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Electrical Activation of Na/K Pumps Can Increase Ionic Concentration Gradient and Membrane Resting Potential

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Abstract. It has been previously demonstrated by our group that our specifically designed synchronization modulation electric field can dynamically entrain the Na/K ATPase molecules, effectively accelerating the pumping action of these molecules. The ATPase molecules are first synchronized by the field, and subsequently their pumping rates are gradually modulated in a stepwise pattern to progressively higher and higher levels. Here, we present results obtained on application of the field to intact twitch skeletal muscle fibers. The ionic concentration gradient across the cell membrane was monitored, with the membrane potential extrapolated using a slow fluorescent probe with a confocal microimaging technique. The applied synchronization-modulation electric field is able to slowly but consistently increase the ionic concentration gradient across the membrane and, hence, hyperpolarize the membrane potential. All of these results were fully eliminated if ouabain was applied to the bathing solution, indicating a correlation with the action of the Na/K pump molecules. These results in combination with our previous results into the entrainment of the pump molecules show that the synchronization-modulation electric field-induced activation of the Na/K pump functions can effectively increase the ionic concentration gradient and the membrane potential.

Key words: Na/K pump molecule — Electric field — Synchronization — Modulation — Activation — Ionic concentration gradient — Membrane potential

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Introduction

The Na/K-ATPase pump molecule, extruding three Na ions from the cell via the exchange of two K ions alongside the consumption of one adenosine triphosphate (ATP) molecule, is one of the most common as well as one of the best-characterized active transporters found within the cell membrane. Function of the pump molecules is critical to innumerable cellular processes, including those involved in signal generation, energy supply and homeostasis. The Na/K pump molecules have become a central target for acute long-term regulation as well as for therapeutic intervention.

Because of the well-documented voltage dependence of the functions of these molecules, it is logical to consider using an external electric field to manipulate the pump function. However, Na/K pumps show a shallow, sigmoidally shaped *I-V* curve, exhibiting saturation behavior and a possible negative slope (Pedemonte, 1988; De Weer, Gadsby & Rakowski, 1988a; Naka & Gadsby, 1989; Rakowski, Gadsby & De Weer, 1997; Chen & Wu, 2002; Apell, 2003); therefore, a simple depolarization of the membrane potential cannot effectively increase the pump currents.

Significant work has been previously undertaken into the possible application of an oscillating electric field in order to activate the function of the ATPase pump molecules. Early work by Teissie & Tsong (1980) used a megahertz AC field to activate the Na/K pump molecules in erythrocytes. Later, several theoretical models were developed, including resonance frequency windows in which an electric field can increase the pump currents (Markin et al., 1992; Astumian & Robertson, 1989), the Brownian motion model (Astumian, 1997; Tsong, 2002) and the adiabatic pump model (Astumian, 2003). The location of these resonance frequencies or frequency windows has not been experimentally identified.

We recently developed a new approach to electrically activate the Na/K pump molecules: dynamic entrainment of the pump molecules (Chen, 2006, 2007). In the first portion of this work, we experimentally demonstrated that an oscillating electric field with a frequency comparable to the pumps' turnover rate can synchronize the pump molecules to work at the same pace (Chen & Zhang, 2006; Chen, Zhang & Huang, 2007). The characteristics of the synchronized pump molecules include a distinguishable inward component of the pump current being revealed, alternating with that of the outward component; magnitude of the outward pump current shown to be around threefold that of the randomly paced, unidirectional outward pump currents; and the magnitude ratio of the outward over inward pump currents is close to 3:2, reflecting the classically proven stoichiometric ration of the ATPase pump molecules (Chen & Zhang, 2006; Chen, Zhang & Huang, 2007).

In the next step, we designed a two-step, synchronization-modulation electric field in order to successfully accelerate the action of the pump functions (Chen & Dando, 2006). The underlying mechanisms involved in this approach are as follows. We first applied a sequence of oscillating electric pulses, having a frequency comparable to the physiological turnover rate of the Na/K pumps, in order to force them to work at this same pace, exploiting the voltage dependence of the Na,K-ATPase molecules. Once synchronized, the field frequency was slowly increased in a stepwise pattern. At each step, there were enough pulses to synchronize the pumping rate to the new higher frequency. In this way, the pumps can be modulated, or accelerated, to sequentially higher and higher pumping rates. Manyfold increases in the pump currents have been shown by the synchronization-modulation electric field. All these experiments were conducted in skeletal muscle fibers under a voltage clamp.

Using the voltage-clamp technique to alternate the membrane potential, transient changes in the pump currents can be simultaneously monitored. The disadvantage inherent under this arrangement is that the cells were not under physiological conditions. In addition, it is not clear whether the field-induced activation of the pump molecules can influence the cells' ionic concentration and, hence, the membrane potential. To answer this question, we recently studied intact skeletal muscle fibers under the influence of the electric field. Changes in the ionic concentration gradient and the membrane potential were monitored by spectrofluorescent imaging techniques using a confocal microscope. Our results showed that the synchronization-modulation electric field could effectively increase the ionic concentration gradient across the cell membrane and, hence, hyperpolarize the membrane potential.

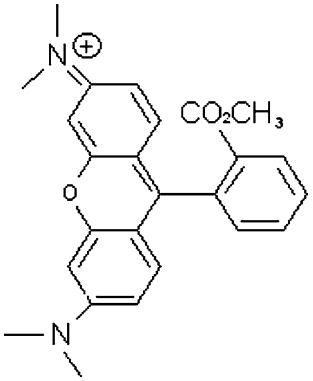

Fig. 1. Chemical structure of TMRE. The double bond on the upper nitrogen can be thought of as delocalized over the three-ring structure, resonating between the nitrogen bonds, which are covered by hydrophobic methyl groups. This combined with the molecule's ester group covers the partial positive charge,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

Materials and Methods

Selection of Fluorescent Dye

Ionic concentration gradients throughout the diameter of the skeletal muscle fibers used in these studies and across the cell membrane were measured using a confocal microscope, utilizing a suitable fluorescent probe. Using a fluorescent indicator to determine the cell membrane potential discloses several advantages to the electrical measurements made using voltage-clamp or micropipette impalement techniques (Gross & Loew, 1989). The dye selected for the study of global variation in membrane potential was tetramethyl rhodamine ethyl ester (TMRE) (Fig. 1).

This dye belongs to a class known as Nernstian dyes, initially developed by Waggoner (Sims et al., 1974; Waggoner, 1979). In contrast to many fluorescent dyes, which exhibit fluorescence only when binding with specific molecules, resulting in structural rearrangement, typically involving charge shift, TMRE will always fluoresce. TMRE molecules are positively charged, exhibiting a high sensitivity to membrane potential, albeit over slow time scales. The lipophilicity of these molecules combined with the delocalization of positive charge renders them membrane-permeant (Tsien & Waggoner, 1990). The high permeability allows the redistribution of TMRE across the membrane when the membrane potential changes. Therefore, the ratio of the equilibrium distribution of the dye molecules across the membrane is governed by the Nernst equation (Sims et al., 1974; Waggoner, 1979):

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

When the muscle fibers are exposed to an applied oscillating electric field, the field-induced oscillating membrane potential will be superimposed upon the existing membrane resting potential. This fast oscillating applied component is not our interest except when calibrating the magnitude of the field-induced membrane potential. We focus on the change in the baseline membrane resting potential, which mainly depends on the extracellular and cytoplasmic K concentration. Activation of the pump molecules can increase the ionic concentration gradients across the cell membrane and, therefore, hyperpolarize the membrane potential. However, it takes time for the pump molecules to build the ionic concentration gradient, through the increased ionic pump current, and time will be

taken for the dye to redistribute throughout the fibers. In other words, we are interested in the slow change, or the DC component in the membrane potential, not the fast alternating component, which we ourselves are applying. TMRE, a so-called slow dye, therefore fits our requirements very well.

Moreover, in contrast to fast membrane potential dyes, which typically show a low sensitivity to membrane potential changes, slow dyes tend to exhibit superior potential sensitivity to their faster counterparts. For example, fast dyes, such as di-4-ANNEPPS or di-8-ANEPPS, show approximately only as high as a 10% change in response to a membrane potential variation of 100 mV. TMRE shows orders of magnitude higher fluorescence variation 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 (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 factors such as pH or, in our case, membrane potential. Compartmentalization of the dye has been reported on longer time scales; however, in all of our scans, measurements were taken immediately after staining, with an analysis region large enough that the mitochondria would not form a significant portion of the window.

Fiber Preparation and Confocal Imagining

Twitch skeletal muscles, semitendinosus and iliofibularis, were dissected from the leopard frog *Rana pipiens*, in relaxing solution, as in our prior work (Chen & Wu, 2002; Chen & Lee, 1994). Single muscle fibers were isolated and then transferred to the experimental chamber filled with relaxing solution. The fibers were held by two clips, with the distance between the two clips being about 3 mm. The two clips were moved slightly apart to avoid contraction and movement during the experiment. A coverslip was placed on the top of the two clips, reducing the depth of the bathing solution around the fiber to about 300 μm. The purpose of this was to increase the resistance of the bathing solution in order to reduce Joule heating effects. The chamber with fiber was then mounted on the confocal microscope for background measurement. The background subtraction from both inside the fiber 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. Finally, the fibers were incubated with dye solution (2 μM of TMRE) in normal Ringer's allowing a maximum intensity of fluorescence to be reached under controlled conditions. To do so, the fiber was washed by the dye solutions six times underneath the coverslip to ensure complete interchange of constituents before the excitation protocol was executed.

The confocal microscope is able to focus on a single slice of the fiber in order to accurately and efficiently monitor the field-induced changes in the fluorescent intensity. The technique used followed that used in other labs (Loew, 1993). An Olympus (Tokyo, Japan) IX81 inverting confocal microscope utilizing the Fluoview FV500 Tiempo V4.3 analysis package was employed for data collection, with a x10 dry objective and a confocal aperture of 80 nm giving a resolution in the x and y directions of 0.621 μm and a z resolution of 3.09 μm. Standard rhodamine optics of excitation under green HeNe at 543 nm and detection with a photomultiplier and barrier filter at 560 nm were employed to graph the observed fluorescence as a two-dimensional map, varying with time. After the six-time change of the bathing solution to that containing TMRE dye, the fluorescent intensity was measured at a time resolution of 1 min. When dye staining had reached a maximal level, reflecting an equilibrium state, application of the stimulation protocol was initiated.

Stimulation with Synchronization-Modulation Electric Field

The oscillating electric field discussed was applied to the fiber by a custom-modified TENMA UTC 72–5085 (Centerville, OH) function generator connected through two agar bridges and Ag/AgCl wires. The small cross section of the bathing solution surrounding the fiber in comparison to the long distance between two agar bridges (3 cm) makes the applied electric field relatively uniform, and the increased resistance of the bathing solution helps to reduce any Joule heating effects. The applied electric potential was 24 V, peak-to-peak, which generated a field strength of 8 V/cm. For a fiber with a diameter of 100 μm, the field-induced membrane potential was estimated as 40 mV, peak-to-peak. We would like to use a higher field strength; however, considering the field-induced side effects in the solution inevitable at applied fields of this level, we selected 40 mV, peak-to-peak, as an acceptable compromise. Under this field strength, the Joule heating effects measured by the changes in temperature and pH value are not noticeable.

The synchronization-modulation field used in our work has been described previously (Chen, Zhang & Huang, 2007; Chen & Dando, 2006) and can be briefly summarized as follows. The electric field has an oscillating square waveform with an initial frequency of 50 Hz, which is assumed to be close to the natural physiological frequency of the Na,K-ATPase pump molecules. Our previous studies showed that at physiological conditions 100 pulsed symmetric 50-Hz oscillations of the membrane potential can effectively synchronize the Na/K pump molecules (Chen, Zhang & Huang, 2007; Chen & Dando, 2006) to this 50-Hz membrane potential oscillation. After a finite duration of 10 s of this stimulation, the frequency was gradually modulated up to a final value of 200 Hz in a stepwise pattern, taking approximately 2 min to reach this final value.

This 200-Hz alternating electric field lasted for another 3 min, before the electric field was removed completely. The fluorescence both in the fiber and in the bathing solution was continuously measured every minute. The data were stored in the hard disk for further analysis.

The compositions of solutions are as follows: relaxing solution, 120 mM potassium glutamate, 5 mM K_2PIPES, 1 mM $MgSO_4$ and 0.1 mM K_2 ethylenglycoltetraacetic acid (EGTA); Ringer's solution, 120 mM NaCl, 2.5 mM KCl, 2.15 mM Na_2HPO_4, 0.85 mM $NaH_2PO_4.H_2O$, 1.8 mM $CaCl_2$, 1 μM tetrodotoxin (TTX) and 3 mM 3,4-diaminopyridine (3,4-DAP); dye solution, same as Ringer's solution with 2 μm TMRE. All solutions were titrated to a working pH of 7.0.

We used the channel blockers TTX and 3,4-DAP to block the Na and K channels, respectively. We had tested that all of the Na channels and most of the K channels were blocked. Even though it is not possible to eliminate some channel currents, the residual currents are passive, which cannot build up any ionic concentration gradient. Under this proviso, any increment in the membrane potential can only be attributed to the active transporters, which in this case must refer to the pump molecules.

Results

In the figures obtained from scans taken as described, a usual time taken to maximal staining intensity at the beginning of the experiment would be around 20 min. This would represent an equilibrium state, whereby the driving force to pull the dye molecules into the cell has reached equilibrium and the concentration ratio in:out is representative of the con-

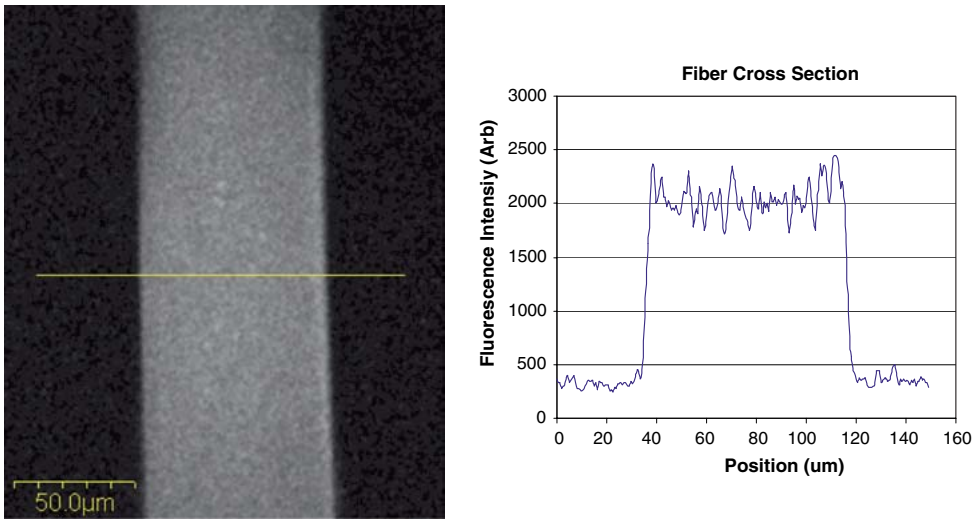

Fig. 2. Cross section of skeletal muscle fiber, semitendinosus, from *Rana pipiens*, stained with 2 µM TMRE. The figure shows the potential dependent rearrangement of dye into the fiber, resulting in an elevated concentration and consequently a higher fluorescence, from the negatively charged intracellular region. Position in the scan field is in micrometers on the abscissa, and fluorescence is represented in arbitrary units on the ordinate, dependent on the amplification of the system. Ordinate and abscissa are as here for all subsequent figures.

centration gradient of potassium ions. The panel to the left in Figure 2 shows a recorded fluorescent image of a cross section of a fiber 30 min after the fluorescent stain, when this equilibrium should have been reached. The horizontal line represents the data acquisition line, a one-dimensional plot of fluorescence intensity, which is shown in the panel to the right. Here and in the following figures, fluorescent intensities of five neighboring scan lines were averaged, with this average displayed.

It is clearly shown in this figure that the fluorescence intensity inside the cell is significantly higher than that outside the cell, as we would expect due to the intracellular negative membrane potential. Inside the fiber, the fluorescence intensity exhibits significant internal fluctuation in comparison to the intensity variation when outside the fiber. The fluctuation of fluorescence intensity showed a distinct pattern, and this pattern remained invariant along the axis of the fiber. We believe this is indicative of the intracellular structures of the skeletal muscle fibers, which are filled with myofibrils and other intracellular organelles.

Our goal was not to investigate the intracellular organelle distributions; therefore, we attempted to minimize the fluctuation in the fluorescence arising from these organelles within the fiber by smoothing the measured intensity curve through application of a smoothing function in the data analysis procedure. The results are shown in Figure 3. The smoothing function is a simple averaging of each pixel's five neighboring respective pixels in each direction across the fiber. The function was not applied to the membrane region, where a large variation would be expected, as the dye concentration should drop off very steeply from the inside to the outside of the fiber. In

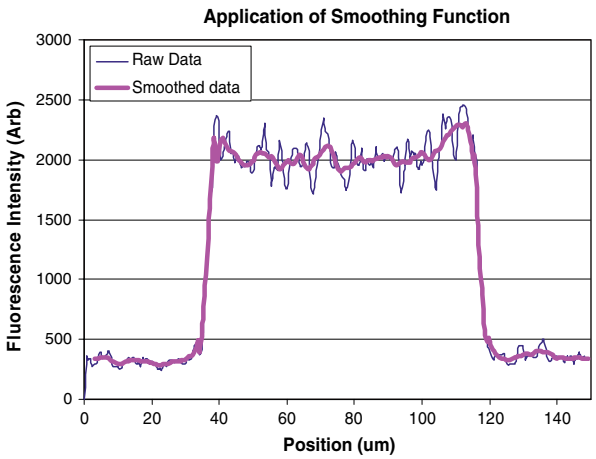

Fig. 3. Smoothing function applied to the fiber cross section in order to eliminate the effects of fluctuations in fluorescence arising from interior organelles in the system.

close proximity to the cell membrane, the averaging function is applied unidirectionally, averaging only medially as lateral averaging would include points outside the fiber, artificially lowering the reading.

To ensure that there is no significant change in the fluorescence intensity of the fiber after dye equilibration across the membrane, we continuously scanned the fluorescent dye. The scans taken at 5 and 10 min after the first scan are shown in Figure 4. There is no discernable variation in fluorescence, discounting minor fluctuations; hence, we assume an equilibrium state has been reached, and there is no variation in dye concentration.

After these control scans were taken, we applied the oscillating electric field to the fiber. Figure 5

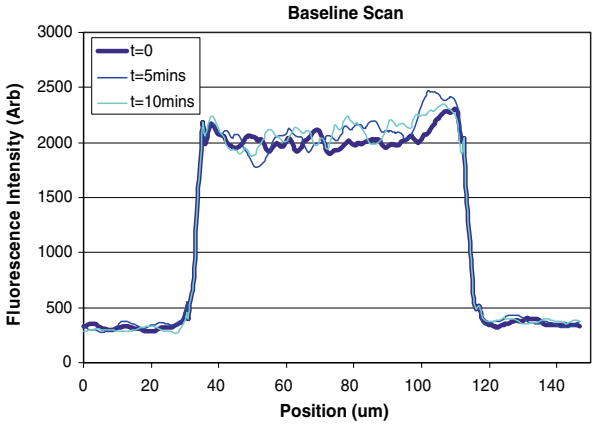

Fig. 4. Time-dependent scan of fluorescence after dye equilibration across membrane. Three scans show initial fluorescence, then at 5-min intervals subsequently, without stimulation.

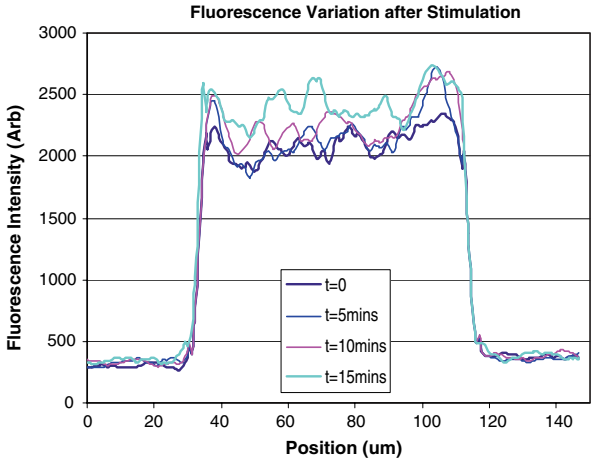

Fig. 5. Fluorescence variation with stimulation via a frequency-modulated oscillating potential up to 200 Hz. The *first trace* was taken without application of the electric field as a control. The *second trace* shows the electric field-induced elevated localized dye concentration in the region of the membrane. Increase in fluorescence, initially at the membrane boundary, is evident, signaling localized membrane potential hyperpolarization induced by the electric field. After stimulation is removed, the fluorescent dye redistributes gradually throughout the cell in the *third trace*. In the *final trace*, it is evident the dye concentration is significantly higher than in the initial scan, indicating an increase in membrane potential of the whole cell.

shows the fluorescent images taken before, right after and every 5 min after application of the electric field. The recorded fluorescence intensity before application of the electric field (t = 0) shows a relatively uniform distribution across the fiber, with the averaged intensity across the fiber being about 2,050 arbitrary units. Right after removal of the oscillating electric field (t = 5), the fluorescence intensity close to the membrane boundary shows a significantly increment; however, those away from the cell membrane remain at a relatively unchanged level. The profile of the fluorescence intensity throughout the fiber shows an elevated localized dye concentration in the region close to the cell membrane.

Comparing this image to the control trace taken before application of the electric field, the dramatic elevation in the intensity near the membrane boundary clearly indicates what must be assumed to be a field-induced effect on the localized ionic concentration measured within the fiber. The fluorescence intensity taken 5 min after removal of the electric field (t = 10 min) indicates the dye molecules' redistribution gradually throughout the fiber. Finally, the trace taken 10 min after removal of the electric field (t = 15 min) shows a significant increment in the fluorescence intensity throughout the profile of the fiber.

We explain this phenomenon as follows. The oscillating electric field activates the Na/K pump molecules, in the manner discussed earlier. As a result, the K concentration gradient across the cell membrane is increased, which in turn hyperpolarizes the membrane potential and attracts more dye molecules into the fiber, across the membrane. The region close to the cell membrane first exhibits an influx of fluorescent dye. Because of the slow diffusion coefficient of skeletal muscle fibers, due to them being filled with myofibrils, the TMRE, which is much larger than a single ion, takes time to diffuse throughout the fiber. Consequently, the measured fluorescence intensity changes always have some time delay corresponding to the application of the oscillating electric field. Later, as the dye's diffusion catches up with the ionic flow and reaches equilibrium, the fluorescence intensity becomes relatively uniformly distributed throughout the fiber.

The influx of dye molecules into the fiber left the cell showing a more negative potential inside the fiber with respect to the outside, after application of the electric field. The average fluorescence intensity across the fiber in the final situation was about 2,400 arbitrary units for the trace taken 10 min after application of the electric field, which is over a 15% increase. The outside intensity remained a constant value of 350 units. After subtracting the background of about 250 units, the potential difference across the cell membrane can be calculated from the Nernst equation (Eq. 1) for both control and post-field application.

$$V_m = \frac{RT}{zF}\ln\left(\frac{c_n^o}{c_n^i}\right) = 26mV\ln\left(\frac{350-250}{2050-250}\right) = -75.15mV$$

$$V_m = \frac{RT}{zF}\ln\left(\frac{c_n^o}{c_n^i}\right) = 26mV\ln\left(\frac{350-250}{2400-250}\right) = -79.77mV$$

where RT/zF is 26 mV for monovalent ions at room temperature. After the field application, the membrane is hyperpolarized to around –80 mV, while the initial membrane potential in the control is about –75 mV, an approximately 7% increase due to application of the electrical field.

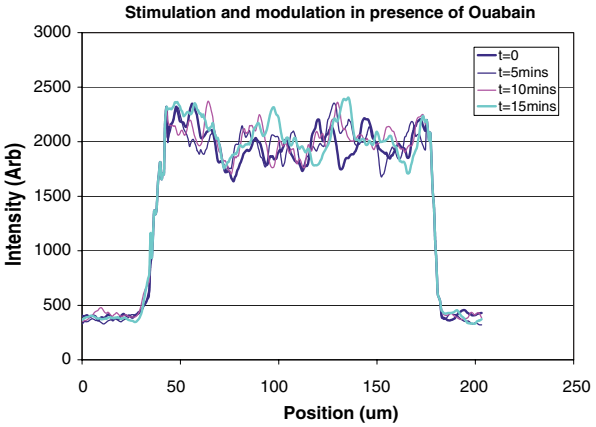

Fig. 6. Scan with 1 mM ouabain added to bathing solution. Fluorescent image was taken at t = 0 as a control. Then, the synchronization-modulation electric field was applied to the fiber for 5 min. Right after removing the field (t = 5 min) and every 5 min after that fluorescent images were taken, and the intensities are plotted here. Again, no discernable variation in the fluorescence, and hence the membrane potential, was detected.

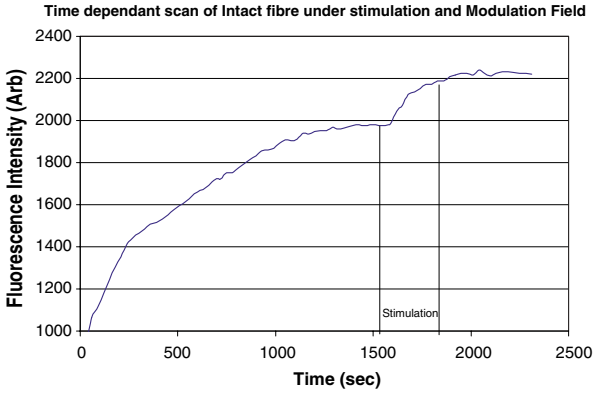

Fig. 7. Intracellular fluorescence intensity as a function of time before, during and after application of the synchronized modulation electric field. As a control, before application of the electric field, the dye intensity exponentially increased and equilibrated. With some time delay, the synchronization-modulation electric field can effectively elevate the dye intensity, and therefore the membrane potential. Due to the fact that this is a slow dye, the fluorescence intensity kept increasing shortly after removal of the electric field.

To verify that the effects which seem to be induced by application of the field are due to activation of the Na/K pump molecules, we repeated the same experiments with 1 mM ouabain, a specific inhibitor for the Na/K pump molecules, in the bathing solution. Again, immediately after taking the first image, the electric field was applied to the fiber for 5 min. The measured results of fluorescent intensities before and after the field application are shown in Figure 6.

Interestingly, all of the four traces show a similar concentration profile of the fluorescent dye across the fiber. There was no discernable variation, which would seem to suggest no significant change in the membrane potential occurred after application of the electric field. From this and Figure 4 we can conclude that changes in the ionic concentration gradient and membrane potential can be attributed to the action of the Na/K pumps.

In addition to using ouabain to inhibit the pump molecules, we repeated the experiments with potassium-free bathing solution in order to eliminate the pump currents in an alterative manner. Again, all traces of fluorescence intensity taken before and after the field application showed a similar profile throughout the fibers. No discernable change could be observed. These results further confirm that the oscillating electric field-induced increase in intracellular fluorescence intensity observed is solely due to the activation of the Na/K pumps.

Further scans were taken to elucidate the time-dependent behavior of the cells on application of the electric field. Images of the same cross section of the fiber were continuously taken immediately after addition of TMRE to the bathing solution. The data acquisition box covered 60 x 20 pixels and was located at the midpoint of the fiber between the center of the fiber and the cell membrane. The fluorescence intensity within the whole of this box was averaged and is plotted in Figure 7 as a function of time. The vertical lines show the starting and terminating points of application of the electric field.

At the beginning of the figure, the fluorescence intensity showed an exponential-like increase, until reaching a plateau, the equilibrium state. This plateau took over 20 min to reach. After the dye intensity within the fiber had stabilized, the electric field was delivered to the fiber. The results show that after a small delay the intensity, and hence concentration, of the dye molecules started to increase. The time delay is probably due to both the time needed for the pumps to build up this ionic concentration gradient and the time needed for the slow dye to diffuse into the cell. After removal of the electric field, the dye intensity further increased for a short time. Our previous studies showed that the desynchronization process is almost instantaneous, with a relaxation time course in the subsecond range after removal of the oscillating membrane potential (Chen, Zhang & Huang, 2007; Chen & Dando, 2006). Therefore, this time delay must mainly be due to diffusion of the slow dye.

As before, to confirm that this effect is due to activation of the Na/K pump molecules, similar experiments were repeated with 1 mM ouabain in the bathing solution, with the results shown in Figure 8. The electric field-induced increase in the fluorescence intensity is no longer apparent. These results indicate that the increment in intracellular fluorescent dye must be associated with activation of the Na/K pump molecules.

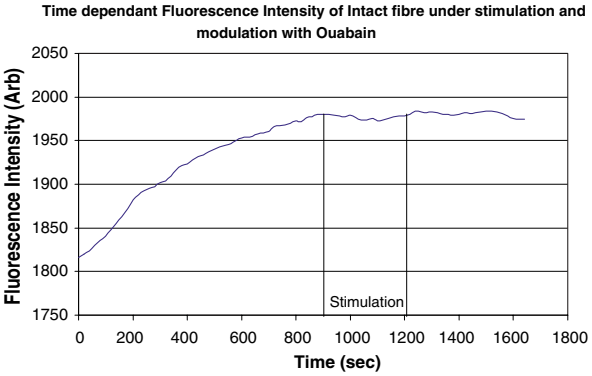

Fig. 8. Intracellular fluorescence intensity as a function of time in the presence of ouabain. Ouabain (1 mM) was used in the bathing solution to inhibit the pump molecules. The oscillating electric field did not show noticeable change in fluorescence intensity.

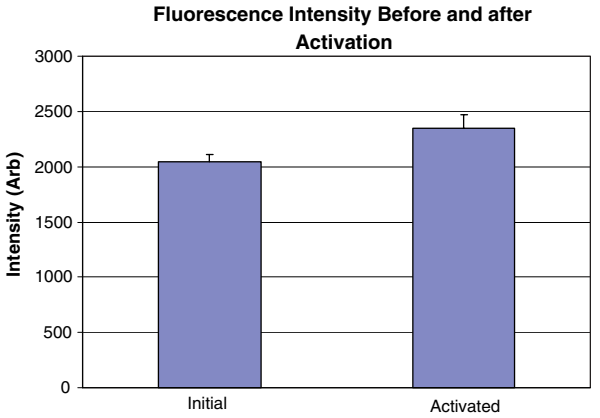

Fig. 9. Statistical study of 10 fibers in electric field-induced increase in ionic concentration gradient. Bars represent standard deviations.

Ten experiments were conducted using 10 different fibers from six frogs. The results consistently showed noticeable increases in the ionic concentration gradient, and therefore the membrane resting potential, even though the absolute values differed from fiber to fiber. This could be expected and is probably due to the variation in diameter of the fibers resulting in different field-induced membrane potential, not to mention an inherent variation in the density of pump molecules from fiber to fiber and from frog to frog. In all of our experiments, the dye concentration in the bathing solution was always 2 μM. Due to the much smaller volume of the fiber in comparison to the bathing chamber, the dye concentration in the solution should remain constant. These results are shown in Figure 9. The mean increase measured is a little over 15% of the ionic concentration gradient, corresponding to a little less than a 5% hyperpolarization in the membrane potential after 5 min of application of the synchronization modulation electric field.

Discussion and Conclusion

In works previously presented, it has been shown that the Na/K pump currents can indeed be increased by a depolarization in the membrane potential. However, the pumps' sigmoidal *I-V* curve indicates a low sensitivity to the membrane potential, which restrains the effectiveness of this particular form of steady-state electrical activation of the pump function. In addition to this, the membrane potential depolarization can only be realized in a laboratory using voltage/patch-clamp techniques. In real situations, it is impossible to simultaneously depolarize the membrane potential on both hemispheres of an intact cell. An electric field depolarizing the membrane potential at one hemisphere and activating the pumps on this side of the cell will inevitably hyperpolarize that on the other hemisphere and inhibit these respective pump molecules. The positive effects produced will hence be canceled, or at least reduced.

In contrast, the technique of synchronization-modulation has been shown in our work to effectively activate the Na/K pump functions. The involved mechanisms have been theoretically studied (Chen, 2006, 2007) and experimentally demonstrated (Chen & Zhang, 2006; Chen, Zhang & Huang, 2007; Chen & Dando, 2006) previously. Briefly, we introduced a concept similar in theory to the synchrotron, whereby electrons can be accelerated gradually turn by turn through a sequential alternating application of force. It has been widely accepted that the stoichiometric numbers of the Na/K pump function remain unchanged at a wide range of membrane potentials (Rakowski, Gadsby & De Weer, 1989; De Weer, Gadsby & Rakowski, 1988b). In order to increase ionic transport across the membrane, the only solution available would be to accelerate the rate at which these molecules move the ions. Based on the Post-Albers kinetic model for the pump, Na extrusion has been shown to be the rate-limiting step, with the pumping in of K ions shown to be the next slowest step. The transport of these two ions across the membrane barrier is in opposite directions; therefore, their voltage dependence will be opposite. Finally, the two ion transports do not occur at the same time but instead in a sequential pattern. Based on these experimental results, we proposed applying an oscillating electric field to the cell membrane with a frequency comparable to the pump's natural turnover rate. The two half-cycles of the applied field would be designed to match the time courses of the two ion transports, respectively. The electric field should therefore be able to facilitate Na transport in one half-cycle and alternatively activate K transport during the others. The times needed for the two transports will become shorter and shorter gradually, loop by loop, as the pump molecules become

synchronized to a greater and greater degree. In other words, the pumping rate can be accelerated by the oscillating electric field.

Our theoretical studies showed that by maintaining this apparent synchronization of the pump molecules, while gradually increasing the frequency imposed upon the cells to induce this synchronization, the pump function can be activated exponentially as a function of the membrane potential (Chen, 2006, 2007). We also experimentally demonstrated synchronization of the Na/K pump molecules and activation of their pumping rate by directly monitoring the pump current the through use of voltage-clamp techniques (Chen & Zhang, 2006; Chen, Zhang & Huang, 2007). The result of this work, and that from our previous studies (Chen & Dando, 2006), shows that our proposed field-induced activation in Na/K pumps can effectively increase the ionic concentration gradient across the membrane and hyperpolarize the membrane resting potential.

In terms of the concern that the field-induced membrane potentials on two hemispheres are opposite, this will not affect our results. As we used a rectangular square waveform, the opposite membrane potentials induced on the cell's two hemispheres only means a phase difference of 180°. Pump molecules on the two hemispheres were synchronized to two paces, respectively, with the same frequency but a 180° phase shift. When the synchronization frequency is increased, it accelerates all of the pumps on both hemispheres at the same rate. Phase shift will not affect ion accumulation in cells.

It is worthwhile to point out that the field strength used in this study is relatively low, only inducing a potential difference of 40 mV, peak-to-peak, across each cell membrane. The maximal unidirectional membrane potential change is only 20 mV. In real terms in fact, not all of the pump molecules within the cell membrane are exposed to a membrane potential even as high as this. Only the pump molecules located in the region of the cell membrane perpendicular to the electric field are exposed to the full potential of 40 mV. Those pumps in the region of the cell membrane parallel to the electric field are exposed to no membrane potential at all. Others are in between. Even at this low field strength and with only a partial amount of the pump molecules fully exposed to the field strength, a significant increment in the ionic concentration gradient and hyperpolarization of the membrane resting potential could be observed.

It is necessary to point out that in our theoretical studies (Chen, 2006, 2007) we were not restricted to the Na/K pumps. Any pump molecules whose ion transport steps are the rate-limiting step to their respective reaction and sensitive to the membrane potential should theoretically experience a form of synchronization by an oscillating electric field with a frequency close to the natural turnover rate.

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