Brown-Shimer, S., Johnson, K. A., Lawrence, J. B., Johnson, C., Bruskin, A., Green, N. R., & Hill, D. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5148-5152.
Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., & Soltoff, S. (1991) *Cell 64*, 281-302.
Charbonneau, H., Tonks, N. K., Walsh, K. A., & Fischer, E. H. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7182-7186.
Cho, H., Ramer, S. E., Itoh, M., Winkler, D. G., Kitas, E., Bannwarth, W., Burn, P., Saito, H., & Walsh, C. T. (1991) *Biochemistry* (in press).
Coleman, J. E., & Gettins, P. (1983) *Adv. Enzymol. Relat. Areas Mol. Biol. 55*, 351-452.
Dixon, J. E., Guan, K., Woodford, T., Rhodes, J., Pot, D., & Qiu, H. (1991) *FASEB J. 5*, Abstr. A784.
Eargle, D. H., Licko, V., & Kenyon, G. L. (1977) *Anal. Biochem. 81*, 186-195.
Faller, L. D., & Diaz, R. A. (1989) *Biochemistry 28*, 6908-6914.
Knorr, R., Trzeciak, A., Bannwarth, W., & Gillessen, D. (1989) *Tetrahedron Lett. 30*, 1927-1930.
Krueger, N. X., Streuli, M., & Saito, H. (1990) *EMBO J. 9*, 3241-3252.
Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., & Candia, O. A. (1979) *Anal. Biochem. 100*, 95-97.
Martin, B., Pallen, C. J., Wang, J. H., & Graves, D. J. (1985) *J. Biol. Chem. 260*, 14932-14937.
Ramer, S. E., Winkler, D. G., Carrera, A., Roberts, T., & Walsh, C. T. (1991) *Proc. Natl. Acad. Sci. U.S.A.* (submitted for publication).
Risley, J. M., & Van Etten, R. L. (1978) *J. Labelled Compd. Radiopharm. 15*, 533-537.
Saito, H., & Streuli, M. (1991) *Cell Growth Differ. 2*, 59-65.
Schwartz, J. H., Crestfield, A., & Lipmann, F. (1963) *Proc. Natl. Acad. Sci. U.S.A. 49*, 722-729.
Streuli, M., Hall, L. R., Saga, Y., Schlossman, S. F., & Saito, H. (1987) *J. Exp. Med. 166*, 1548-1566.
Streuli, M., Krueger, N. X., Hall, L. R., Schlossman, S. F., & Saito, H. (1988) *J. Exp. Med. 168*, 1523-1530.
Streuli, M., Krueger, N. X., Tsai, A. Y. M., & Saito, H. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8698-8702.
Streuli, M., Krueger, N. X., Thai, T., Tang, M., & Saito, H. (1990) *EMBO J. 9*, 2399-2407.
Thomas, M. L. (1989) *Annu. Rev. Immunol. 7*, 339-369.
Tsai, A. Y. M., Itoh, M., Streuli, M., Thai, T., & Saito, H. (1991) *J. Biol. Chem.* (in press).
Van Etten, R. L., & Hickey, M. E. (1977) *Arch. Biochem. Biophys. 183*, 150-159.
Zhang, Z.-Y., & VanEtten, R. L. (1991) *J. Biol. Chem. 266*, 1516-1525.

Interpretation of the Effect of an Oscillating Electric Field on Membrane Enzymes

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ABSTRACT: Theoretical expressions for the frequency and amplitude dependence of the rate of a catalyzed reaction are fitted to the data of Graziana et al. (1990) [Graziana, A., Ranjeva, R., & Teissié, J. (1990) *Biochemistry 29*, 8313-8318] for Ca^{2+} uptake by carrot protoplasts in an oscillating electric field. This uptake is a direct (linear) measure of the rate of increase of ATP caused by a plasma membrane enzyme in the oscillating field. The fit gives 20 ms and 33 μs for the relaxation times of the enzyme and roughly 3 for the effective number of elementary changes displaced across the membrane by a conformational change of the enzyme in its catalytic cycle. Additional experiments are suggested to define further the mechanism of the enzymatic reaction.

Graziana et al. (1990) have observed that an applied 100-Hz oscillating electric field of 25 V/cm peak amplitude stimulates Ca^{2+} uptake by carrot protoplasts by as much as a factor of 3. The Ca^{2+} influx increased with electric treatment duration up to 5 min and then decreased slowly to approximately its initial value after 15 min. Remarkably, the stimulation elicited by the electric field was still effective after the field was switched off. After an initial 5-min treatment was turned off, the influx dramatically continued to increase for 5 min and then decreased slowly. The Ca^{2+} influx after 5-min treatment also was found to depend on the oscillating field amplitude and frequency. Thus, the effect could not be due to joule heating, which is independent of frequency. During electric stimulation, the amount of internal ATP increased by more than 30%. After the field was turned off, the extra amount of ATP was almost completely consumed within 5 min, corresponding to the time of maximum influx of Ca^{2+}. Addition of 50 μM dicyclohexylcarbodiimide (DCCD), an inhibitor of ATP synthesis, suppressed all the electric field induced effects, i.e., the increase in ATP concentration and Ca^{2+} influx. The authors conclude that the oscillating electric field stimulates an increase in ATP that drives the influx of Ca^{2+}.

They report that for a constant-amplitude and constant-frequency field applied for up to 5 min both the rate of Ca^{2+} influx and the amount of additional ATP are proportional to the time of application of the oscillating field. Thus, their amplitude- and frequency-dependent Ca^{2+} influx measurements (which were obtained by applying a steady oscillating field for 5 min) are proportional to the amplitude- and frequency-dependent amount of additional ATP.

Transmembrane proteins respond to applied electric fields (Witt et al., 1976; Teissié et al., 1981; Teissié, 1986) because (1) the field is strongly concentrated in the plasma membrane, (2) the conformational changes of many membrane proteins involve a large displacement charge, and (3) the membrane prevents the protein from rotating and thereby evading the effect of the field (Tsong & Astumian, 1986).

Membrane enzymes undergo conformational changes in their catalytic cycle. The motion of charge during this conformational change provides a coupling between an electric

field and the conformational state. This electroconformational coupling permits an oscillating field to affect the reactions catalyzed by the enzyme (Tsong & Astumian, 1986; Westerhoff et al., 1986; Tsong, 1990). A qualitative mechanistic understanding of the effect of this has been obtained by considering a large oscillating field interacting with the enzyme. This allows an analytical solution for the flux (Markin et al., 1990) and gives a physical picture of how the oscillating field causes the enzyme to cycle (Robertson & Astumian, 1990a).

Recently the relaxation kinetics for the effect of a weak oscillating field on these reactions have been developed (Robertson & Astumian, 1991). The average rates versus the frequency are sums of Lorentzian curves. Since the amplitudes of the Lorentzians can be either positive or negative, frequency windows are possible. The frequency dependence can be used to determine the relaxation times of the system. The theory is valid for weak oscillating fields, for which the response is proportional to the square of the amplitude of the oscillating field. The catalyzed reactions are not limited to being near-equilibrium.

If all but one of the relaxation times are fast, the amplitudes of the Lorentzians satisfy a generalized Michaelis–Menten equation that describes the dependence on the oscillating field amplitude as well as on substrate and product concentrations (Robertson & Astumian, 1990b). The amplitude dependence can be used to calculate the effective displacement charge of the enzyme.

The predicted curve has been found to agree with the frequency dependence of unidirectional Na^+ and Rb^+ transport by Na^+–K^+-ATPase in erythrocytes measured by Liu et al. (1990). However, the amplitude dependence they observed exhibited a window, which cannot be described by a theory that is limited to weak fields. In contrast, the amplitude dependence of Ca^{2+} uptake by carrot protoplasts measured by Graziana et al. is monotonic.

In this paper, the theoretical frequency and amplitude dependences of the ATP increase (reflected in Ca^{2+} influx) are compared with the experimental data for carrot protoplasts, and the relaxation times and effective displacement charge of the plasma membrane enzyme that cause this increase are obtained. This provides insight and suggests what experimental information may help narrow mechanistic possibilities.

Materials and Methods

Parameters in eq 1 and 2 were adjusted to fit the Ca^{2+} uptake measurements of Graziana et al. using the algorithm for least-squares estimation of nonlinear parameters of Marquardt (1963). This is a combined gradient search and linearized fit technique.

Results

The average rate of a reaction catalyzed by an enzyme in an oscillating field versus the frequency of the field is a sum of Lorentzians (Robertson & Astumian, 1991). Two Lorentzian terms are sufficient to fit the Ca^{2+} uptake versus frequency measured by Graziana et al. (1990). Thus, the average rate of the catalyzed reaction is

$$\bar{V} = \bar{V}_\infty + \frac{V_1}{1 + \tau_1^2\omega^2} + \frac{V_2}{1 + \tau_2^2\omega^2} \qquad (1)$$

where V_∞ is the average rate at infinite frequency, $\omega/2\pi$ is the frequency, τ_k is the relaxation time of the kth normal mode, and V_k is the rate for the kth normal mode for $k = 1$ and 2. This equation describes the response to a sinusoidal field, whereas the field applied in the experiment is a square wave. However, a square wave is a sum of sinusoids whose frequencies are the odd multiples of the fundamental frequency $\omega/2\pi$ and whose amplitudes decrease like 1, 1/3, 1/5, 1/7, ... The Lorentzian amplitudes are proportional to the squares of the amplitude of the applied wave, and so the Lorentzian amplitudes corresponding to the odd harmonics decrease like 1, 1/9, 1/25, 1/49, ... Thus, the square wave can be approximated by a sinusoid, and the higher harmonics give rise to a negligible correction. When the parameters of eq 1 are adjusted to fit the experimental data, the values in Table I are obtained. Comparison of the resulting expression with experiment is shown in Figure 1a.

Table I: Lorentzian Parameters for Ca^{2+} Uptake by Carrot Protoplasts

k	1	2
$1/2\pi\tau_k$ (s^{-1})	8.1	4900
τ_k (ms)	20	0.033
V_k (dpm/filter)	−3700	7000

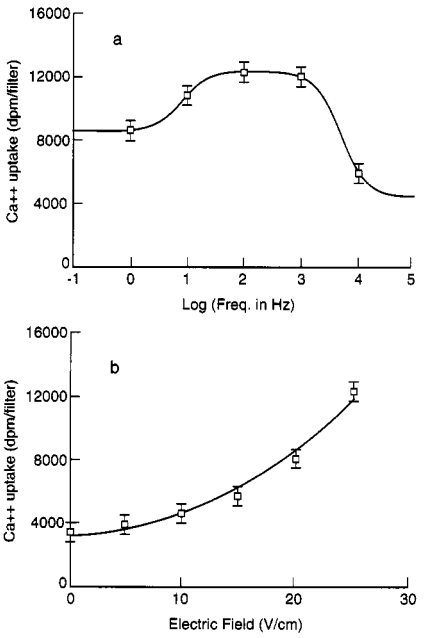

FIGURE 1: Theoretical effect of oscillating electric field compared with the experiment of Graziana et al. (1990) on Ca^{2+} uptake by carrot protoplasts. Graziana et al. applied the electric field for 5 min and then added labeled Ca^{2+}, and after 1 min measured the uptake, which is a direct measure on the rate of increase of ATP caused by a plasma membrane enzyme. The bars represent the 1 standard deviation uncertainty reported in the experimental paper. (a) Effect of increasing frequency at constant amplitude of the oscillating field (25 V/cm). The curve (eq 1) is a sum of two Lorentzians fitted to the experimental data. The best-fit Lorentzian parameters are given in Table I. (b) Effect of increasing field at constant frequency (100 Hz). The curve (eq 2) is a constant plus a term proportional to the square of the amplitude of the oscillating field fitted to the experimental data. From this fit, the effective number of elementary charges displaced across the membrane by a conformational change is estimated to be roughly 3.

The experimental rate versus the amplitude of the oscillating field can be fitted by a constant plus a term proportional to the square of the oscillating amplitude as shown in Figure 1b, in agreement with theory.

The fit parameters can be used along with the generalized Michaelis–Menten expression (Robertson & Astumian, 1990b) to calculate the charge displacement of the enzyme that causes the ATP increase. Unfortunately, the substrate and product concentrations in the carrot protoplasts are not available. However, an estimate can be obtained by assuming that the

product concentration is small and the substrate concentration is large compared with the respective Michaelis constants. Then for weak fields, the peak rate is

$$\bar{V} \approx [1 + (ze\psi_1/kT)^2/4]k_{cat}[E_T] \quad (2)$$

where $k_{cat}[E_T]$ is the rate in the absence of the field, ψ_1 is the amplitude of the oscillating membrane potential, e is the elementary charge, and z is the effective number of elementary charges displaced across the membrane by the conformational change.

When averaged over a spherical surface, the square of the amplitude of the oscillating membrane potential is given by

$$\langle\psi_1^2\rangle = 3E_1^2r^2/4 \quad (3)$$

where E_1 is the amplitude of the oscillating field and r is the radius of the protoplast (Astumian & Robertson, 1989). Teissié reports that the diameter of the carrot protoplasts used by Graziana et al. is $2r = 21 \pm 2$ μm. The amplitude of the first harmonic component of a square wave is $4/\pi$ times the amplitude of the square wave. These give $z \approx 3$ for the effective number of elementary charges displaced across the membrane.

Discussion

Graziana et al. have described the frequency- and amplitude-dependent effect of an oscillating electric field on the increase of ATP in plant protoplasts. The Ca^{2+} influx they measured directly is proportional to the amount of additional ATP that results from applying the oscillating field for 5 min. The proportionality can be deduced from their observation that for a constant-amplitude and constant-frequency field applied for up to 5 min, both the rate of Ca^{2+} influx and the amount of additional ATP are proportional to the length of time the oscillating field is applied. (When under the same conditions two quantities are each proportional to time, they must be proportional to each other.) It is reasonable to assume that the proportionality is valid whatever the frequency and amplitude as long as the amplitude is not too large. Since the (frequency- and amplitude-dependent) Ca^{2+} influx is proportional to the amount of additional ATP that results from applying the field for 5 min, it must be proportional to the (frequency- and amplitude-dependent) rate of ATP increase.

An applied field results in a very large field in the plasma membrane and essentially zero field everywhere inside the protoplast (Tsong & Astumian, 1986). This is explained as follows. The high ionic strength of the cytosol permits ionic currents to flow throughout all the connected regions accessible to the cytosol and thus build up a charge distribution that cancels out any electric field inside the protoplast, at least for frequencies well below 1 MHz. The cytosol is a good ionic conductor and so has the same electric potential throughout. On the other hand, the plasma membrane is a poor ionic conductor, and the charge accumulates on its surface. The entire potential drop across the protoplast due to an applied field must appear across just the plasma membrane. Thus, the cause of the oscillating field dependent ATP increase in the plant protoplasts must be a plasma membrane molecule with an appreciable displacement charge.

We interpret the data using our previous theory of the effect of a weak oscillating field on membrane enzymes (Robertson & Astumian, 1991). The theory predicts that when a weak oscillating field is applied, the average rate of increase of ATP versus frequency is a sum of Lorentzian curves whose amplitude is quadratic in the applied field strength (see eq 1). The theory is compared with experiment in Figure 1. The fit of the sum of the two Lorentzian curves of eq 1 to the experimental average rate is shown in Figure 1a. The fit of a constant plus a term proportional to the square of the amplitude of the oscillating electric field to the experimental average rate is shown in Figure 1b. The agreement between theory and experiment is excellent.

The theory is testable even with data at just five frequencies. One can imagine five data points that could not be fitted at all with just two Lorentzians, e.g., the five rates 10, 1, 10, 1, and 10 at the five frequencies, respectively. These five points would require the sum to contain at least four Lorentzians to fit the data.

The theory gives values for the relaxation times and the displacement charge of the membrane enzyme affected by the oscillating field. The relaxation times of the system are obtained from the fit to the measured frequency dependence and are listed in Table I. The effective number of elementary charges that move across the membrane during a conformational change of the enzyme is obtained from the fit to the measured amplitude dependence and is estimated to be roughly 3. These numerical values are reasonable. For comparison, we note that the magnitude of the displacement charge is similar to the magnitude of the gating or displacement charge for a sodium channel (Hartshorne et al., 1985; Furman et al., 1986). The diameter of the protoplast must be known in order to determine the magnitude of the displacement charge by our method.

The theory suggests further experiments that will provide a more stringent test of the assumptions. In particular, measurements at additional frequencies—especially over a broader range of frequencies—are needed to test whether two Lorentzians are sufficient.

A measured enzyme-catalyzed average reaction rate that is the sum of N distinct Lorentzians requires a mechanism with at least $N + 1$ kinetically significant states. A similar statement is true for a transient rate in conventional linear relaxation kinetics that is the sum of N distinct exponentials. The data of Graziana et al., which can be fitted with two Lorentzian frequencies, are consistent with a mechanism having three kinetically significant states. This does not mean that there are actually just three states of the enzyme system.

The Lorentzian amplitudes and frequencies can be calculated from the kinetic parameters of a postulated mechanism (Robertson & Astumian, 1991). The amplitude of the Lorentzian depends on substrate and product concentrations as described by a Michaelis–Menten equation (Robertson & Astumian, 1990b). This can be used to design experiments and determine kinetic constants in the same way as is done with the usual Michaelis–Menten equation.

The theory we have compared with experiment is an extension of relaxation kinetics (Eigen & DeMaeyer, 1974; Hammes & Schimmel, 1967) to second order, where the first-order correction to the average catalyzed flux is zero; it is a quadratic response theory. When combined with experiment, it provides a powerful method for determining the kinetic parameters of membrane enzymes, which are essential for signal and energy transduction, but which have been technically very difficult to study experimentally. The method we described permits biochemists to use average rate measurements to do rapid reaction kinetics on these difficult systems.

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Registry No. ATP, 56-65-5; Ca^{2+}, 7440-70-2; ATP synthase, 37205-63-3.

REFERENCES

Astumian, R. D., & Robertson, B. (1989) *J. Chem. Phys. 91*, 4891-4901.
Eigen, M., & DeMaeyer, L. (1974) in *Techniques of Chemistry* (Hammes, G. G., Ed.) Vol. VI, Part II, pp 63-146, Wiley, New York.
Furman, R. E., Tanaka, J. C., Mueller, P., & Barchi, R. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 488-492.
Graziana, A., Ranjeva, R., & Teissié, J. (1990) *Biochemistry 29*, 8313-8318.
Hammes, G. G., & Schimmel, P. R. (1967) *J. Phys. Chem. 71*, 917-923.
Hartshorne, R. P., Keller, B. U., Talvenheimo, J. A., Catterall, W. A., & Montal, M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 240-244.
Liu, D.-S., Astumian, R. D., & Tsong, T. Y. (1990) *J. Biol. Chem. 265*, 7260-7267.
Markin, V. S., Tsong, T. Y., Astumian, R. D., & Robertson, B. (1990) *J. Chem. Phys. 93*, 5062-5066.
Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431-441.
Robertson, B., & Astumian, R. D. (1990a) *Biophys. J. 57*, 689-696.
Robertson, B., & Astumian, R. D. (1990b) *Biophys. J. 58*, 969-974.
Robertson, B., & Astumian, R. D. (1991) *J. Chem. Phys. 94*, 7414-7419.
Teissié, J. (1986) *Biochemistry 25*, 368-373.
Teissié, J., Knox, B. E., Tsong, T. Y., & Wehrle, J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7473-7477.
Tsong, T. Y. (1990) *Annu. Rev. Biophys. Biophys. Chem. 19*, 83-106.
Tsong, T. Y., & Astumian, R. D. (1986) *Bioelectrochem. Bioenerg. 15*, 457-476.
Westerhoff, H. V., Tsong, T. Y., Chock, P. B., Chen, Y.-D., & Astumian, R. D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4734-4738.
Witt, H. T., Schlodder, E., & Graber, P. (1976) *FEBS Lett. 69*, 272-276.

Regulation of Fatty Acid ^{18}O Exchange Catalyzed by Pancreatic Carboxylester Lipase. 1. Mechanism and Kinetic Properties†

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ABSTRACT: The exchange of ^{18}O between H_2O and long-chain free fatty acids is catalyzed by pancreatic carboxylester lipase (EC 1.1.1.13). For palmitic, oleic, and arachidonic acid in aqueous suspension and for 13,16-*cis*,*cis*-docosadienoic acid (DA) in monomolecular films, carboxyl oxygens were completely exchanged with water oxygens of the bulk aqueous phase. With enzyme at either substrate or catalytic concentrations in the argon-buffer interface, the exchange of DA oxygens obeyed a random sequential mechanism, i.e., $^{18}O,^{18}O$-DA $\rightleftharpoons$ $^{18}O,^{16}O$-DA $\rightleftharpoons$ $^{16}O,^{16}O$-DA. This indicates that the dissociation of the enzyme·DA complex is much faster than the rate-limiting step in the overall exchange reaction. Kinetic analysis of ^{18}O exchange showed a first-order dependence on surface enzyme and DA concentrations, i.e., the reaction was limited by the acylation rate. The values of k_{cat}/K_m, 0.118 cm^2 pmol^{-1} s^{-1}, for the exchange reaction was comparable to that for methyl oleate hydrolysis and 5-fold higher than that for cholesteryl oleate hydrolysis in monolayers [Bhat, S., & Brockman, H. L. (1982) *Biochemistry 21*, 1547]. Thus, fatty acids are good "substrates" for carboxylester lipase. With substrate levels of carboxylester lipase in the interfacial phase, the acylation rate constant k_{cat}/K_m was 200-fold lower than that obtained with catalytic levels of enzyme. This suggests a possible restriction of substrate diffusion in the protein-covered substrate monolayer.

Pancreatic carboxylester lipase (CEL,[1] EC 1.1.1.13) catalyzes the hydrolysis of simple glycerides, lysophospholipids, and vitamin esters in the intestinal lumen (Rudd & Brockman, 1984). Related enzymes are found in the liver (Camulli et al., 1989) and milk of humans and other mammals (Hui & Kissel, 1990). In the digestive process, pancreatic carboxylester lipase functions after the partial digestion of dietary fats by lingual lipase and pancreatic colipase-dependent lipase (Lindstrom et al., 1988; Bernbäck et al., 1990) to effect the complete release of fatty acyl groups from ingested lipids. In addition to this well-recognized role in fat breakdown, carboxylester lipase has been implicated in the catalysis of cholesterol reesterification in the intestinal cell [e.g., Gallo et al. (1984) and Williams et al., (1989)] and, possibly, on the luminal side of the villus membrane (Bhat & Brockman, 1982a). However, these roles for the enzyme are not univ-

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[1] Abbreviations: DA, 13,16-*cis*,*cis*-docosadienoic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CEL, monomeric porcine pancreatic carboxylester lipase; GC-MS, gas chromatography-mass spectrometry.

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