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Quick and effective hyperpolarization of the membrane potential in intact smooth muscle cells of blood vessels by synchronization modulation electric field

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Abstract Blood vessel dilation starts from activation of the Na/K pumps and inward rectifier K channels in the vessel smooth muscle cells, which hyperpolarizes the cell membrane potential and closes the Ca channels. As a result, the intracellular Ca concentration reduces, and the smooth muscle cells relax and the blood vessel dilates. Activation of the Na/K pumps and the membrane potential hyperpolarization plays a critical role in blood vessel functions. Previously, we developed a new technique, synchronization modulation, to control the pump functions by electrically entraining the pump molecules. We have applied the synchronization modulation electric field noninvasively to various intact cells and demonstrated the field-induced membrane potential hyperpolarization. We further applied the electric field to blood vessels and investigated the field induced functional changes of the vessels. In this paper, we report the results in a study of the membrane potential change in the smooth muscle cells of mesenteric blood vessels in response to the oscillating electric field. We found that the synchronization modulation electric field can effectively hyperpolarize the muscle membrane potential quickly in seconds under physiological conditions.

Keywords Na/K pump · Membrane potential · Hyperpolarization · Synchronization modulation

Introduction

Membrane potential in smooth muscle cells of blood vessels play a significant role in regulation of the vessel contractile activity (Edwards et al. 1988; Haddy 1983; Hermsmeyer 1983; Knot et al. 1996). Membrane potential hyperpolarization closes dihydropyridine-sensitive L-type voltage-gated Ca channels (Jackson 2000; Welsh et al. 1998), and therefore decreases the intracellular Ca concentration. As a result, the muscle cells relax and the blood vessels dilate. The membrane potential not only regulates Ca influx through Ca channels in the plasma membrane but also influences Ca release from the intracellular organelles and Ca-sensitivity of the contractile apparatus (Jackson 2000).

Potassium is an important regulator of the membrane potential of the vessel smooth muscle cells. Activation of skeletal muscles and nerves are accompanied with opening of the ion channels and efflux of potassium ions, which elevates the local extracellular K concentration (Nelson and Quayle 1995; Quayle et al 1997). Potassium can also be released through charybdotoxin- and apamin-sensitive K channels on endothelial cells into myoendothelial space, functioning as an endothelium-derived hyperpolarizing factor (EDHF) (Edwards et al. 1998). The elevated K concentration activates the Na/K pumps in smooth muscle cells of the adjacent blood vessels (Duling 1975; Edwards et al. 1988). Pump activation hyperpolarizes the membrane potential (Duling 1975; Johansson and Somlyo 1980; Sparks 1980; Haddy 1983) that stimulates the inward rectifier K channels (McCarron and Halpern 1990; Robertson et al. 1996; Burns et al. 2004) which further hyperpolarizes the membrane potential.

Clearly, the pump activation and membrane potential hyperpolarization are the critical steps in the smooth muscle contractile activities and vascular functions. Ouabain, the pump inhibitor, has shown inhibition of the K-induced dilation of cremasteric arterioles (Lombard and Stekiel 1995), cerebral arteries (McCarron and Halpern 1990) and hepatic arteries (Edwards et al. 1988) of rats.

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In order to dilate blood vessels and increase blood flow, the pump function needs to be activated. Previously, significant efforts have been made to physically activate the Na/K pumps. Pioneering works include those by Teissie and Tsong (1980) in a study of Rb accumulation in erythrocytes showing that the Na- and K-transports of the pump molecules can be activated separately by two resonance oscillating electric fields, and those by Blank and Soo (1989) showing that an alternating current can either stimulate or inhibit ATP hydrolysis activity of the pumps depending on the concentration ratio of Na and K ions. Later, several theoretical models including resonance (Markin et al. 1992), Brownian motion (Tsong and Chang 2003), and adiabatic (Astumian 2003) were developed. To date, no practical approach is available to effectively activate the Na/K pump functions.

Recently, we developed a new technique, so called synchronization modulation, to control the pumps' turnover rate by introducing a new concept, entrainment of the pump molecules. The technique was developed based on the observation of the Na/K pumps: i) the Na- and K-transports occur sequentially in each pumping cycle; ii) both transports move cations against their electrochemical potential differences but to the opposite directions, and therefore, have reversal voltage-dependence. We can imagine a single pump molecule exposed to a special oscillating electric field so that during the Na-transport the field is always in the positive half-cycle depolarizing the membrane potential while during the K-transport the field is in the negative half-cycle hyperpolarizing the membrane potential. The electric field facilitates the two transports in two half-cycles, respectively. Assuming we can keep the field oscillating frequency matching the pump's turnover rate, the field will alternatively activate the two transports and accelerate the pumping rate.

In order to do so, the oscillating electric field has to match the pumping cycle in phase and a real-time monitor of the pumping cycle is necessary. However, it is impractical to detect the pump cycle of a single pump because only a few ions are transported in each cycle. In addition, there are hundreds and thousands of pump molecules on a cell membrane, each of which runs at a different pumping rate and a random phase. It is impossible to use one electric field to match all the pump molecules.

We introduced the concept from an electronic synchrotron accelerator to the biological system. By a specially designed oscillating electric field, we first force all the individual pumps to run at the same pace including pumping rate and phase. Then, we treat the group of synchronized pumps as a single pump to modulate their pumping rates.

The waveform and parameters of the oscillating electric field were specifically designed based on the pump physiological features including the time courses and energy barriers of the two transports, especially the reaction rates of the corresponding voltage-dependent steps. The electric field is initially oscillated with a frequency comparable to the natural turnover rates of the Na/K pumps. As the field oscillating, the Na transports from all the pump molecules are entrapped into the positive half-cycle, whilst all the K transports are similarly entrapped into the negative half-cycle. Then, by gradually changing (either increase or decrease) the synchronization frequency and carefully maintaining the pump synchronization, the pump molecules can be entrained (either accelerated or decelerated) to a defined pumping rate.

The underlying mechanisms involved in the technique (Chen 2008), computer simulations in study of the synchronization procedure (Chen and Huang 2008; Huang et al 2009), and experiment studies in direct measurements of the pump currents (Chen et al. 2007; 2008) have been reported, previously. Currently, the pumping rate can be accurately accelerated to a defined value up to 10 fold quickly in tens of seconds. Relative long (in minutes) application of the electric field non-invasively to skeletal muscle fibers (Chen and Dando 2006, 2007a), cardiomyocytes (Chen and Dando 2007b) and tissue slices (Dando et al. 2012) can elevate the ionic concentration gradients and hyperpolarize the membrane resting potential.

A question remained is whether the technique can activate the pump functions in aggregated cell tissues to hyperpolarize the membrane potential and influence the tissue functions. Bearing this question in mind, we applied the oscillating electric field non-invasively to intact mesenteric blood vessels of rats and studied the field-induced effects on the membrane potential of the vessel's smooth muscle cells and the vessel's functions. In this paper, we report the first part of the results, if the electric field can hyperpolarize the membrane potential beyond the physiological value, the efficiency of the potential changes, and how far we can go. The second part of the study in terms of the field-induced functional changes of the blood vessels will be reported separately.

Methodology

Animals and tissue preparation

All animals used in our lab was in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Research Council (USA) and approved by the University Institutional Animal Care and Use Committee. Male Sprague Dawley rats (80–150 g, Charles River Laboratories, Wilmington, MA) were euthanized with 1 μM inaction (>150 mg/kg body weight). Pieces of mesenteric intestines were removed and washed in cold Ca-free physiological salt solution (PSS: 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.4). The tissue was then placed in a

water-jacketed dissection chamber maintained at 4 °C, filled with Ca^{2+}-free PSS containing 0.1 % bovine serum albumin (BSA) and 1 mM EGTA.

The low Ca^{2+} concentration and 1 mM EGTA were used to prevent vasospasm that may occur during dissection. The mesenteric intestine was then pinned to a Sylgard 184 (Dow Corning Corp., Midland, MI) pad in a Petri dish. Third-order superior arterioles, 1–2 mm long and devoid of side branches, were isolated by hand dissection under a stereomicroscope (Nikon, Melville, NY), and transferred to a custom-built cannulation chamber. The bottom of the chamber was formed by a coverslip, and the chamber was filled with PSS containing $CaCl_2$ (1.8 mM). Procedure of vessel cannulation follows those described previously (Burns et al. 2004). Briefly, the arteriole was gently pulled onto the tips of two parallel pipettes of diameter 30–50 μm, and tied in place with two 11–0 nylon monofilament knots at each side. The length of the arteriole segments was adjusted to approximate the *in vivo* length of the arterioles. If no leaks were detected, the bath solution was slowly warmed to 34 °C with a continuous flow (2 mL/min) of PSS containing $CaCl_2$ (1.8 mM). The arterioles were pressurized to 20 cm H_2O for 30–60 min. When the arterioles reach physiological temperature the pressure was then gradually increased to 70–80 cm H_2O. It took 15–30 more minutes to stabilize the arterioles before the experiments.

Measurement of membrane potential

Membrane potential of smooth muscle cells in the walls of arterioles was measured using a glass microelectrode. Electrodes were pulled on a pipette puller (Narishige PB-7, East Meadow, NY) from borosilicate glass (1.2 mm O.D., World Precision Instruments, Sarasota, FL). The pipettes were filled with 200 mM KCl and the tip resistances is about 200–300 MΩ. The microelectrode was connected to the head-stage of a patch clamp (Dagon, Clamp One 2200) in record mode through a Lucite pipette holder by a silver wire. The head-stage and microelectrode were mounted on a 3-way electrical controlled mechanical micromanipulator. The reference electrode was Ag-AgCl wire placed in the outflow portion of the chamber. Bathing solution was continuously flowed through a micro-pump (Cole-Parmer Instrument Co. Masterflex). Labview (National Instruments, Austin Texas) data acquisition system was used to record the output of the microelectrode. The experiments were conducted under an inverted microscope (Zeiss Invertoskop, Thornwood, NY).

Before the impalement, the reading of the microelectrode was adjusted to be zero. To confirm a proper impalement of the microelectrode into the smooth muscle cells, a criterion was that the reading from the microelectrode should exhibit rapid development of a potential in a range from −30 to −40 mV with intracellular negative which returned back to zero or baseline upon withdrawal of the electrode from the cell.

To verify that the potential we recorded is from smooth muscle cells, in some experiments the tips of pipettes were filled with 200 mM KCl containing 10 mM Alexa Fluor 488. This highly fluorescent dye served as an excellent marker of cell impalement which appeared to label only single smooth muscle cells (Emerson and Segal 2000; Welsh et al. 1998; Burns et al. 2004).

Field application

Synchronization modulation electric field was generated through a purpose designed LabVIEW (Texas Instruments, Dallas, TX) program, and applied to a pair of platinum electrodes in the bathing solution 1 cm apart and parallel to the axis of the blood vessel. Strength of the pulsed oscillating electric field was adjusted from cell to cell so that the field-induced membrane potential measured by the microelectrode is about 60 mV. This potential difference is within the physiological range that is far below the thresholds of membrane electroporation [Lislie and 1992; Chen and Lee, 1994] or membrane protein denature [Chen and Lee, 1994]. The field-strength was maintained the same throughout the experiments.

Several kinds of electric fields were used, forward and backward modulations, as well as fixed frequencies. Both forward and backward modulation electric fields start from a synchronization stage consisting of 100 oscillating pulses with an initial frequency close to 50 Hz comparable to the natural pumping rates of the Na/K pumps (Rakowski et al. 1989). Our early works have shown that 100 pulses are more than enough to synchronize the pump molecules (Chen et al. 2007). In the following modulation stage, the field frequency or the pulse duration changes 3% for every 10 pulses. For the forward modulation the field frequency is gradually increased to a final value of 500 Hz in about 8 s, while for the backward modulation the field is progressively reduced to 5 Hz in tens of seconds. The last stage is the maintenance stage where the field frequency remains at the same value at the end of modulation stage until removal of the electric field. The total field application time may be slightly different from cell to cell, as indicated in the individual figures.

An electric field with a constant frequency (50 Hz or 500 Hz) was also used in some experiments where the waveform and magnitude of the electric field are exactly the same as the modulation electric field.

Our previous voltage-clamp studies showed that a pulsed waveform of the oscillating electric field has the best results in synchronization of the pump molecules. Considering the time course in charging the membrane capacitance, at the beginning of each pulse has a narrow overshoot so that the main pulses always remain a pulsed waveform.

Results and analysis

Figure 1 shows the profile of the field-induced oscillating membrane potential measured by the microelectrode from a smooth muscle cell of a blood vessel when the vessel is exposed to the forward synchronization modulation electric field. Before the field application, the natural membrane potential is about −30 mV that is consistent to the value measured from other labs (Burns et al. 2004). When the oscillating electric field is applied to the blood vessel, the membrane potential oscillates with respect to the natural membrane potential. At the beginning, due to the low frequencies, the overshoot for individual pulses is somehow distinguishable. At higher frequency, the overshoot of individual pulses is no longer distinguishable.

Interestingly, during the whole synchronization stage (the first 2 s) and the early modulation stage (up to the 6th or 7th second), the profile of the oscillating membrane potential remained the same, or average of the oscillating pulses stayed at about −30 mV, with little change from the natural membrane potential regardless of the field frequency changes. Then, the profile of the oscillating membrane potential quickly shifted to the negative direction, or the average of the oscillating pulses became more and more negative until the end of the modulation stage (at about the 9th second). It is necessary to point out that the field's waveform and magnitude remained the same except the frequency increase. At the time a little over the 9th second or at the end of the modulation stage where the frequency became the maxim, the profile of the oscillating membrane potential reached the maximum negative value. Then, the potential profile stopped shifting as the field frequency was no longer increased.

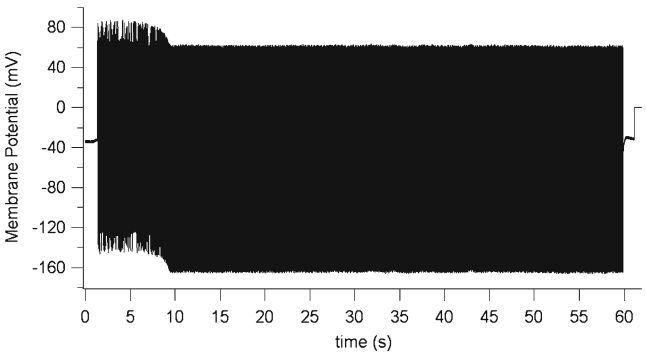

Fig. 1 The profile of the field-induced membrane potential in response to the oscillating electric field. Because of large amount (over 1000) pulses, we can only observe the profile but cannot identify individual pulses. Before the field application, the natural membrane potential is about −30 mV. At the synchronization stage and early modulation stage, the average value of the oscillating membrane potential is the same as the control value. Then, the profile is gradually shifted to the negative direction indicating the membrane potential hyperpolarization. As long as the field frequency stops increasing at about the 9th second, the potential profile remains the same

Figure 2 shows the details of the oscillating membrane potential at different stages. Figure 2a is at the beginning of the field application in the synchronization stage. The membrane potential oscillated from about 2 mV to −58 mV with an average of −30 mV, the same as that before the field application. Figure 2b shows the oscillating membrane potential measured at about the 8th second in the later stage of the modulation, oscillating from - 8 mV to - 63 mV with an average of 36 mV which was shifted down approximately 6 mV. Figure 2c shows the membrane potential measured at a little over the 9th second or the end of the modulation stage where the frequency reached the maximum value of 500 Hz. The membrane potential oscillated from −22 mV to −78 mV with an average of −50 mV, negatively shifted for 20 mV. Finally, Fig. 2d is the membrane potential measured at about 59 s, the end of the maintaining stage, oscillating from −23 mV to −79 mV, almost the same as those measured at the end of modulation stage shown in Fig. 2c.

The results showed that due to the field application, the membrane potential was shifted to the negative direction, or hyperpolarized for about 20 mV. As long as the field frequency stopped increasing, the membrane potential remained at the same value. In order to prove that the membrane potential hyperpolarization is due to the Na/K pump acceleration by the synchronization modulation electric field, we have to clarify i) it is attributed to the Na/K pumps, and ii) solely depends on the frequency modulation.

In order to confirm that the membrane potential hyperpolarization is contributed by the Na/K pumps, 1 mM ouabain, a specific inhibitor of the Na/K pump was used in bathing solution to block the pump functions. We re-applied the forward modulation electric field and re-measured the field-induced membrane potential oscillation. The results are shown in Fig. 3. Again, at the beginning of the field application, due to the low frequency, the overshoot of each pulse is somehow distinguishable. Later, as the frequency increased, the overshoot can no longer be distinguished. The details of the oscillating membrane potential measured at the beginning of the field application of 50 Hz and at the end of the field-application of 500 Hz are shown in Fig. 3b and c, respectively. The magnitude of the oscillating membrane potential remains the same regardless of the frequency change. Even though the frequency increased for 10 times, the average of the oscillating membrane potential remained at the same about −30 mV. The field-induced negative shift of the membrane potential disappeared.

Inhibition of the Na/K pump functions eliminating the field-induced membrane potential hyperpolarization confirms that the potential hyperpolarization is due to the Na/K pumps.

One of the key concepts of the technique is gradual entrainment of the pump molecules by the forward frequency modulation so that the pumping rate can be accelerated step-by-step. In other words, the pattern of the frequency

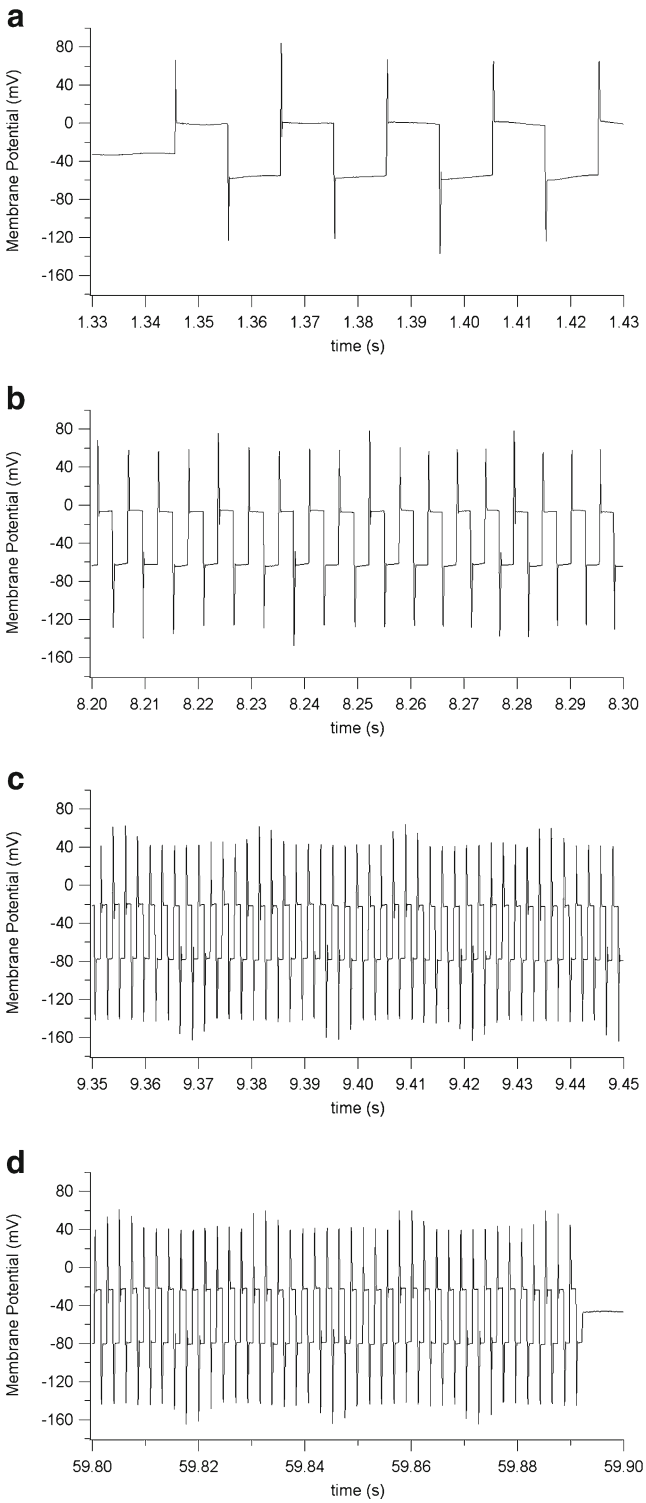

Fig. 2 Details of the field-induced oscillating membrane potential: **a** at the beginning of the field application, the synchronization stage; **b** during the modulation stage; **c**: at end of the modulation, and **d** at the end of field-application. The transient overshoot current at the beginning of each half-pulse is due to over compensation of the membrane capacitance currents by the overshoot pre-pulse

change is critical. To clarify this concept, we applied the oscillating electric field to the blood vessel with exactly the same waveform and magnitude, but i) with a fixed oscillating frequency without modulations or ii) with backward modulations. Figure 4a shows the results of the oscillating membrane potential in response to a 50 Hz electric field. Because of the low frequency, the overshoot of individual pulses can somehow be distinguished throughout the field application. The profile of the oscillating membrane potential exhibits no shift. Figure 4b is the membrane potential measured at the beginning of the field application oscillating from −5 mV to −75 mV with an average of −40 mV. At the end of the field application, about the 60th second, the

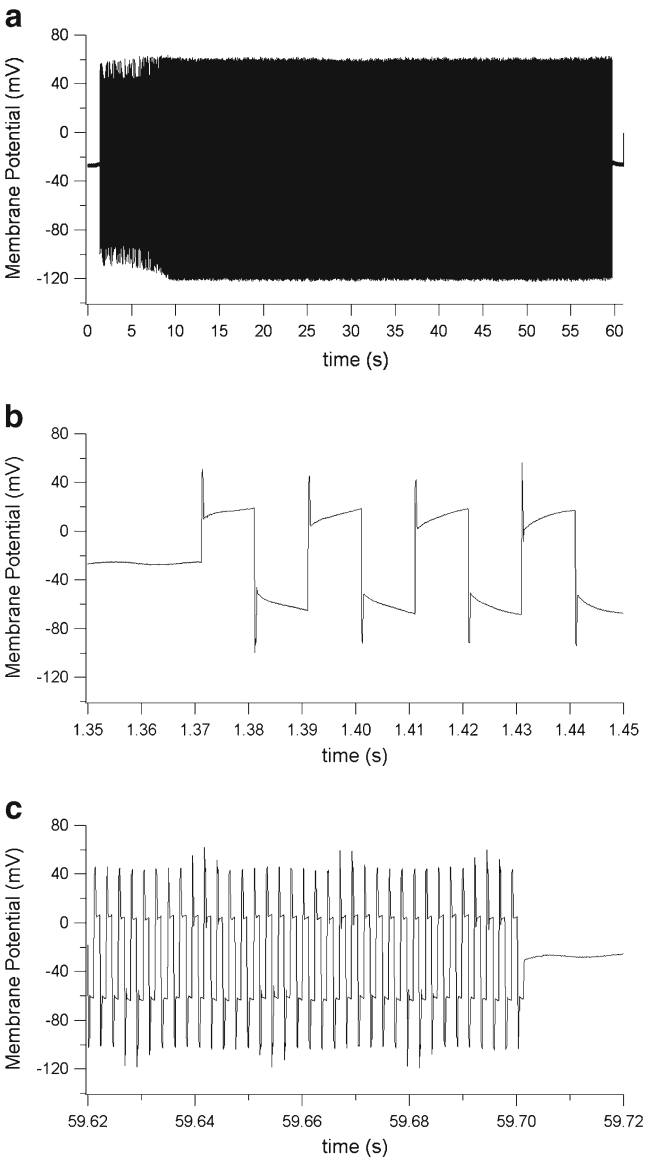

Fig. 3 **a** The profile of the field-induced oscillating membrane potential in the presence of 1 mM ouabain in response to the same forward modulation electric field as in Fig. 1. **b** and **c**: details of the measured oscillating membrane potential at the beginning and the end of the field-application, respectively. The profile of the oscillating membrane potential remains the same regardless of the frequency changes

membrane potential was oscillated from −2 mV to −74 mV with an average of −38 mV, as shown in Fig. 4c. Clearly, a fixed 50 Hz oscillating electric field cannot hyperpolarize the membrane potential. The small change may be due to drifting of the membrane potential during the measurement.

We also applied the same oscillating electric field with a fixed frequency of 500 Hz to the blood vessels. The field-induced oscillating membrane potential is shown in Fig. 5. Due to a relatively high frequency, the overshoots of individual pulses are not distinguishable in the figure. The profile of the membrane potential oscillation remains unchanged throughout the field application. This result shows that direct application of a high frequency oscillating electric field cannot hyperpolarize the membrane potential, which is consistent to our previous results that the Na/K pumping rate can only be progressively entrained to a higher value.

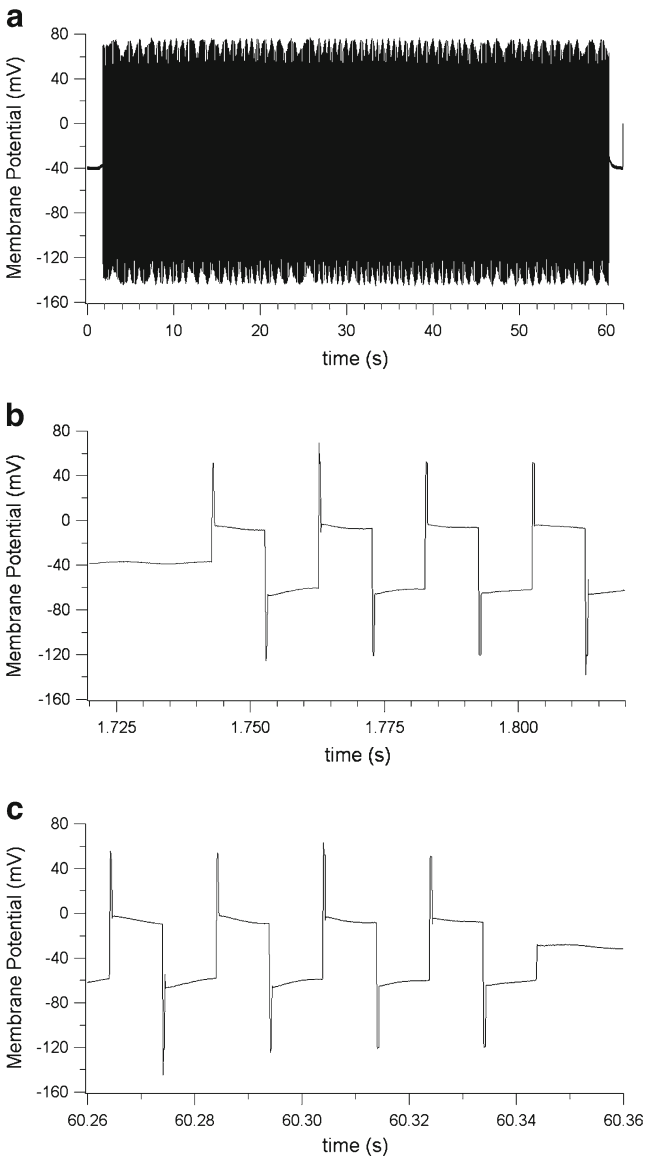

Fig. 4 **a** Profile of the field-induced membrane potential in response to the same oscillating electric field but with a constant frequency of 50 Hz. **b** and **c** are the membrane potential measured at the beginning and the end of the field application, respectively. A 50 Hz oscillating electric field cannot hyperpolarize the membrane potential

In contrast to the forward modulation electric field, we also applied the backward modulation electric field to the blood vessels, and monitored the field-induced membrane potential changes. Again, the synchronization stage consists of 100 pulses of 50 Hz. Then, the frequency was gradually reduced to 5 Hz with the same 3% step-change for every 10 pulses followed by a constant frequency of 5 Hz until removal of the field. Figure 6a shows the field-induced oscillating membrane potential throughout the field application. Because of the frequency gradually reducing, the overshoot of individual pulses becomes more and more distinguishable. Again, no obvious shift or hyperpolarization of the membrane potential is observed. Figure 6b shows the oscillating membrane potential measured at the beginning of the field application in the synchronization stage. The averaged membrane potential is about −38 mV very close to the value in the control before the field application, which remains relatively unchanged until the end of the field application where the frequency is only 5 Hz, as shown in Figure 6c. Please notice the scale in Figure 6c is ten times larger than that of 6b.

The results shown in Figs. 4, 5 and 6 are functioning as another kind of control. All the electric fields have the same waveform and magnitude with exactly the same field-application protocol, but cannot hyperpolarize the membrane potential at all. Those results rule out the possibility that the membrane potential hyperpolarization shown in Fig. 1 is simply due to the field application. Instead, only the specially designed frequency modulation can entrain the pump molecules to run at higher and higher pumping rates, and therefore hyperpolarize the membrane potential.

Two more questions still remain: i) how can we quantitatively prove that the membrane hyperpolarization is due to the activation of the Na/K pump by the synchronization modulation electric field? And why does the hyperpolarization mainly occur at later stage of the modulation? ii) If the backward modulation decelerates the pumping rate as show previously in our whole cell voltage-clamp experiments, why cannot it depolarize the membrane potential?

In order to answer the first question, we can simply compare the time course of the membrane potential change with the time course of the field frequency change. We rescale the synchronization and modulation stages in Fig. 1 and reprint them in Fig. 7a. Averages of the oscillating membrane potential in each cycle are plotted in Fig. 7b, which represents the membrane potential change in response to the synchronization modulation electric field.

There are two aspects that contributed to the membrane potential, the ionic equilibrium potential and the Na/K pump currents induced voltage-drop on the cell membrane. The

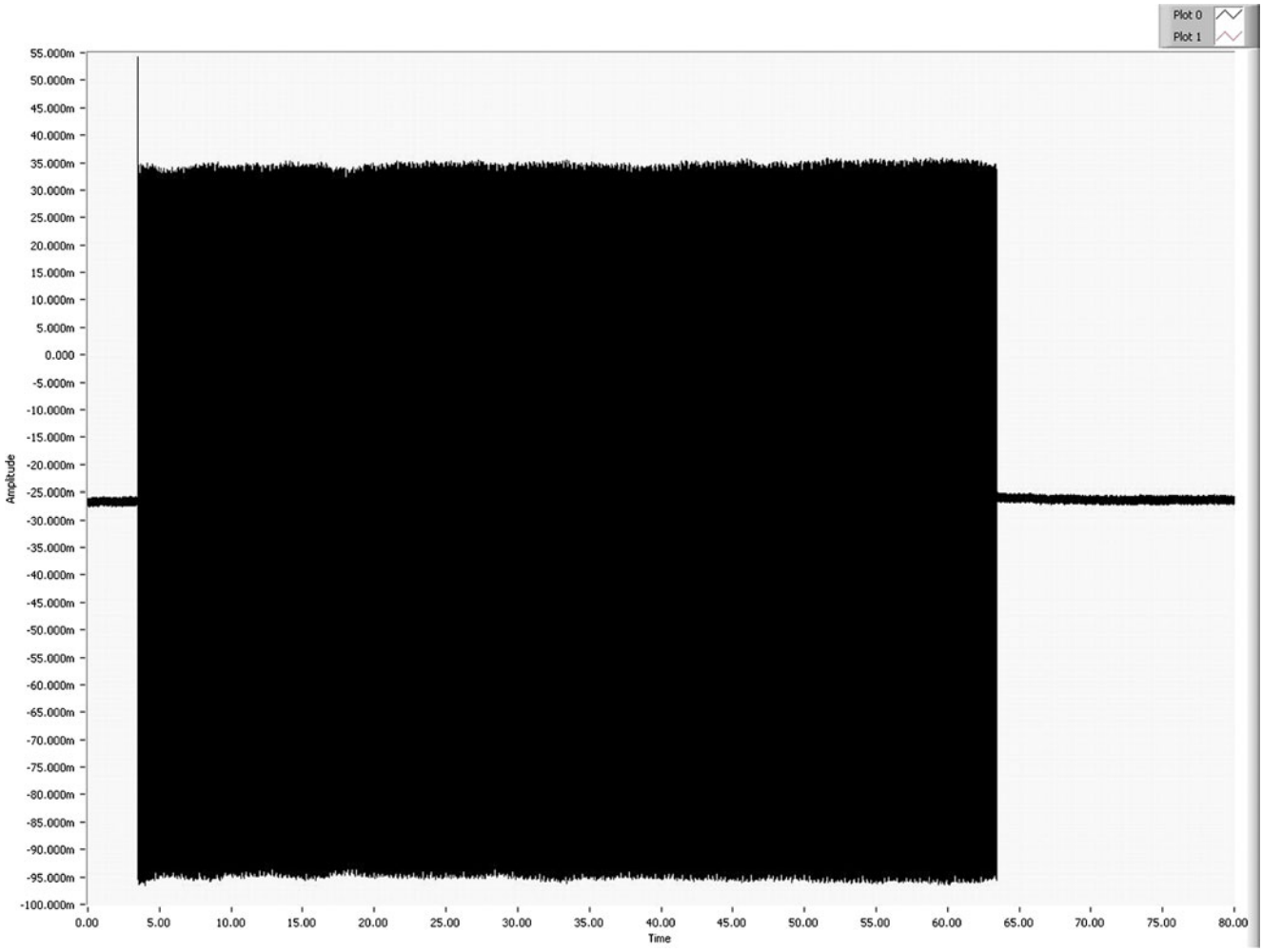

Fig. 5 Profile of the field-induced oscillating membrane potential in response to a 500 Hz oscillating electric field. Even though the frequency is ten times higher than the pump natural turnover rate, the electric field cannot influence the membrane potential

former depends on the ionic concentration gradients. Due to only 3 Na and 2 K ions being transported in each pump cycle compared to a huge number of ions in the cells, it takes time for the pump molecules to accumulate the effects in changing the ionic concentrations. In contrast, the latter is a transient effect without need of accumulation, which can be calculated by multiplying the pump currents and the membrane resistance. Considering extruding 3 Na ions by exchanging 2 K ions in each cycle, the net pump current is always outward hyperpolarizing the membrane potential. With a physiological pumping rate the pump current-induced membrane potential hyperpolarization is only few (1–3) mV (Fromter 1979; Weiss 1996). This value is trivial comparing to the ionic equilibrium potential in a range of tens of mV. That is why scientists often use an ionic concentration gradient to calculate the membrane potential. However, when the pumping rate is significantly accelerated or the pump currents are dramatically increased by the synchronization modulation electric field, the pump-current induced membrane hyperpolarization can no longer be negligible.

Based on the control value of the membrane potential of −30 mV, assume that the membrane potential due to the pump-current is about −2 mV and therefore the ionic concentration gradient contribution is −28 mV. When the pumping rates are increased 10 fold, the contribution of the pump-current to the membrane potential should also increase to −20 mV. As a result, the total membrane should hyperpolarize to −48 mV, which is very consistent with the membrane potential we measured at the end of the modulation, -50 mV. After that, the field frequency remains unchanged, the contribution of the pump current to the membrane potential also keeps the same.

The detailed changes in the membrane potential as a function of time can be estimated based on the changes in the pumping rate. During the synchronization stage (in the first 2 s), the pumps are only forced to run at the same pace without acceleration, the pump currents and therefore, the membrane potential should remain the same as those in the control. Then,

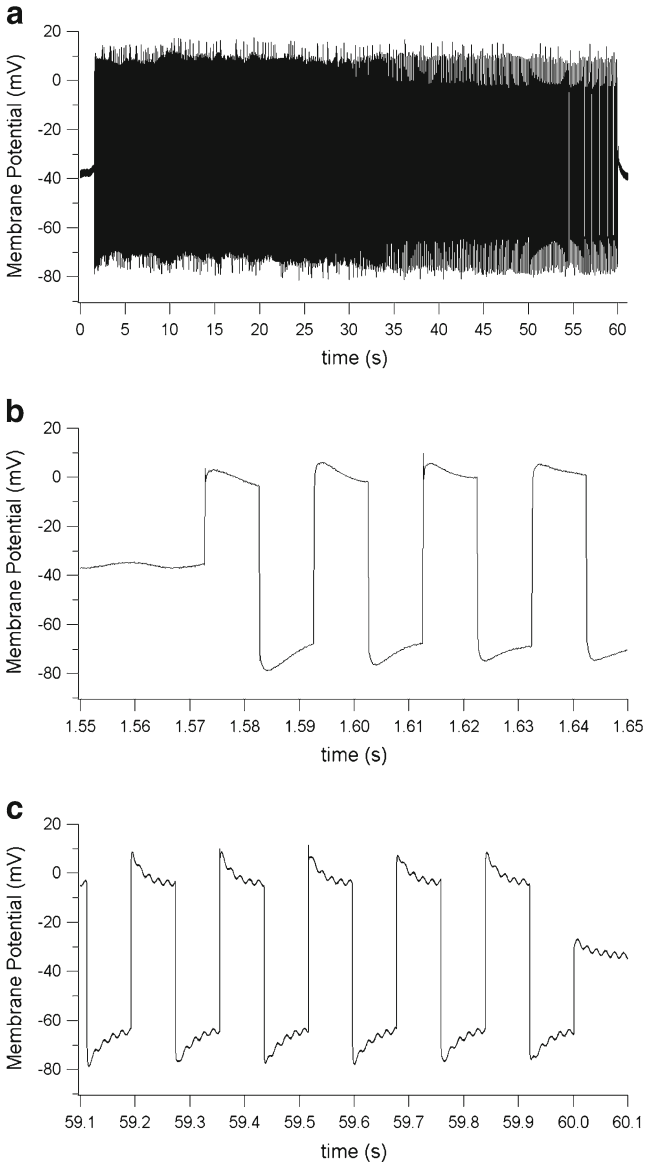

Fig. 6 The backward modulation electric field does not show noticeable depolarization of the membrane potential. **a** Profile of the field-induced oscillating membrane potential in response to a backward modulation electric field (see the text). The irregular edge of the profile is due to interference of the oscillating electric field with power line electric field. **b** and **c** are the oscillating membrane potential measured at the beginning and end of the field application, respectively

the field frequency is increased by 3% becoming 51.5 Hz, or the full-cycle is decreased from 20 to 19.4 ms in the first step, which accelerates the pumping rate. The pump-current contribution to the membrane potential should also increase 3% from −2 to −2.06 mV, resulting in the membrane potential of about −30.06 mV. Due to 10 pulses in each step, this value lasts from 2 to 2.0194 s. In the next step, the pumping rate is accelerated for another 3% reaching 53 Hz and the membrane potential becomes −30.12 mV lasting from 2.194 to 2.382 s. Similarly, we can estimate the membrane potential changes for all individual steps throughout the modulation stage based on the field oscillating frequency or the pumping rate. The results are shown in Fig. 8.

Comparison of Figs. 8 and 7b shows that the trends of the membrane potential hyperpolarization estimated based on the changes in the field frequency and pumping rate, are very consistent to the average membrane potential we measured in the experiments. This comparison indicates that the membrane potential hyperpolarization is solely due to the pumping-rate acceleration.

Due to gradually reducing the pulse-duration, the duration of each modulation step (10 pulses) becomes shorter and shorter. At the beginning of the modulation, 10 pulses of 50 Hz take 200 ms while at the end, 10 pulses of 500 Hz take only 20 ms. Whilst the percentage change in the pumping rate and therefore in the pump-current contribution to the membrane potential remains the same of 3%, the time-course of the membrane potential hyperpolarization becomes more and more steep towards the end of the modulation stage.

In terms of the second question, why did not the membrane potential significantly depolarize in response to the backward modulation electric field, where the pumping rate was reduced 10 times from 50 Hz to 5 Hz. That is because under physiological conditions, the pump current induced membrane potential is only about −2 mV which is much smaller than the equilibrium potential. Reduction in the pumping rate makes the contribution even smaller such that it may not be observable due to the membrane potential drifting.

Conclusions

In our previous whole-cell patch clamp studies, we demonstrated the synchronization and modulation of the Na/K pumps by directly measuring the pump currents. Here, we further showed that the synchronization modulation electric field can effectively hyperpolarize the membrane potential in smooth muscle cells of mesenteric blood vessels under physiological conditions. The trends and time courses of the membrane potential hyperpolarization are consistent with the time course of the pumping rate acceleration. This result along with those using ouabain to block the pump functions and using constant frequencies or backward modulation provide further evidence that the transient membrane potential hyperpolarization is due to the pumping rate modulation of the Na/K pumps.

The significant increase in the membrane hyperpolarization confirms our previous studies in direct measurement of the pump currents that the synchronization modulation technique can drastically accelerate the pumping rate. Currently, we are able to increase the pumping rate ten fold. To the best of our knowledge, to date, this is the first time a practical technique has been shown to significantly accelerate the pumping rate of the Na/K pumps. In a landmark work, Teissie and Tsong

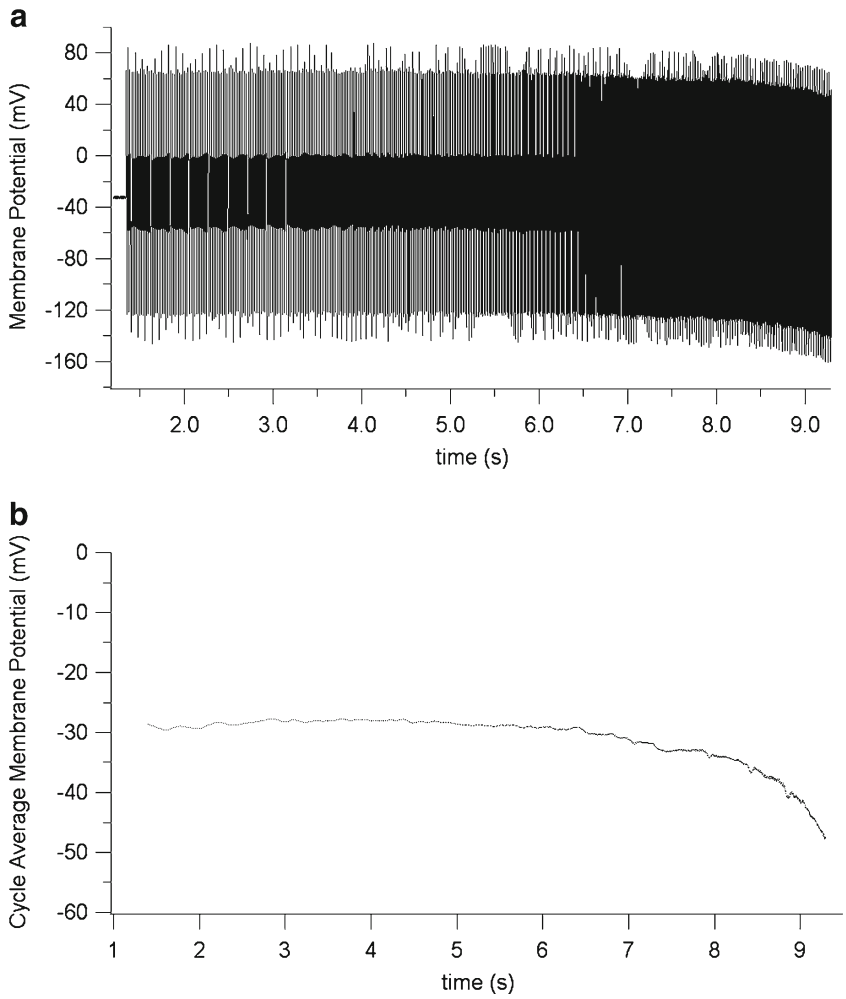

Fig. 7 **a** Redrawn of the field-induced oscillating membrane potential during the synchronization and modulation stages as shown in Fig. 1. **b** Average of the oscillating membrane potential as a function of time, which shows a gradual hyperpolarization in response to the forward synchronization modulation electric field

(1980) reported that a weak oscillating electric field with mega-Hz frequency can activate the Na transport while a field with kilo-Hz frequency can activate the K transport for about 30%, respectively. No other experimental results have demonstrated significant acceleration of the whole pumping rate.

In general, membrane potential is a characteristic property of cells, which remains relatively constant due to natural control mechanisms. Whenever, the membrane potential is depolarized, Na/K pumps will be activated to reinstate the physiological value. As long as the membrane potential is closer to the physiological value, the negative feedback mechanism slows down the pumping rates. Therefore, the membrane potential can be restored but seldom hyperpolarized.

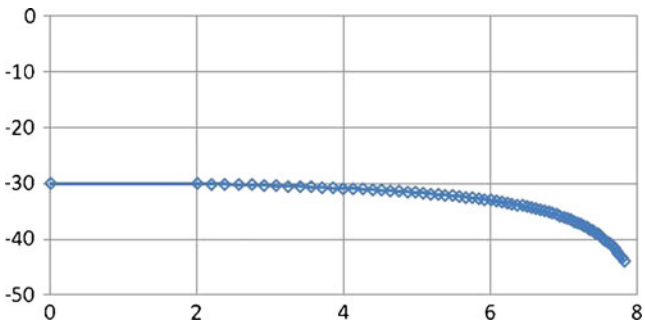

Fig. 8 Estimation of the membrane potential change in response to the forward synchronization modulation electric field based on the changes in the pumping rates. The trend is very similar to the experimental results shown in Fig. 7b

In previous studies of single skeletal muscle fibers, we demonstrated that the synchronization modulation electric field can facilitate the process of the membrane potential reinstallation for the electroporated cells (Chen and Dando 2008). Results from this study in smooth muscle cells of mesenteric blood vessels show that even under physiological conditions the synchronization modulation technique can override the natural mechanisms, continuously accelerating the pumping rate and quickly hyperpolarizing the membrane potential.

This is not surprising based on the mechanisms involved in the synchronization modulation technique. Most other techniques in activating the pump functions are passive, mainly providing a favorable environment for the pump molecules. For example, increasing in the intracellular Na or extracellular K ions raises the ion availability and binding probability to the

pump molecules which is a favorable condition for the pump running. Within minutes of elevated insulin secretion, pumps containing alpha-1 and 2 isoforms have increased affinity for sodium ions, and therefore accelerate their turnover rates (Jorgensen et al. 2003). In either way, no energy is provided to the pump molecules. Nakao and Gadsby (1989) have found that varying the concentration of extracellular K or intracellular Na merely leads to an up- or down-shifting of the *I-V* curve without appreciably changing the sigmoidal shape of the *I-V* curve. In contrast, the synchronization modulation electric field provides energy to the pump molecules to reduce the energy barriers of the two transports during the two half-cycles, respectively. By this way, the electric field forces the pumps running at the same turnover rate as the field oscillating frequency. Therefore, the technique can override the negative feedback mechanisms in accelerating the pumping rates even through the membrane potential is already hyperpolarized beyond the natural membrane potential.

In addition, other techniques or chemical treatments can only qualitatively influence the pump functions, but cannot accurately control the pumping rate. In contrast, the synchronization modulation technique can accelerate or decelerate the pumping rate accurately to a defined value simply by changing the parameters of the oscillating electric field.

Finally, the response is transient having no comparison with any chemical treatment. The synchronization modulation technique can quickly or transiently hyperpolarize the membrane potential in seconds, which is much more efficient than any chemical treatments. For example, even for single cells in bathing solution it takes minutes to half-hour for ouabain to show blockage of the Na/K pump currents.

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