Harmonic Response of Cellular Membrane Pumps to Low Frequency Electric Fields

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We report on harmonic generation by budding yeast cells in response to a sinusoidal electric field, which is seen to be minimal when the field amplitude is less than a threshold value. Surprisingly, sodium metavanadate, an inhibitor of *P*-type ATPases reportedly responsible for nonlinear response in yeast, reduces the threshold field amplitude, increasing harmonic generation at low amplitudes while reducing it at large amplitudes, whereas the addition of glucose dramatically increases the production of even harmonics. Finally, a simple model is proposed to interpret the observed behavior.

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Among the forces of nature, electromagnetic interactions play a dominant role in biological processes. An applied low-frequency electric field, for example, polarizes live cells in suspension, resulting in an enormous dielectric response [1], and also modulates each cell's membrane potential by an amount given by, for a spherical cell [2], $U_m(\omega, \theta) = 1.5E_cR\cos\theta[1 + i\omega\tau_m]^{-1}$, where E_c is the external field amplitude, R is the cell radius, θ is the polar angle from the field direction, and τ_m is a relaxation time. Thus, a field of only 5 V/cm modulates the normal membrane potential, typically around -70 mV, of a 10 μm radius cell by up to 7.5 mV, creating a net field modulation of $\sim$0.75 MV/m across a 10 nm thick plasma membrane of a resting cell.

Membrane proteins [3] are candidates for nonlinear behavior [4] since they cannot rotate within the membrane and dissipate energy through Debye-like relaxation, and also since any transmembrane domains with dipole moments interact with the greatly amplified ac field. The modulated transmembrane potential drives membrane proteins to change their conformational states, and can even induce pumps to transport ions [5,6]. This combination of protein conformational changes and ion translocation creates a nonlinear response manifested by the generation of harmonics [7,8]. An externally modulated membrane potential may therefore affect enzyme activity, transport, and conformational behavior. Cyclic processes, such as ion transport and ATP consumption, are of special interest because they are likely to be sensitive to synchronization by an oscillatory field.

In yeast (*S. cerevisiae*) cells, the observation [9] that harmonic generation by a mutant strain containing vandate-resistant H^+-ATPase is also highly resistant to sodium metavanadate, as compared to normal cells in which vanadate is an H^+-ATPase inhibitor, strongly suggests that these membranous cation pumps are major sources of nonlinear harmonic production at low frequencies. H^+-ATPase shows nonlinear behavior due to its structural and mechanistic similarity to Na, K-ATPase, and related *P*-type ATPases [10,11], and is thus expected to transduce exogenous electric fields in a manner similar to that demonstrated by Tsong and colleagues [12]. The best understood *P*-type ATPase is Ca-ATPase, which exists in two main conformational states, E_1 and E_2, while H^+-ATPase and other *P*-type pumps are believed to be similar. The structure [13] is like that of a hand in a mitten that opens and closes, as shown in Fig. 1(a). An oscillatory potential across the membrane can induce time-dependent conformational changes that alter the energy landscape [Fig. 1(b)] seen by the cations [14]. Once the ions become trapped in the well, a conformational change facilitated by ATP hydrolysis or an external field takes place. The modulated potential U_m affects the potential barrier seen by the ion, thus increasing (or decreasing) the probability for the ion to hop into the adjacent well.

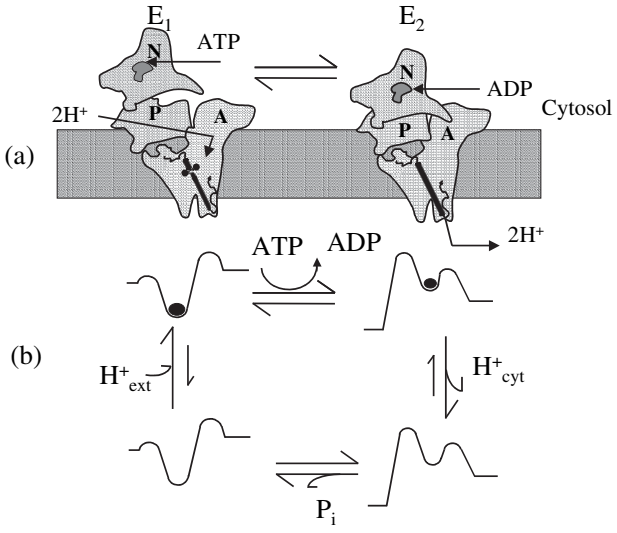

FIG. 1. (a) Primary open and closed conformational states, E_1 and E_2, of a *P*-type ATPase membrane pump. (b) Potential energy landscapes, showing ions trapped in a potential well for certain configurations, as the pump cycles through its conformational states [adapted from Ref. [6]].

Frequencies well below 1 kHz are required to obtain sufficient transmembrane field amplification for the nonlinear response to be dominated by complexes in the plasma membrane. A problem that plagues measurements at low frequencies is the generation of harmonics by the electrical double layer at each electrode [15,16]. In order to reduce such spurious harmonics, we use only two electrodes to apply the electric field and employ a high-T_c superconducting quantum interference device (SQUID) magnetometer to probe the time-dependent magnetic field generated by the resulting currents, while the output of the SQUID electronics is fed into a spectrum analyzer. The SQUID magnetometer eliminates the need for measurement electrodes and any additional interface effects, but does not completely eliminate harmonics produced at the source electrodes. Harmonics induced by the cell suspension are measured by applying a sinusoidal signal in the frequency range 10–100 Hz and field amplitude range 0–5 V/cm, and the response of a conductivity-matched reference medium is subtracted to further reduce any spurious harmonics. Further details, including the measurements and preparation of budding yeast suspensions (*S. cerevisiae*, 10^8 cells/ml) are described elsewhere [17].

For sufficient field amplitudes, a series of harmonics are observed, the largest of which, in the absence of glucose, are the odd harmonics. Figure 2 shows the measured third harmonic response versus applied frequency for fixed amplitude of 3 V/cm, before and after adding 0.8 mM of sodium metavanadate. Note that the inhibitor suppresses the third harmonic response for the same amplitude, consistent with previous observations. Figure 3 depicts the amplitude-dependent third harmonic response for a fixed applied frequency of 23 Hz, before (closed circles) and after (open circles) adding sodium metavanadate. The data before adding the inhibitor exhibit a prominent peak centered around 2.75 V/cm amplitude and a major threshold field of around 2.2 V/cm, below which little response is observed except for a minor peak at around 1.4 V/cm. In addition, the sodium metavanadate appears to reduce this threshold field and greatly increase the third harmonic response for field amplitudes of around 1.8 V/cm, while the third harmonic response is suppressed somewhat for amplitudes in the range 2.5–3.5 V/cm.

The increase in harmonic response at lower field amplitudes after adding sodium metavanadate is especially surprising, because previous studies [9] suggest that H^+-ATPase, of which sodium metavanadate is an inhibitor, is a major contributor to the nonlinear harmonic response of these organisms. Vanadate is known to interact with E_2 conformation the enzyme in the absence of the cation (e.g., H^+ or Ca^{2+}) by acting as a phosphate group (P_i) analog. It has been suggested [18] that uncoupled (e.g., E_2-E_2) cycles can occur in addition to the coupled E_1-E_2 cycle. Thus, although ATP hydrolyzing reactions will be inhibited by vanadate, since it stabilizes the E_2 state it may also enable an oscillatory field-driven E_2-E_2 cycle to take place. If the major threshold field for the E_1-E_2 cycle was due to the energy barrier for driving cations from the potential well, then perhaps the threshold field for driving cations back and forth over the E_2 barrier in a field-driven E_2-E_2 cycle might be reduced. Moreover, as suggested by previous studies [5,6], oscillatory fields can drive conformational state changes and cation transport even in the absence of ATP. Since these measurements were done without glucose, it is likely that the E_1-E_2 changes in conformational state prior to adding vanadate were indeed field driven rather than ATP driven. The potential energy barrier to drive the E_1-E_2 conformational change and cation transport without ATP hydrolysis is likely larger than that to simply drive cation transport over the barriers in the E_2 conformation, as suggested by the energy landscapes of Fig. 1, which would lead to a larger threshold field in the absence of either vanadate or glucose.

The addition of glucose to yeast cells has been shown [19] to cause a two to threefold increase in plasma membrane ATPase activity. In order to study the effects of glucose in real time, we automated the data acquisition so that specific harmonics could be plotted as a function of time. Figure 4 shows measured second and third harmonics as functions of time after adding D glucose, for a fixed

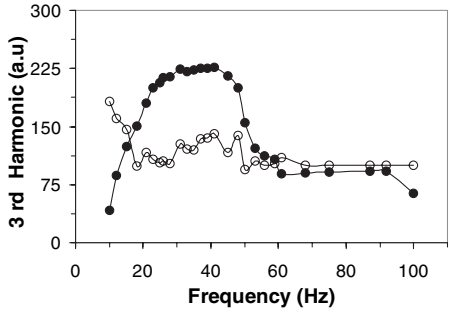

FIG. 2. Induced third harmonic response of budding yeast (*S. cerevisiae*, 10^8 cells/ml) as a function of applied fundamental frequency before (closed circles) and after (open circles) adding 0.8 mM of the *P*-type ATPase inhibitor sodium metavanadate. The field amplitude was fixed at 3 V/cm.

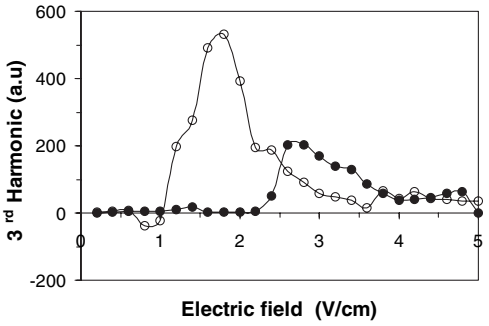

FIG. 3. Third harmonic response induced by budding yeast cells (*S. cerevisiae*, 10^8 cells/ml) as a function of applied field amplitude before (closed circles) and after (open circles) adding sodium metavanadate. Here the frequency was fixed at 23 Hz.

frequency of 45 Hz and amplitude of 3 V/cm, where $t = 0$ represents the time when glucose was added. Note that, after several minutes, the second harmonic increases dramatically, by over 400% after 10 minutes, but then reduces in amplitude after about 25–30 minutes as the glucose concentration becomes depleted. By contrast, the third harmonic actually reduces in amplitude over the same time period when the second harmonic is larger, and then recovers to about its original size after 30 minutes.

Several features of the above harmonic generation experiments warrant explanation, including the strong frequency dependence, with thresholdlike behavior in the amplitude dependence, and the observed increase of even harmonics when ATP production is taking place. In order to capture the essential behavior, we propose a model that explicitly introduces a threshold field, similar to those observed in density waves, where fields above threshold drive charge transport through an energy landscape with multiple wells [20,21], and in Coulomb blockade tunnel junctions, recently exploited to define the current standard [22].

In our model, a *P*-type enzyme complex is represented as a junction, through which cation (e.g., H^+ or Ca^{2+}) transfer can be driven by a sufficiently large voltage, with the following properties. First, the displacement current is taken to be *zero* when the modulated voltage across the membrane is in the range $-V_2 \leq V \leq V_1$, where V_1 and V_2 are threshold voltages and $V_2 > 0$ [23]. When V falls outside this range, the displacement current is assumed to persist for a time $\tau_{1,2}$ reflecting the time for the system to change conformational states and for cation transfer to take place. The displacement current is taken to be:

$$I = \frac{dQ}{dt} \sim \pm f(\nu_\pm) g(t_\pm), \tag{1}$$

where $\nu_+ = (V - V_1)$ when $V > V_1$, $\nu_- = (|V| - V_2)/V_2$ when $V < -V_2$, and $t_\pm = [t - t(V_{1,2})]/\tau_{1,2}$ is the normalized time period after the voltage crosses the threshold value in either the positive or the negative direction. We take $f(\nu)$ to peak for a finite ν, in this case using the simple form [24]:

$$f \sim \nu \exp[-\nu]\Theta(\nu), \tag{2}$$

where $\Theta(\nu)$ is the unit step function. In order to reflect the time required for conformational changes and cation transfer to take place, and to avoid discontinuities, the function $g(t_\pm)$ is taken to be:

$$g \sim \{1 - \cos[2\pi t_\pm]\}\Theta(1 - t_\pm). \tag{3}$$

Finally, taking the voltage $V(t) = V_0 \sin\omega t$ to be sinusoidal, the displacement current $I(t)$ is determined using the above expressions and the various Fourier components of the current response are computed.

We find that the dc current and all even harmonics are zero in this model, as expected, when the junction is perfectly symmetric, i.e., when $V_1 = V_2$ and $\tau_1 = \tau_2$. However, the theoretical third harmonic response is found to be nonvanishing, and to exhibit a threshold voltage in its amplitude dependence. Figure 5 (top) shows representative theoretical plots of third harmonic versus amplitude and frequency. Note that, for certain parameters, the plots are qualitatively consistent with the experimental data shown in Figs. 2 and 3.

When the junction becomes asymmetric, both a dc current and second harmonic begin to appear in the model predictions. The bottom plots of Fig. 5 show theoretical second harmonic response versus amplitude and frequency for several values of τ_2/τ_1 when $V_2/V_1 = 1$. Note that the predicted second harmonic response increases significantly as the asymmetry in time scales increases. We observed similar behavior for the theoretical dc current, which is not shown here. As pointed out in Ref. [13], an asymmetry in time scales is sufficient for an ac field to drive ion transport through a membrane pump. We also find (not shown in Fig. 5) that an asymmetry in threshold voltages, V_1 and V_2, also enables an ac field to induce both a second harmonic response and a dc current in our model. These results suggest that the production of ATP from glucose makes the pump more asymmetric by enabling ATP induced ion transport along a preferred direction.

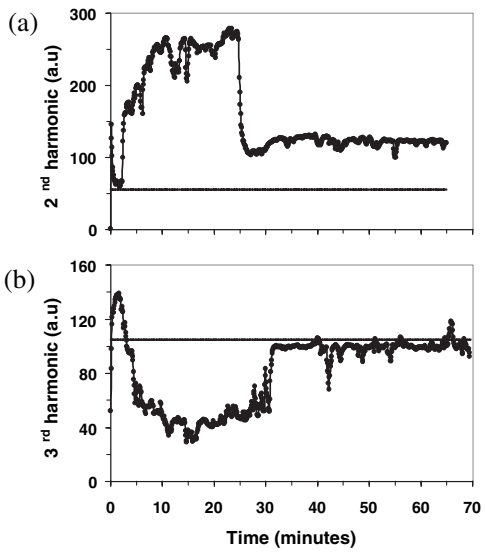

FIG. 4. Time-dependent second (top) and third (bottom) harmonic responses of a yeast cell suspension (10^8 cells/ml) after adding 0.1 M of *D* glucose. The glucose was added to the resting cell suspension a few minutes before the measurements (Time = 0 minutes). H^+-ATPase and other transporters apparently increase activity in the presence of glucose, as reflected by an increase in the second harmonic response and decreased (by about 60%) third harmonic response. After about 25–30 minutes the second harmonic drops significantly, while the third harmonic increases back to its original value, as the glucose concentration becomes depleted. The frequency and amplitude of the applied signal was set to 45 Hz and 3 V/cm, respectively. The horizontal lines show the original second and third harmonic responses without any added glucose.

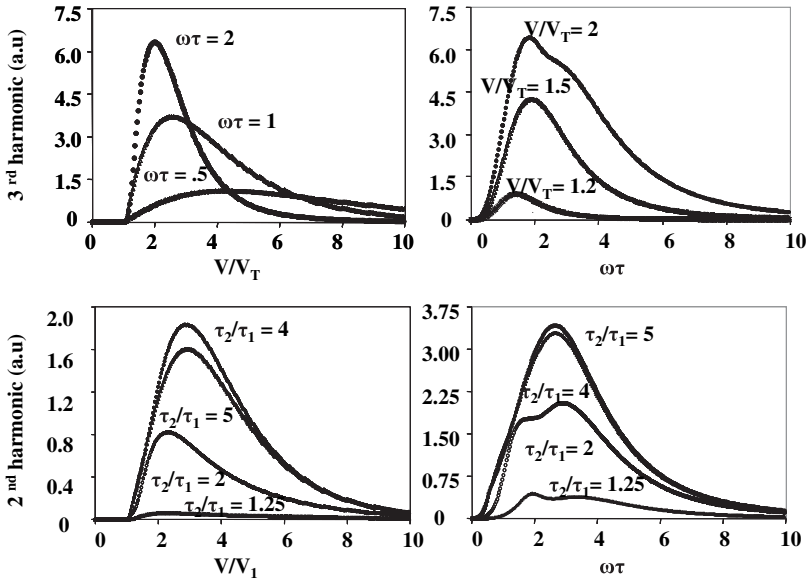

FIG. 5. Theoretical plots of third harmonic response (top) vs amplitude and frequency for a symmetric junction, where the second harmonic response is *zero* as discussed in the text. The bottom figures show theoretical second harmonic response curves vs amplitude (when $\omega\tau = 1$) and frequency (taking $V/V_T = 2$) for an asymmetric junction for several values of τ_2/τ_1 when $V_1 = V_2 = V_T$.

The ability to noninvasively monitor active physiological processes *in vivo* is of potential importance for biophysics, biomedicine, and the pharmaceutical development. For example, proton pump inhibitors are widely employed to treat acid reflux disease and related stomach disorders. Additional enzymes in the plasma membrane that may contribute to harmonic generation include *ABC* transporters. In addition, we have recently observed, in the nonlinear harmonic responses at kilohertz frequencies of whole cells, mitochondria, and chloroplasts, evidence that the technique can monitor internal metabolic events that would be extremely difficult to study using invasive (e.g., patch clamp) methods. These results will be presented in a forthcoming paper.

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[23] We originally took $Q(t) = CV(t)$ when $-V_1 \leq V \leq V_1$, but found that taking C to be zero does not alter the results when calculating any of the harmonics except for the fundamental.
[24] Following Feynman's expression [see, e.g., Ref. [6]] for the Brownian ratchet: net rate $\propto \Delta U \exp[-\Delta U/kT]$.