cells from six experiments. Bars represent standard deviation

the electric field–induced increase in the membrane potential may be the result of activation of these Na/K pumps by our applied field.

The fundamental mechanism involved in this technique is to dynamically entrain the pump molecules to higher pumping rates. In other words, we expect that the pump molecules were initially synchronized by the 20-Hz pulse train and later gradually modulated to a pumping rate of 400 Hz. If that was true, when we reverse the modulation frequency, in order to modulate the pumps to lower pumping rates, the membrane potential hyperpolarization should disappear, while still subjecting the cells to a field of identical magnitude and duration. To confirm our hypothesis, we conducted the following experiments using an electrical stimulation which we refer to from this point as "backward modulation."

The waveform used was similar to the forward modulation electric field except the sequence of frequency change was reversed. The initial pulse frequency was 400 Hz and lasted for 10 s, followed by a gradual frequency decrease to 20 Hz in a stepwise pattern, 1% every 0.1 s. The field strength remained unchanged, generating an 80-m*V* peak-to-peak magnitude of membrane potential.

With the same method, the intracellular fluorescence intensity was measured when the backward modulation electric field was applied to the cells. For all of the six experiments, the previously shown increase in fluorescence intensity was eliminated even though the field strength and the individual pulse waveforms remained the same. Instead, the backward electric field caused a slight reduction in fluorescence intensity and, therefore, a depolarization of the membrane potential.

The statistics of the results from the six experiments are shown in Fig. 6, with standard deviations represented by bars. This result shows that the direction of the frequency

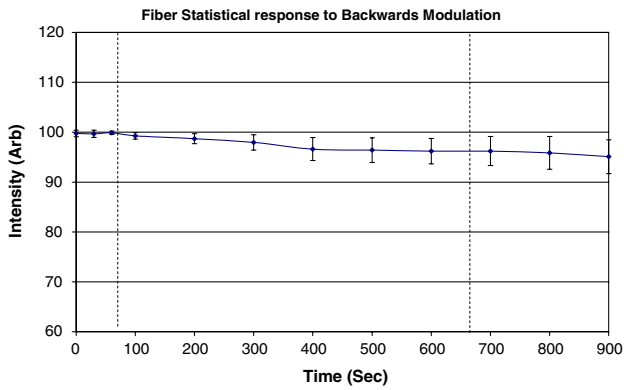


**Fig. 6** Statistical average of intracellular fluorescence intensities induced by the backward modulation electric field from six experiments. The pulsed waveform was the same as that in the forward modulation electric field except the frequency modulation was reversed. Bars represent standard deviation

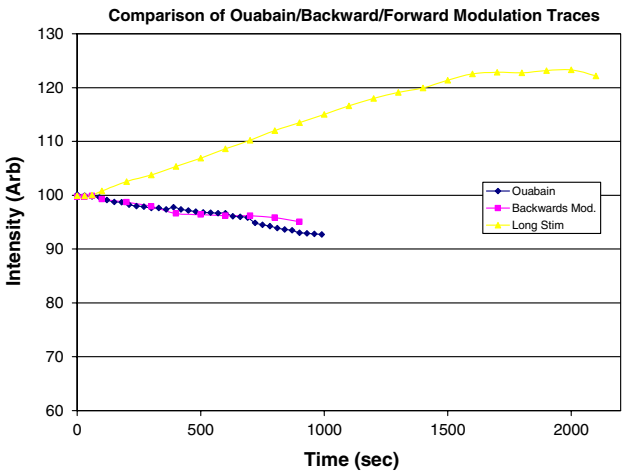


**Fig. 7** Comparison of intracellular fluorescence intensities induced by the forward/backward modulation electric fields and from ouabain-treated cells

modulation is critical to the effect observed. Only the forward modulating electric field can accelerate the Na/K pumping rates, which is consistent with results measured previously, through direct monitoring of the pump current using a voltage clamp. As a result, we conclude that our specific forward modulated electric field can increase the ionic concentration gradient across the cell membrane and subsequently hyperpolarize the membrane potential.

To compare the results from forward and backward modulation as well as from the ouabain-treated cells, the intracellular fluorescence intensity traces were plotted in the same coordinates, as shown in Fig. 7. It is clearly shown that the forward synchronization modulation electric field can significantly increase the membrane potential.

## Discussion and Conclusion

When an oscillating electric field is applied to cells, the electric field will inevitably affect other membrane proteins, in addition to the Na/K pump molecules, such as activation of $Na^+$ and $K^+$ ion channels and of $Ca^{2+}$ signaling and, hence, alteration of the number of plasma sodium pumps by membrane trafficking. In order to identify the effects of activation of the pump molecules, it is necessary to discuss the effects on those proteins. The sodium channel currents, as explained previously, were entirely removed by the blocker TTX. The potassium currents, however, were not removed. In our previous studies, included with this response we showed that a stimulation of this duration, with a high enough frequency to be considered continuous, will rapidly show inactivation, removing the K channel current almost entirely. It is worth noting also that any current from these channels

would show a net decrease in membrane potential and the opposite effect to the one we observed. In terms of membrane trafficking, previous studies showed that the time of action is significantly larger than our experimental duration, usually overnight (Bertorello et al., 1999), as opposed to 30 min. Also, any change in membrane constituents would be present also in the control scans. In addition, these trafficking events are in response to the addition of a factor, e.g., epinephrine (Bertorello et al., 1999) and ß-agonists (Pesce et al., 2000). No such addition was made, and efforts were taken to ensure that the control fibers encountered as near to identical conditions as the experimental fibers, so any change would also be observed here. Most importantly, comparison of the results in the presence of ouabain and those with reverse frequency modulation showed that the field-induced membrane potential hyperpolarization is mainly from activation of the Na/K pumps.

The concepts and the mechanisms involved in this technique differ significantly from the theory of resonance-frequency-windows and the excitation-stimulation technique. The resonance-frequency-windows theory considers the existence of windows in which the pump molecules can absorb energy from a fixed, relatively high-frequency field, while we consider activation of the pump molecules to be a dynamic process of entrainment. The detailed comparisons have been discussed in our previous report (Chen & Zhang, 2006; Chen & Dando, 2008a, 2008b).

In terms of excitation-stimulation induced activation of the Na/K pumps, Clausen & Nielsen (1998), in an excellent review, summarized the involved mechanisms. Activation of the Na/K pumps elicited by excitation is most likely to reflect a rapid but slowly reversible increase in the affinity of the Na/K pump for intracellular Na ions, possibly elicited by depolarization during the action potentials. This would allow for more efficient clearance of Na from the cytoplasm and of K from the extracellular phase. Another possible mechanism is the excitation-induced leakage of Na and K ions, which increases the availability of ions to bind with the pump molecules (Buchanan, Nielsen & Clausen, 2002; Clausen & Nielsen, 1998). All of these explanations would allow more efficient binding and clearance of Na and K ions.

We focus on the Na- and K-transport steps instead of ionic availability and binding affinity. In fact, in our experiment, we blocked Na channels so that the effects of any changes in Na ion availability or binding affinity were eliminated, or at least significantly reduced. Indeed, the oscillating electric field, in both forward and backward modulations, inevitably elicited K-channel currents, resulting in a reduction in the K concentration gradient and, hence, the membrane potential depolarization. However, the two modulations showed significantly different results even with the same magnitude and frequencies. The only difference we imposed upon the system was to the sequence of frequency change. The backward modulation resulted in a slight depolarization of the membrane potential, while the forward modulation not only reinstated but also hyperpolarized the membrane potential. Clearly, the observed phenomena cannot be due to changes in ion availability or binding affinity but must be related in some way to the modulation direction.

For the forward modulation, the pump molecules were initially synchronized to 20 Hz and then gradually modulated to a pumping rate of 400 Hz in a stepwise pattern. Synchronization of pump molecules and frequency modulation have been demonstrated previously, by direct measurement of the pump current using voltage-/patch-clamp techniques (Chen & Zhang, 2006, 2008; Chen & Dando, 2008a). Due to this significant acceleration in the pumping rates, the membrane potential could be quickly recovered and even hyperpolarized (Fig. 4). In contrast, the backward stimulation had an initial frequency of 400 Hz and then was gradually modulated to a pumping frequency of 20 Hz. Reductions in the pumping rates resulted in a decrease in the pump currents. As a result, the backward stimulation could not restore the membrane resting potential (Fig. 6). Instead, the membrane potential is slightly depolarized (Fig. 5).

It is necessary to point out that stimulation at an initial frequency of either 20 or 400 Hz cannot synchronize all the pumps. Our study has shown some kinds of distribution of the pumping rates as a function of local environmental parameters such as membrane potential, ionic concentration and temperature (Huang, Rabson & Chen, unpublished data). Meanwhile, we also showed that an oscillating electric field can only synchronize the pumps whose pumping rates are comparable to the field frequency (Chen, unpublished data). In order to synchronize as many pumps as possible, we need to scan the field frequency. Forward frequency scanning starting from a low frequency of 20 Hz can synchronize most of the pumps and force them to eventually run at a high frequency of 400 Hz. Backward scanning can also synchronize most of the pumps but forces them to finally run at a low frequency of 20 Hz.

In fact, the underlying mechanism involved in excitation-stimulation involves triggering the natural physiological mechanisms used in living systems to maintain the cellular functions. Because the desired goal is to maintain the membrane potential, there is a negative feedback in the process. The less the depolarization in the membrane potential, the less the change in ion availability and binding affinity and, therefore, the less activated the pump molecules become. Consequently, as long as the membrane potential is restored, the pump molecules are no longer activated, so the membrane potential can never become hyperpolarized in normal conditions. Using our technique, the electric field directly affects the pump

molecules. The pumping rates are controlled by the frequency of the synchronization field. As a result, this technique not only can restore but also can hyperpolarize the membrane potential, which is difficult to realize using excitation-stimulation.

Furthermore, in terms of energy consumed within the pumping loop, this technique is also different from excitation-stimulation. Excitation-stimulation does not directly affect the pump molecules. Instead, it changes the environment by opening ion channels or affecting other processes, which in turn triggers activation of the pump molecules by increasing ion availability and binding affinity. Excitation-stimulation does not provide energy directly to the pump molecules. Therefore, the process is indirect and passive.

In contrast, in our technique, the synchronization-modulation electric field activates pump functions by directly providing electric energy to the pump molecules to overcome the energy barriers for both Na and K transport. This is a direct and active process. In fact, we have shown that the pump turnover rate can be controlled by the synchronization modulation electric field going up or going down.

In terms of the concerns of opposing polarity of membrane potentials induced by the electric field on the two hemispheres, which we mentioned earlier, the synchronization modulation effects on the pump molecules will no longer be cancelled. That is beneficial from our design of using a symmetric oscillating waveform. As long as the pump molecules are synchronized to the oscillating electric field, the pump molecules on the two hemispheres are restrained to two pumping paces, respectively, having the exact same rate but a $180^{o}$ phase shift. As the synchronization frequency increases, all of the pumping rates are accelerated. The phase shift does not affect the ion accumulation.

In summary, this technique significantly differs from the current theories and techniques. Excitation-stimulation triggers the Na/K pumps by intrinsic mechanisms within the body designed to maintain cellular membrane potential, which is sufficient for normal everyday physiological situations. However, in response to nonphysiological conditions, such as injury, hypoxia and some diseases, this system may prove inadequate. In contrast, our technique is to actively entrain the pumping rates by directly providing energy to overcome the relevant energy barriers. We have previously shown that this technique can accelerate the pumping rate and therefore hyperpolarize the membrane potential in frog skeletal muscle fibers (Chen & Dando, 2008b). In this study, we extend our studies to mammalian cardiomyocytes and further confirm our results.

The synchronization modulation technique provides a new method to study the pump molecules. Many functions of the Na/K pumps, which are difficult to study using traditional methods, can be easily investigated using the synchronization modulation technique. Once synchronized, all the pumps extrude Na ions at the same time and then pump in K ions. Therefore, detailed information on Na and K transport can be revealed, separately. For example, it took many years of work by several labs to recognize the pumps' stoichiometric ratio of 3:2. Once synchronized, the measured current magnitude ratio clearly reflects the stoichiometric ratio. When running the Na/Na exchange mode, because three Na ions are exchanged by the same amount of Na ions, no pump currents can be measured in the traditional manner even though ion movements across the cell membrane are involved in each limb. When reaching synchronization, the inward and outward Na currents can be clearly identified, showing a magnitude ratio of 1:1. In addition, synchronization significantly increases the signal/noise ratio, which will allow us to study the proteins' structure–function relationship.

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