Recognition and Processing of Randomly Fluctuating Electric Signals by Na,K-ATPase

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ABSTRACT Previous work has shown that Na,K-ATPase of human erythrocytes can extract free energy from sinusoidal electric fields to pump cations up their respective concentration gradients. Because regularly oscillating waveform is not a feature of the transmembrane electric potential of cells, questions have been raised whether these observed effects are biologically relevant. Here we show that a random-telegraph fluctuating electric field (RTF) consisting of alternating square electric pulses with random lifetimes can also stimulate the Rb^+-pumping mode of the Na,K-ATPase. The net RTF-stimulated, ouabain-sensitive Rb^+ pumping was monitored with $^{86}Rb^+$. The tracer-measured, Rb^+ influx exhibited frequency and amplitude dependencies that peaked at the mean frequency of 1.0 kHz and amplitude of 20 V/cm. At 4°C, the maximal pumping activity under these optimal conditions was 28 Rb^+/RBC-hr, which is approximately 50% higher than that obtained with the sinusoidal electric field. These findings indicate that Na,K-ATPase can recognize an electric signal, either regularly oscillatory or randomly fluctuating, for energy coupling, with high fidelity. The use of RTF for activation also allowed a quantitative theoretical analysis of kinetics of a membrane transport model of any complexity according to the theory of electroconformational coupling (ECC) by the diagram methods. A four-state ECC model was shown to produce the amplitude and the frequency windows of the Rb^+-pumping if the free energy of interaction of the transporter with the membrane potential was to include a nonlinear quadratic term. Kinetic constants for the ECC model have been derived. These results indicate that the ECC is a plausible mechanism for the recognition and processing of electric signals by proteins of the cell membrane.

INTRODUCTION

Time-dependent oscillation of chemical reactions has been found in many biological systems and is postulated to be an effective molecular mechanism for cellular transduction of energy and signals (Kalmijn, 1982; Bullock and Heilingenberg, 1986; Tsong, 1989, 1992). For example, in response to binding of an agonist to a membrane receptor, many hormone-secreting cells display oscillations in cytoplasmic calcium, $[Ca^{2+}]_i$, and transmembrane electric potential, $\Delta\psi_m$ (Atwater et al., 1979; Berridge, 1990; Tsien and Tsien, 1990; Meyer and Stryer, 1991). The concentration of cAMP in culture medium also shows periodic patterns during certain phases of the cell congregation in the slime mold (Tomchik and Devreotes, 1981), and there are oscillations of filamental structures at the level of molecular assembling. Do these oscillations of physical properties or concentrations have special effects to the cellular reactions, and if so, how? In vitro, alternating electric or electromagnetic fields have been shown to affect many biological reactions inside a cell (Becker, 1981; Blank and Findl, 1987; Tsong, 1989, 1992). Effects of an electric field on a cell are most apparent on plasma membrane because an external field is greatly amplified at the plasma membrane (Cevc, 1990; Tsong and Astumian, 1987; Tsong, 1991). For a spherical cell, an applied electric field of strength E can generate a maximal $\Delta\psi_m$ of 1.5 $R_{cell}E$. The effective transmembrane electric field, V, is equal to $(1.5R_{cell}/d_m)E$ at the site of the plasma membrane (R_{cell} being the radius of cell and d_m the thickness of the bilayer, or roughly 5 nm). The amplification of E is approximately 1,000 for a cell the size of a human red cell (Cevc, 1990; Tsong and Astumian, 1987; Tsong, 1991). Thus, plasma membrane is a logical place to search for the molecular basis of electric field interactions with living cells.

Previous studies have shown that Na,K-ATPase of human erythrocytes is one of many membrane enzymes that are affected by sinusoidal (AC) electric fields (Serpersu and Tsong, 1983, 1984; Liu et al., 1990). It was found that the K^+- and the Na^+-pumping modes of the enzyme were activated by the AC fields: ouabain-sensitive pumping of these ions up their respective concentration gradients was detected by the radioactive tracers, $^{45}K^+$, $^{86}Rb^+$, and $^{22}Na^+$. Because these pumping activities were not dependent on the cellular ATP concentration in the range 10 μM to 1 mM, AC stimulation of ATP hydrolysis was discounted, and it was concluded that the enzyme must be able to absorb free energy directly from the AC field and convert it to the chemical potential energy of an ion. This interpretation was supported by the analysis of a four-state membrane transport model based on the theory of electroconformational coupling (ECC) (Tsong and Astumian, 1986, 1988; Westerhoff et al., 1986; Astumian et al., 1989; Chen, 1987; Tsong, 1990). According to the ECC, a transmembrane enzyme that possesses two

Received for publication 15 November 1993 and in final form 8 June 1994.

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0006-3495/94/09/1247/05 $2.00

conformational forms with different charge distributions, or electric moments, can convert from one form to another under the influence of a $\Delta\psi_{tm}$. An AC field of appropriate amplitude and frequency can drive the catalytic wheel of the membrane transport system to turn in unidirection, and in so doing, transfer its energy to pump a ligand, or ion, across the membrane (Tsong and Astumian, 1986, 1988; Tsong, 1990, 1992). In experiments, this means an optimal frequency and amplitude of AC for the stimulated transport of each ion (Serpersu and Tsong, 1983, 1984; Liu et al., 1990).

The use of electric fields with a sinusoidal waveform (Fig. 1 *a*) for experiments has certain limitations, and constraints must be applied to analyze the results (Tsong and Astumian, 1986, 1988; Westerhoff et al., 1986; Astumian et al., 1989; Chen, 1987). There are several reasons to examine other types of oscillating electric fields. First, while in a cell, the transmembrane electric potential can be fluctuating, at least locally, but a sinusoidal waveform is too regular to be a feature of such fluctuations (DeFelice, 1981). Second, with the sinusoidal waveform, an analytical solution for a four-state membrane transport model cannot be derived (Westerhoff et al., 1986; Astumian et al., 1989; Chen, 1987). Most previous calculations were performed under limiting conditions, and many approximations were required to simulate experimental data (Tsong and Astumian, 1986, 1988; Astumian et al., 1987; Markin et al., 1990; Markin and Tsong, 1991a, b, 1993). Third, according to the ECC model, an enzyme, which can perform energy coupling with a sinusoidal AC field, should also be able to perform similar tasks with other forms of fluctuating electric fields (Astumian et al., 1987; Chen, 1987). This prediction of the ECC model has never been tested experimentally. We report the stimulation of the Rb^+ pumping mode of the Na,K-ATPase by using random telegraph fluctuation (RTF) electric fields and the analysis of these results by the four-state ECC model.

MATERIALS AND METHODS

Generation of RTF field

An RTF field is characterized by a series of square wave electric pulses between $+n$ V/cm to $-n$ V/cm, with pulse duration (t) distributed randomly according to an exponential distribution function (Astumian et al., 1987), $t = -\tau^* \ln(R)$, in which τ^* is the mean lifetime, ln, the natural logarithmic function, and R, a random number between 0 to 1 (in actual experiment, only random numbers between 0.01 and 0.99 were used). The mean frequency, $f^* = 1/(2\tau^*)$, were chosen at several values in the range 100 Hz to 50 kHz. First, an IBM compatible PC (Dell Computer System 310, CPU 80386, CPU speed 20 MHz) was used to generate a train of triggering signals using TurboPascal random number generator. These signals were used to drive a Wavetek Model 166 50 MHz Pulse/Function Generator through an interfacing board, CyberResearch, Model PC-66A (2 μs setting time, 12-bit resolution). In Fig. 1 *a*, a short segment of the RTF ($f^* = 1$ kHz) used in the experiment is shown. The frequency distribution spectrum (or the histogram of lifetime distribution) of this signal is given in Fig. 1 *b*. In Fig. 1 *a*, the sinusoidal and the regular square waves ($f = 1$ kHz) are also given to compare with the RTF signal. For the two regularly oscillatory fields, the frequency distribution is a delta function at 1 kHz, as shown in Fig. 1 *b*.

Rb^+ transport measurement

$^{86}Rb^+$ was used to measure the ouabain-sensitive influx and efflux of the ion, as described previously (Serpersu and Tsong, 1983, 1984; Liu et al., 1990). Freshly prepared human erythrocyte was incubated in an isotonic solution containing RbCl to pre-load Rb^+ into the cytoplasm. The erythrocyte samples used for the electric stimulation experiment contained 5 mM Na^+, 16 mM Rb^+, and 105 mM K^+. The external medium contained 10 mM Rb^+, 0.5 mM $MgCl_2$, 10 mM Tris-HCl at pH 7.4 and 5–140 mM NaCl, plus a suitable amount of sucrose to make the solution isotonic, and some $^{86}Rb^+$ tracer. For a given set of conditions, Rb^+ influx was measured for four samples: the first one without ouabain and no electric stimulation (NS), the second one in the presence of 0.2 mM ouabain and no electric stimulation (ONS), the third one treated with electric field in the presence of 0.2 mM ouabain (OS), and the fourth one treated with electric field in the absence of ouabain (S). The ion flux was measured at 3 ± 1°C for 30 min, and Rb^+ influx was expressed in attomole (10^{-18} mol) per red cell per hour to compare with our previous experiments using sinusoidal electric fields. The quantity NS measured the normal, nonspecific leak current of Rb^+; (NS − ONS) measured the ATP-dependent Rb^+ pumping by the Na,K-ATPase; and (S − OS) measured the net RTF-stimulated Rb^+ pumping activity.

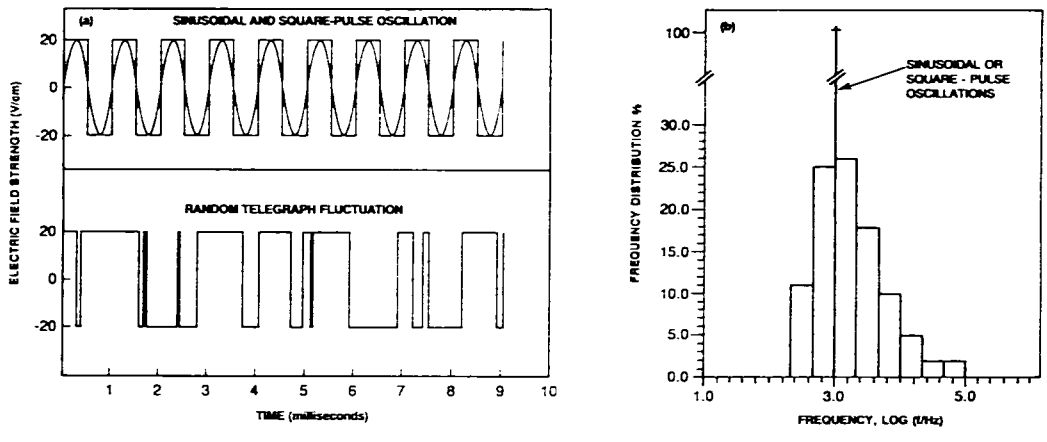

FIGURE 1 (*a*) Three waveforms used for the electric activation of Na,K-ATPase are shown. The sinusoidal, and the square waves (*top*) have the frequency of 1.00 kHz. The RTF waveform (*bottom*) has a mean frequency of 1.00 kHz. (*b*) The histogram of the RTF signal shown in *a* is given. The histogram shows fractions of time occupied by pulses in a certain range of the mean lifetime, by integrating occurrences against time. The delta function occurring at 1.00 kHz is the frequency of the sinusoidal and the square waveforms.

The chamber for electric stimulation has been described elsewhere (Serpersu and Tsong, 1983, 1984; Liu et al., 1990). Two platinized platinum sheets spaced at 2–3 mm were used as electrodes. The sample volume was 150 μl. The temperature of the sample was maintained at 3 ± 1°C by a refrigerated water circulator and was monitored frequently with a thermistor probe with a time constant of less than 100 ms. After the electric stimulation, 3 aliquots from each sample were drawn and Rb^+ influx was determined by radioactivity counting. When efflux of Rb^+ was to be measured, erythrocytes were pre-loaded with $^{86}Rb^+$ tracer. Other procedures were identical to the published procedures (Serpersu and Tsong, 1983, 1984; Liu et al., 1990).

RESULTS

RTF-stimulated Rb^+ influx

As was predicted by the analysis of theory of the electroconformational coupling, RTF stimulated the Rb^+-pumping mode of the Na,K-ATPase (Astumian et al., 1987; Chen, 1987). The RTF-stimulated Rb^+ influx was completely blocked by 0.2 mM ouabain, indicating that the RTF-induced ion transport was mediated by the Na,K-ATPase. The net RTF stimulated activity, (S − OS), was approximately 50% higher than that obtained with the sinusoidal electric field under comparable experimental conditions. It was found that the RTF stimulated only the influx of Rb^+, but not the efflux, as was the case with the sinusoidal electric fields.

The RTF-stimulated Rb^+ pumping was also dependent on the amplitude of the electric field and the mean frequency of the RTF, as was found with the sinusoidal electric field. Fig. 2 *a* presents data obtained with an RTF of $f^* = 1.00$ kHz, at different values for amplitude (one-half of the peak-to-peak value), and Fig. 2 *b* presents data obtained with a field amplitude of 20 V/cm at varied f^*. The maximal, net electric field-stimulated, Na,K-ATPase-mediated Rb^+ pumping activity, when an RTF of 20 V/cm^{-1} kHz was used, was 28 ± 4 attomole per cell per hour (U) ($n = 4$). Experiment with the same erythrocyte sample stimulated with a sinusoidal electric field of 20 V/cm^{-1} kHz, gave a value of 20 ± 4 U ($n = 4$). These values are to be compared with the maximal activity of 15 ± 5 U in previous experiments with sinusoidal fields under identical solvent conditions but with erythrocytes from several individuals (Serpersu and Tsong, 1983, 1984; Liu et al., 1990). The RTF-stimulated activity is roughly 50% higher than the average stimulated activity with the sinusoidal electric field.

Analysis by electroconformational coupling model

Aside from some considerations discussed above, one advantage of using RTF fields for the experiments is that transport kinetics at steady state can be solved analytically using the diagram method (Chen, 1987; Hill and Chen, 1972). The model we used to simulate the data in Fig. 2 is schematically shown in Fig. 3 *a*. This is the usual four-state electroconformational coupling model (Tsong and Astumian, 1986, 1988; Astumian et al., 1987, 1989; Chen, 1987; Markin and Tsong, 1991a, b; 1993). The corresponding kinetic diagrams in the presence of a constant, nonoscillating membrane potential and an RTF field are shown, respectively, in Fig. 3, *b* and *c*. For a given set of rate parameters, the transport flux can be calculated using the standard matrix method (Westerhoff et al., 1986; Chen, 1987). Note that the four-state model we used for analysis is a truncated model that allows simplification of analysis for the sake of clarity. This means that the Na^+-pumping mode was excluded from consider-

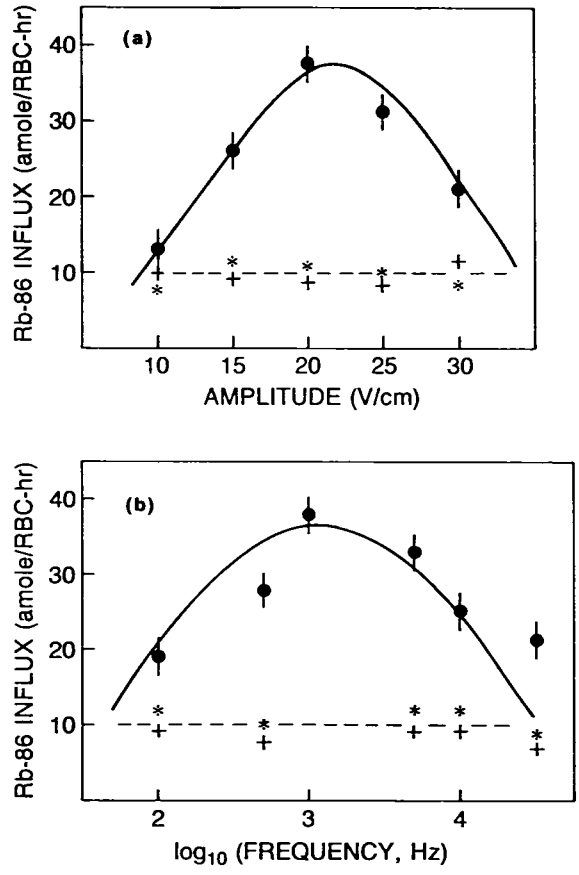

FIGURE 2 (*a*) The RTF-stimulated Rb^+ uptake is plotted against the amplitude (one-half the peak-to-peak V/cm) of an RTF with a mean frequency of 1.00 kHz. The erythrocyte sample contained approximately 5 mM Na^+, 16 mM Rb^+ (loaded by incubation or electroporation method (see Tsong, 1991) and 105 mM K^+. The external medium contained 10 mM Rb^+, 0.5 mM $MgCl_2$, 10 mM Tris-HCl at pH 7.4, 5–140 mM NaCl (plus suitable amount of sucrose to make the solution isotonic), and some $^{86}Rb^+$ tracer. In each experiment, four samples were done simultaneously, one RTF-stimulated sample (●), one RTF-stimulated sample, in the presence of 0.2 mM ouabain (+), one nonstimulated sample (data similar to +), and the fourth, nonstimulated sample, in the presence of 0.2 mM ouabain (*). The average value for the nonstimulated samples was 10.5 ± 1.1 amol/(RBC-h) ($n = 4$). The temperature for experiments was 3 ± 1°C. In these experiments, no RTF-stimulated, ouabain-sensitive Rb^+ efflux was detected, as was the case in previous experiments with sinusoidal electric fields (Serpersu and Tsong, 1983; 1984; Liu et al., 1990). Data are expressed as attomole per red cell per hour, to be consistent with previous publications (Serpersu and Tsong, 1983, 1984; Liu et al., 1990). (*b*) RTFs of the amplitude 20 V/cm, with varying mean frequencies were used for experiments. Symbols are the same as in *a*. In both *a* and *b*, the solid lines are simulations of data according to the ECC model, using a four-state transport kinetic scheme, as discussed in Fig. 3. In a similar experiment using the sinusoidal electric field of 20 V/cm^{-1} kHz for stimulation, the net stimulated Rb^+ pumping was 20 ± 4 amol/(RBC-h) ($n = 4$). See text for details.

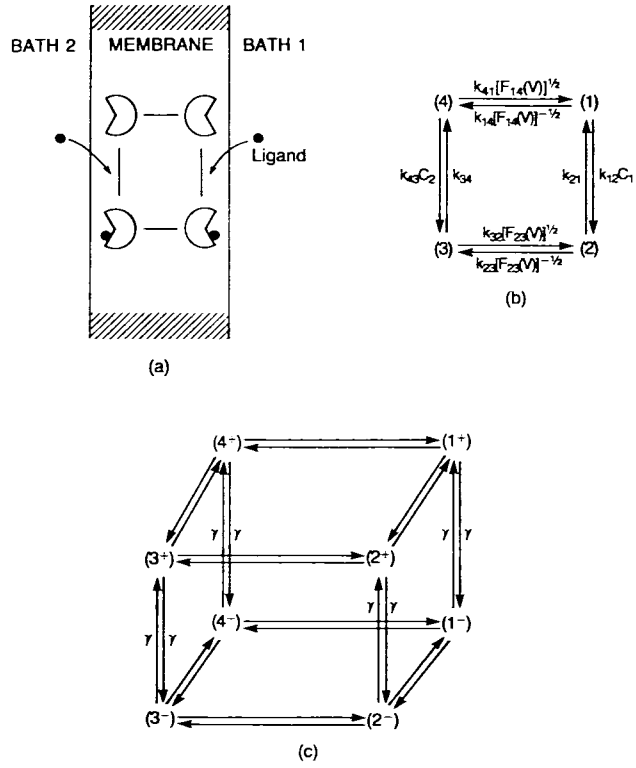

FIGURE 3 (*a*) A schematic of a four-state electroconformational coupling model used in our analysis of data. The transporter is charged so that the equilibrium distribution of the states, as well as the transitions between the two states, depends on the electric field applied across the membrane, V. (*b*) The kinetic diagram of the model in the presence of a constant $\Delta\psi_{tm}$. If the concentrations of ligand on the two sides are equal and the ligand is uncharged, no net transport across the membrane is observed at steady state. The k values are the transition rate constants of the model in the absence of a $\Delta\psi_{tm}$. The values of k must satisfy the "detail balance" condition at all time, i.e., $k_{12}k_{23}k_{34}k_{41} = k_{21}k_{32}k_{43}k_{14}$. $F_{ij}(V)$ represents the differential effects the $\Delta\psi_{tm}$ on the free energy of a transporter between states i and j. In general, it can be expressed as a polynomial in V. In this calculation, terms higher than V^2 were truncated. Thus, $F_{ij}(V) = \exp-[a_{ij}\Phi + b_{ij}\Phi^2]$, where $\Phi \cong \Delta\psi_{tm}\, FRT = d_m V\, FRT$. F, R, and T are the Faraday constant, the gas constant, and the Kelvin temperature, respectively. Both a_{ij} and b_{ij} are constants and can be of either sign (+ or −) (Hill and Chen, 1972). C_1 and C_2 are the concentrations of ligand in the two solutions separated by the membrane. (*c*) The kinetic diagram of the model in the presence of an RTF (Chen, 1987). The ligand can be transported across the membrane up its concentration gradient even if it is uncharged (via electroconformational coupling). The plus (+) sign in the diagram means that the potential in bath 1 is higher than that in bath 2. The minus (−) sign means the reverse. $\gamma = 2f^*$. The simulations (—— in Fig. 2) were produced with these parameters: number of Na,K-ATPase per red cell = 360; C_1 = 10 mM; C_2 = 16 mM; $a_{14} = 4$; $a_{23} = 5$; $b_{14} = -7.5$; $b_{23} = -7.5$; k_{12}, k_{21}, k_{43}, k_{34}, k_{14}, k_{41}, k_{23}, and k_{32} are, respectively, $2 \times 10^4\ s^{-1}\ M^{-1}$, $200\ s^{-1}$, $2 \times 10^3\ s^{-1}\ M^{-1}$, $2 \times 10^3\ s^{-1}$, $5 \times 10^4\ s^{-1}$, $5 \times 10^3\ s^{-1}$, $5 \times 10^3\ s^{-1}$, and $5 \times 10^4\ s^{-1}$. Note that the value for a_{23} is one unit greater than the value for a_{14}. This reflects the fact that the transporter/Rb^+ complex has one extra positive charge.

ation and only one Rb^+ was allowed to translocate the membrane per enzyme turnover. This assumption implies either that kinetics of Na^+ transport are much faster than that of Rb^+ transport or that the coupling of the Na^+- and the Rb^+-pumping modes is mediated by the ATP-dependent phosphorylation of enzyme. The latter has been suggested previously (Tsong and Astumian, 1986, 1988; Tsong, 1990, 1992).

The behavior of this four-state electroconformational coupling has been studied in some detail for regularly oscillating electric fields (Tsong and Astumian, 1986; Westerhoff et al., 1986; Astumian et al., 1989; Chen, 1987; Markin and Tsong, 1991a, b, 1993). In most of these previous calculations, the difference of free energy changes (increase or decrease) of the two transporter states, caused by the presence of a transmembrane potential, was assumed to be linearly proportional to $\Delta\psi_{tm}$. However, to account for the amplitude dependence in transport flux (Fig. 2 *a*), the higher order terms in membrane potential have to be considered (Tsong and Astumian, 1986, 1988; Tsong, 1990). The origin of these higher order terms can be traced to the existence of polarizability in protein, the "Second Wien" effect, rotation of permanent dipole of the transporter against a restoring force, etc. (Tsong and Astumian, 1986, 1988; Hill and Chen, 1972). For example, the free energy change of interaction between and transmembrane electric field $V\,(=\Delta\psi_{tm}/d_m)$ and a conformational equilibrium involving two states differing in molar electric moment by ΔM_{elc} is $\Delta M_{elc} \cdot V$. An induced dipole term would have the form $\Delta\alpha \cdot V^2$, where $\Delta\alpha$ is the difference in polarizabilities of the two conformational states of the transporter. To account for these higher order effects, a quadratic term was included in the function $F_{ij}(V)$, which expresses effects of field interactions with the transporter (Tsong and Astumian, 1986; Chen, 1987). With this inclusion (see legend of Fig. 3), the four-state model in Fig. 3 can easily reproduce both the frequency- and amplitude dependencies of the measured flux data presented in Fig. 2. The kinetic parameters used to fit the data are listed in the legend of Fig. 3. One must note that the fitting is not unique; many other sets of parameters can also fit the data similarly well. Other considerations that treat Na,K-ATPase as a channel-like enzyme can also simulate the field strength dependence of the transport data, but only with limited success (Markin et al., 1992).

DISCUSSION

The ability of a transmembrane enzyme to recognize oscillating electric fields of a particular amplitude and frequency, and absorb free energy transmitted through these fields to perform chemical work, has been suggested to be an effective mechanism for cellular recognition and processing of electric signals (Tsong, 1989, 1992). This idea is made even more credible with the data presented here, because by the same mechanisms, an enzyme can also transduce energy from randomly fluctuating electric fields. Such fields mimic local transmembrane electric fields of a cell membrane. Note that these fields were generated, or sustained by energy input. No enzyme can transduce energy from the equilibrium electric noise (Chen, 1987; Astumian et al., 1987). Doing so would be in violation of the thermodynamic principles. Recently, harmonic oscillation analysis has been applied to interpret

electric activation data (Astumian and Robertson, 1989; Robertson and Astumian, 1991, 1992; Horn, 1993). This allows derivation of kinetic information of several membrane enzymes, under their naturally exiting state. By the conventional relaxation methods, these enzymes would have to be solubilized, or reconstituted into liposomes for study. However, if an enzyme can also recognize randomly fluctuating electric fields, RTF analysis might prove to be more convenient than the harmonic oscillation analysis because kinetics in an RTF can be solved analytically, but kinetics in a regular periodic field cannot. If an enzyme can respond to very weak electromagnetic fields, the mechanisms discussed here could also explain biological effects of environmental electromagnetic fields (Tsong, 1989, 1992; Weaver and Astumian, 1990). In this regard, one should point out that an ion activation model proposed by Blank (Blank and Soo, 1990; Blank, 1992) can also explain the frequency optimum of the electric field induced cation transport when sinusoidal waveform was used for experiment. Whether the model can also reproduce the present data by RTF stimulation remains to be investigated.

We thank Carol J. Gross for help with the manuscript.

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