Kinetics for Development of Gramicidin-Induced Ion Permeability in Unilamellar Phospholipid Vesicles†

Nancy R. Clement and J. Michael Gould*

ABSTRACT: The kinetics for the development of gramicidin-dependent cation permeability in small unilamellar vesicles have been studied by using a vesicle-entrapped, pH-sensitive fluorescence probe to continuously report changes in intravesicular pH. The incoporation of 4–5 gramicidin dimers/vesicle was sufficient to increase the proton and counterion permeability of that vesicle by several orders of magnitude, so that ionic equilibration following a perturbation of the external medium pH occurred in <1 s. Once a functional gramicidin dimer has become incorporated into one vesicle, it does not readily exchange into another, so that the effects of gramicidin with regard to an individual vesicle can be considered to be essentially "all or none". The rate at which transmembrane ion permeability develops in a vesicle suspension was found to depend upon the degree of fluidity of the membrane hydrocarbon interior, being much lower at low temperatures or when cholesterol was present in the bilayer. Low temperatures and increasing bilayer cholesterol content also decreased the number of vesicles affected by a given gramicidin concentration, indicating a decreased membrane solubility for the ionophore at low bilayer fluidities.

Protein-mediated ion translocation across phospholipid bilayer membranes can occur by one of two principal mechanisms. The carrier mechanism, exemplified by cyclic depsipeptide ionophores such as valinomycin and the macrotetralide actins, involves complexation of the ion with the carrier molecule, followed by diffusion of the ion–carrier complex across the membrane and dissociation of the complex on the other side (Szabo et al., 1969; Stark & Benz, 1971; Benz et al., 1973). Because carrier-type ionophores must diffuse across the membrane hydrocarbon interior, the rate at which these ionophores catalyze transmembrane ion movements is very sensitive to the microviscosity of this region.

Ions may also cross phospholipid bilayers via transmembrane, hydrophilic channels formed by various proteins. Perhaps the most widely studied protein in this class is the antibiotic ionophore gramicidin,[1] a linear pentadecapeptide with an unusual arrangement of alternating L and D hydrophobic amino acids, beginning and terminating with formyl and ethanolamine groups, respectively (Sarges & Witkop, 1964). In hydrophobic environments, gramicidin forms a cylindrical, left-handed β helix containing an ~4 Å diameter hydrophilic channel down the center (Urry, 1971). The end to end dimerization of two gramicidin molecules incorporated into a bilayer membrane results in the formation of a transmembrane hydrophilic channel (Goodall, 1970; Urry et al., 1971) capable of conducting a variety of monovalent cations (Mueller & Rudin, 1967) at flux rates approaching the mobility of these ions in water (Hladky & Haydon, 1970). While the rate of ion transfer through an individual gramicidin channel is largely unaffected by changes in membrane microviscosity (Krasne et al., 1971; Boheim et al., 1980), the microviscosity could influence the overall efficacy of a given gramicidin concentration to mediate ion transport in one of two ways: (1) in highly ordered, less fluid membranes, gramicidin molecules could be effectively excluded from the bilayer (Racker & Hinkle, 1974), and (2) variations in the motional freedoms of the phospholipid molecules comprising the bilayer should result in concomitant variations in the ability of individual gramicidin monomers to undergo lateral diffusion necessary for the formation of functional dimers.

These effects have been investigated in this study by using small unilamellar vesicles of the type often employed in membrane reconstitution experiments (Racker, 1973). Using a recently developed technique for continuously monitoring the internal aqueous pH of phospholipid vesicles (Clement & Gould, 1981a), we have measured, under a variety of conditions, the kinetics by which exogenously added gramicidin molecules collapse an existing transmembrane proton gradient.

Experimental Methods

Preparation of Vesicles and Measurement of Ion Transport. Unilamellar vesicles were prepared from purified soybean phospholipids (asolectin)[2] by a sonication procedure as described in a preceding paper (Clement & Gould, 1981a). The diffusion of hydrogen ions into the vesicles following an abrupt change in the external (medium) pH was monitored as changes in the fluorescence intensity of the pH-sensitive fluorescence probe 8-hydroxy-1,3,6-pyrenetrisulfonate (pyranine) trapped within the vesicle internal aqueous compartment as detailed elsewhere (Clement & Gould, 1981a,b).

Fluorescence Polarization. The fluidity of the hydrocarbon region of the phospholipid bilayer was estimated from the fluorescence polarization of the hydrophobic membrane probe 1,6-diphenyl-1,3,5-hexatriene (Cogan et al., 1973) by using an Elscint MV-1a polarimeter. For these experiments pyranine was omitted from the vesicle preparation solution.

Results

A rapid decrease in the external (medium) pH of a suspension of pyranine-containing asolectin vesicles equilibrated at alkaline pH is followed by a biphasic decrease in the vesicle

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[1] The commercial preparation of gramicidin used in this study (Sigma Chemical Co., St. Louis, MO.) contained a mixture of gramicidins A, B, and C (approximately 70%, 10% and 20%, respectively; Glickson et al., 1972). Gramicidins B and C differ from gramicidin A only in the identity of the hydrophobic amino acid at position 11.

[2] Abbreviations used: pyranine, 8-hydroxy-1,3,6-pyrenetrisulfonate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; tricine, *N*-tris(hydroxymethyl)methylglycine.

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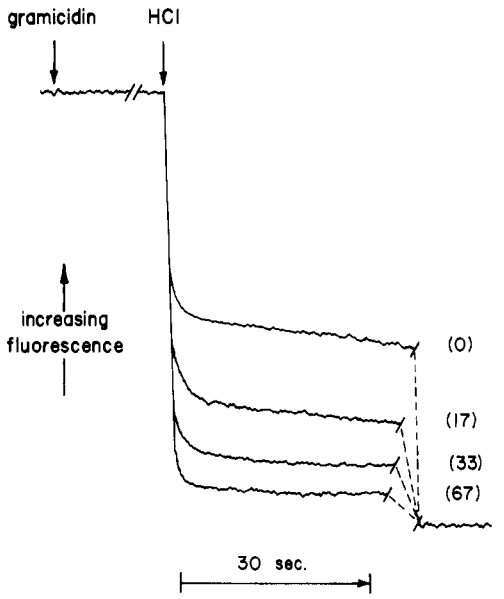

FIGURE 1: Passive hydrogen ion diffusion into phospholipid vesicles in the presence and absence of gramicidin. Unilamellar asolectin vesicles containing entrapped pyranine were prepared as described under Experimental Methods. The 3-mL reaction mixture contained 0.2 M sucrose, 0.1 M KCl, 5 mM tricine/KOH, 5 mM Mes/KOH (pH 8.2), and vesicles equivalent to 53 μg of phospholipid/mL. The indicated levels of gramicidin (nM) were added 2 min prior to the addition of 12 μL of 1 N HCl, which lowered the external medium pH from 8.2 to 6.6. Note that gramicidin increases the extent of the fast component of the fluorescence decrease (ΔF^{fast}) but has little or no effect upon the slower, counterion-limited component.

internal pH, as monitored by the entrapped probe (Figure 1). An initial, very fast decrease in pyranine fluorescence (limited in these experiments by the sample mixing time, $t_{1/2} \cong 1$ s) has been shown to result from rapid, electrically uncompensated H^+ influx (Clement & Gould, 1981a). The consequent development of a transmembrane electric potential (inside positive) leads to a much slower rate of H^+ influx, limited by the rate of charge-compensating counterion redistributions (Clement & Gould, 1981a,b). Vesicles preincubated with gramicidin exhibit a much larger proportion of the total fluorescence decrease during the initial, fast phase, although there is little or no effect upon the kinetics of the slower counterion-limited portion of the fluorescence change (Figure 1a). This effect, which contrasts sharply with the effects of valinomycin (Clement & Gould, 1981a,b), probably results from two important characteristics of gramicidin's ionophoretic mechanism: (1) the mobility of ions through the transmembrane hydrophilic channel formed by the gramicidin dimer is very high, approaching the mobility in water (Hladky & Haydon, 1970), and (2) once incorporated into a given vesicle, gramicidin molecules do not readily exchange into another vesicle.

This latter point is illustrated in the experiment presented in Table I, in which pyranine-containing vesicles were mixed with varying amounts of nonfluorescent vesicles either before or after the addition of gramicidin and then subjected to a rapid drop in external pH. The ability of a given gramicidin concentration to increase the percent of the fluorescence decrease occurring in the initial fast phase (ΔF^{fast}) was greatly diminished when gramicidin was added to the mixture of nonfluorescent and pyranine-containing vesicles. On the other hand, when gramicidin was added to pyranine-containing vesicles *prior* to the addition of the nonfluorescent vesicles, the effectiveness of that gramicidin concentration was not reduced, even after a 60-min incubation. These findings indicate that, under the conditions of this study, gramicidin does not readily exchange between vesicles. In other words, the effects of gramicidin on these vesicles are essentially all or none; i.e., those vesicles which contain a gramicidin channel or channels exhibit a very high proton and counterion permeability, whereas those vesicles containing no gramicidin are, of course, unaffected. At gramicidin levels greater than about 8–10 gramicidin molecules (or 4–5 dimers) per vesicle [assuming an average vesicle ≅ 3000 phospholipid molecules (Watts et al. 1978)], the proportion of the total fluorescence change occurring in <1 s approached 100%, suggesting that nearly all of the vesicles now contained some functional gramicidin channels.

Table I: Lack of Gramicidin Exchange between Vesicles

expt[a]	empty vesicles[b] (μg of phospholipid/mL)	increase in ΔF^{fast} (arbitrary units): time of HCl pulse (min)[c] 0	2	60
1	0		46	
	50		35	
	250		17	
2	50	41		40
	250	52		55

[a] Reaction conditions are described in the legend to Figure 1. In experiment 1, pyranine-containing vesicles (final concentration 80 μg of phospholipid/mL) were mixed with the indicated amount of empty vesicles prior to the addition of gramicidin (0.42 μg/mL). In experiment 2 gramicidin was added to the pyranine-containing vesicles 2 min before the addition of the indicated amount of empty vesicles. [b] Empty vesicles were prepared by sonication as described under Experimental Methods except that pyranine was omitted from the vesicle preparation buffer. [c] The HCl pulse (12 μL of 1 N HCl), added at the indicated time after the last addition to the samples (gramicidin in experiment 1 and empty vesicles in experiment 2), lowered the medium pH from 8.2 to 6.6. The fast component (ΔF^{fast}), measured in the absence of gramicidin, was 33% of the total fluorescence change.

In preliminary experiments we noticed that, in order for the maximum effectiveness of a given gramicidin concentration to develop, a short (~2-min) incubation period was necessary between the addition of gramicidin to the vesicle suspension and the rapid change in external pH. A variety of events could be occurring during this incubation, including insertion of gramicidin monomers into the lipid bilayer, followed by lateral diffusion and functional dimer formation, or dimer formation in solution followed by insertion of the dimer into the bilayer. While we do not have any direct indication as to which of the above processes is the rate-limiting step, it was nevertheless possible to directly observe the kinetics of development of gramicidin-dependent ion permeability by adding gramicidin to the vesicle suspension *after* a sudden pH shift in the external medium, during the slower, counterion-limited portion of the fluorescence change (Figure 2a). Addition of gramicidin at this point results in a transient increase in the rate of the fluorescence decrease, followed by the return of a slower rate of fluorescence change. Semilogarithmic replots of the changes in fluorescence intensity yielded four essentially linear regions[3] which could be described by the apparent rate constants k_1, k_2, k_3, and k_4 (Figure 2b). The apparent rate constant k_3 was taken to represent the kinetics of the rate-limiting step in the

[3] The relationship between pyranine fluorescence intensity and molar hydrogen ion concentration is not linear over the entire H^+ concentration range bounded by the initial and final pH values used in this experiment. However, the change in H^+ concentration within each region of the fluorescence decay is small enough that the relationship between fluorescence intensity and H^+ concentration can be taken as approximately linear.

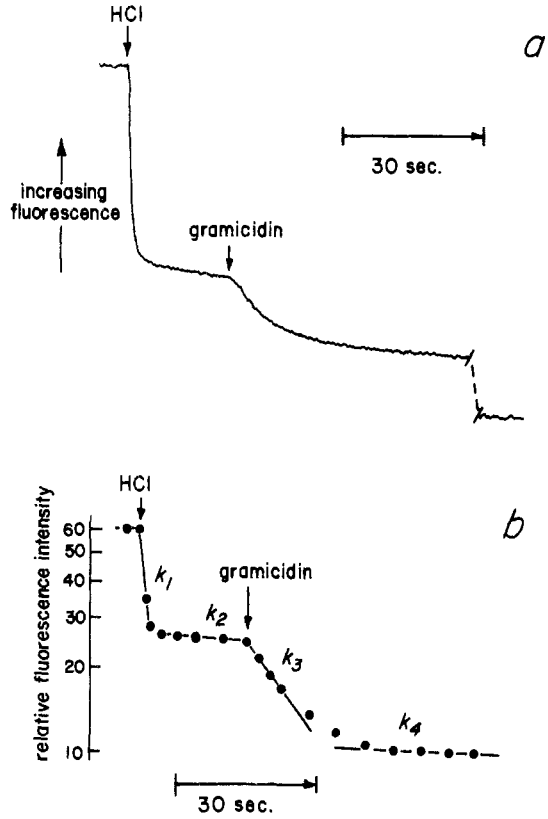

FIGURE 2: Kinetics for the development of gramicidin-dependent ion permeability in unilamellar asolectin vesicles. Reaction conditions were essentially as described in the legend to Figure 1. The vesicle concentration was equivalent to 59 μg of phospholipid/mL. The HCl pulse (12 μL of 1 N HCl) lowered the external medium pH from 8.2 to 6.6. (Panel a) Gramicidin (25 ng/mL final concentration) was added (in 10 μL of ethanol) at the indicated time. (Panel b) Semilogarithmic replot of the data presented in panel a. The four linear regions of the plot, characterized by their apparent rate constants, are defined as k_1, rapid, electrically uncompensated proton influx, k_2, counterion-limited proton influx, k_3, gramicidin-induced rapid collapse of proton and counterion gradients in a portion of the vesicle population (limited in these experiments by the rate of gramicidin insertion and/or dimer formation in the bilayer), and k_4, counterion-limited proton influx in vesicles not containing gramicidin dimers.

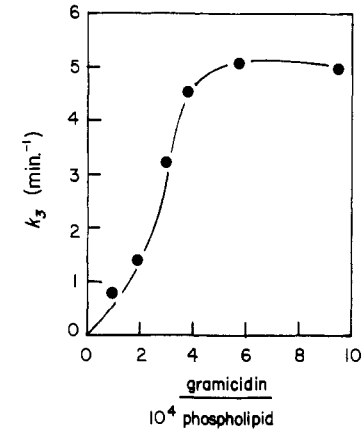

FIGURE 3: Dependence of the apparent rate constant for the development of gramicidin-induced ion permeability (k_3) on the gramicidin/phospholipid ratio. Reaction conditions were as described in the legend to Figure 1, except that gramicidin was added in a small volume of ethanol (<10 μL) 45 s after the HCl pulse. The samples contained vesicles equivalent to 60 μg of phospholipid/mL.

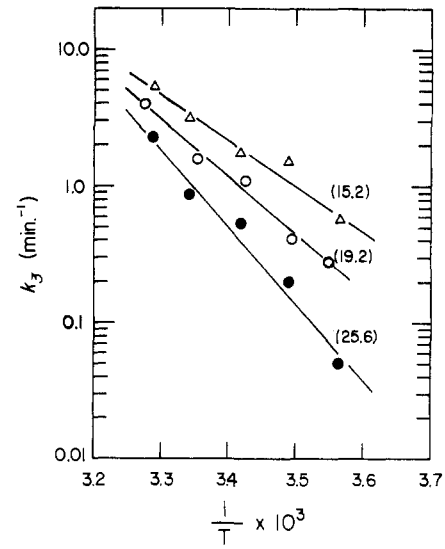

FIGURE 4: Effects of temperature and cholesterol on rate constant for development of gramicidin-induced ion permeability (k_3) in unilamellar asolectin vesicles. Reaction conditions were essentially as described in the legend to Figure 3 except that the temperature was varied as indicated. The vesicle samples contained 59 μg of phospholipid/mL (△), 53 μg of phospholipid plus 20 mol % cholesterol/mL (○), and 48 μg of phospholipid plus 33 mol % cholesterol/mL (●). Gramicidin was added to give a final concentration of ~3 gramicidin molecules/10^4 phospholipid molecules. The numbers in parentheses are the apparent Arrhenius activation energies in kilocalories per mole.

development of functional transmembrane gramicidin channels, since we have observed that, once these channels are in place, the permeability of an individual vesicle to protons and counterions becomes extremely high, with the existing ion gradients in that particular vesicle collapsed in <1 ms (C. M. Biegel and J. M. Gould, unpublished experiments).

As expected k_3 depended strongly upon the gramicidin/phospholipid ratio (Figure 3) and upon the temperature and composition of the membrane (Figure 4). At a given gramicidin/phospholipid ratio, values for k_3 in asolectin vesicles decreased by a factor of 10 as the temperature was lowered from 30 to 5 °C. The dependence of k_3 on temperature was similar in vesicles containing cholesterol, although the absolute values of k_3 became lower at any particular temperature with increasing cholesterol content. The presence of cholesterol in the vesicle bilayer increased the activation energy for the rate-limiting process which determines k_3 from ~15 kcal/mol in the absence of cholesterol to nearly 26 kcal/mol in the presence of 33 mol % cholesterol (Figure 4).

The effects of temperature and cholesterol on k_3 can most likely be ascribed to changes in fluidity of the hydrocarbon interior of the membrane bilayer. Estimates of the fluidity of this region of the asolectin bilayers used in this study were obtained from the fluorescence polarization of the lipophilic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) (Cogan et al., 1973). Both decreasing temperature and/or increasing cholesterol content increased the polarization of DPH (i.e., decreased membrane fluidity) (Figure 5). From these data it is possible to calculate a fluidity term ($\eta V/R$) which relates the membrane microviscosity (η), multiplied by the constant V/R (V is the partial molar volume of the probe and R is the gas constant), to the observed fluorescence polarization (p) by using a rearrangement of the Perrin equation

$$\eta\frac{V}{R} = \frac{\tau p(3 - p_0)T}{3(p_0 - p)}$$

where p_0 is the limiting polarization, τ the fluorescence lifetime, and T the absolute temperature. When the values for k_3 determined under the various conditions of temperature and cholesterol content are normalized to this fluidity term, all of

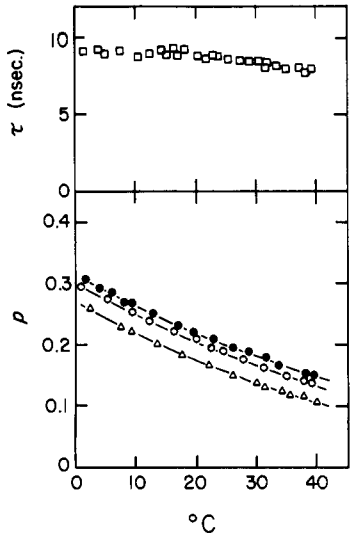

FIGURE 5: Temperature dependence of the fluorescence polarization and lifetime of 1,6-diphenyl-1,3,5-hexatriene in unilamellar asolectin vesicles. Vesicles were formed by sonicating 325 μg of phospholipid containing 10 nmol of 1,6-diphenyl-1,3,5-hexatriene and 0 (△), 20 (○), or 33 (●) mol % cholesterol in 0.5 mL of 0.2 M sucrose, 0.1 M KCl, 5 mM tricine/KOH, and 5 mM Mes/KOH (pH 8.2). After sonication, the sample was diluted to 4 mL in the same buffer. p, polarization; τ, fluorescence lifetime.

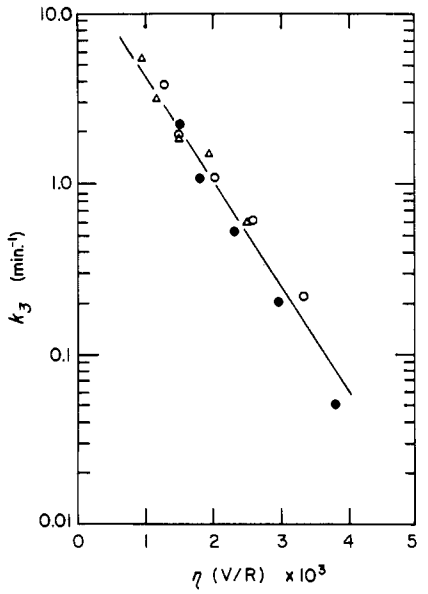

FIGURE 6: Dependence of the rate constant for the development of gramicidin induced ion permeability (k_3) on membrane fluidity in unilamellar asolectin vesicles. Values of k_3 from the data presented in Figure 4 are plotted versus the fluidity term $\eta(V/R)$ from the Perrin equation. Values of $\eta(V/R)$ were derived from the polarization and lifetime data presented in Figure 5.

the points fall on a single line (Figure 6) suggesting rather strongly that the primary determinant for k_3 is membrane fluidity.

In addition to their effects upon the rate of development of gramicidin-induced ion permeability, temperature and cholesterol content also appeared to affect the number of vesicles incorporating functional gramicidin channels. Lowering the temperature or increasing membrane cholesterol diminished both the rate *and* the extent of the gramicidin-induced kinetic transient (Figure 7).

Discussion

It has been known for some time that ion transport by carrier-type ionophores (e.g., valinomycin) is sensitive to the

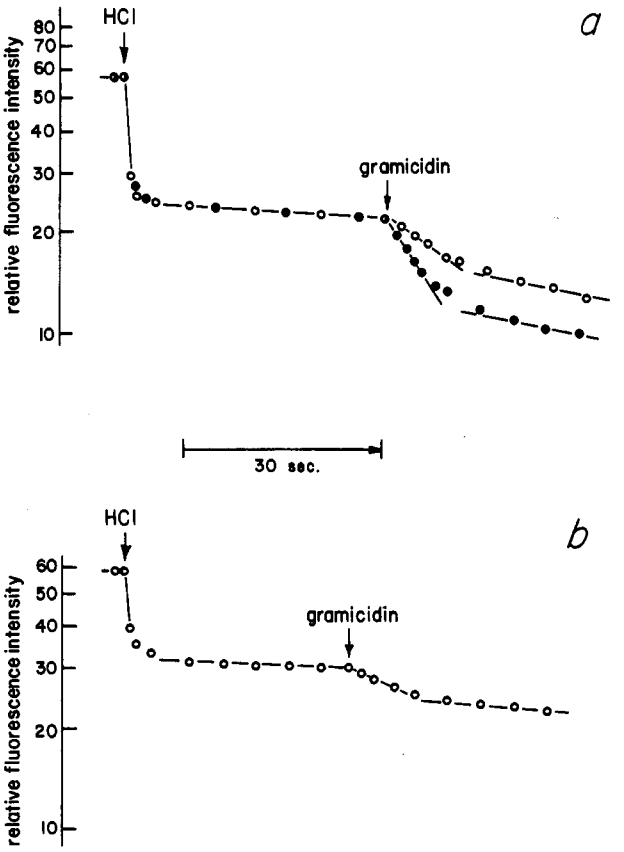

FIGURE 7: Kinetics for the development of gramicidin-induced ion permeability in unilamellar asolectin vesicles: effect of temperature and cholesterol on the number of vesicles affected by gramicidin. Reaction conditions were essentially as described in the legend to Figure 3, except that the temperature was varied. (Trace a) Semilogarithmic replots of the data obtained for the transient fluorescence decrease seen upon addition of 75 ng of gramicidin to vesicle suspensions containing 59 μg of phospholipid; open circules represent data obtained at 19.5 °C, and the closed circles are for data at 26.2 °C. Note the increase in k_3 with increasing temperature. (Trace b) Semilog replot of data obtained in an experiment identical with that shown in trace a (at 26.2 °C), except that 33 mol % cholesterol was incorporated into the phospholipid vesicles.

fluidity of the lipid bilayer (Krasne et al., 1971; Benz et al., 1973, 1977; Papahadjopoulos et al, 1973; Boheim et al., 1980; Clement & Gould, 1981b), while ion transport by channel-forming ionophores (e.g., gramicidin) is largely insensitive to membrane fluidity (Krasne et al., 1971; Racker & Hinkle, 1974). In this study we have shown that the kinetics for development of gramicidin-dependent ion transport in artificial vesicles are very sensitive to membrane fluidity, regardless of how the fluidity is varied. Unfortunately, the experiments reported here provide no direct information on the nature of the fluidity-sensitive step. For instance, membrane fluidity could determine the rate at which gramicidin monomers (or dimers) become incorporated into the bilayer or the rate of functional dimer formation from nonconductive monomers as a result of lateral diffusion within the membrane (Haydon, 1975), or both. Future experiments employing a covalently linked gramicidin dimer (Urry et al., 1971) may provide further insight into this aspect of the problem.

In addition to affecting the mobility of molecules within the bilayer, changes in membrane fluidity can also affect the solubility of foreign molecules, so that at lower temperatures, fewer gramicidin molecules can be accommodated in a given membrane area (Krasne et al., 1971; Boheim et al., 1980). This phenomenon is reflected here as a decrease in the extent of the gramicidin-induced fluorescence transient by lower

temperatures or increased cholesterol content (Figure 7).

Finally, these experiments illustrate an unusual and potentially very useful application of the vesicle-entrapped pyranine technique (Clement & Gould, 1981a) to study the molecular dynamics of membrane–protein interactions. Additional studies on the effects of membrane surface charge and phospholipid composition, as well as studies utilizing larger ionophoretic proteins, such as the hydrophobic CF_0 portion of the chloroplast ATP synthase complex, are in progress.

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Electric Field Induced Transient Pores in Phospholipid Bilayer Vesicles†

Justin Teissie and Tian Yow Tsong*

ABSTRACT: A study of the voltage induction of transient pores in phospholipid bilayer vesicles is reported. Unilamellar vesicles (dipalmitoylphosphatidylcholine), with a size distribution of 100 ± 30 nm, were prepared by the method of Enoch & Strittmatter [Enoch, H., & Strittmatter, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 145]. The vesicles loaded with [^{14}C]sucrose and suspended in a mixture of 150 mM NaCl and 272 mM sucrose (both are the isotonic solvent for erythrocytes) were exposed to an intense electric field in the range of 20–40 kV/cm, with a field decay time of 5–15 μs. A transient leakage of sucrose label was detected when the field strength exceeded 30 kV/cm. After the field was removed, no slow leakage of the tracer molecules occurred during a 65-h incubation period at the room temperature (23 ± 2 °C). The leakage is attributed to the field-induced transmembrane potential, but not other effects such as the Joule heating or the shock wave associated with the voltage discharge. When this potential exceeded a threshold value of 200 mV, corresponding to an applied field strength of 30 kV/cm, there was a dielectric breakdown of the bilayer structure. Pores which allowed passage of sucrose were formed, transiently. Experiments show that these pores were fully reversible, and no global and permanent damages to the vesicle bilayer were detected. The implication of this membrane potential triggered conducting state of lipid bilayers to biological functions of cells is discussed.

Transmembrane potentials are believed to play a major role in biological processes. Neurotransmission is linked to the transfer of ionic currents across the nerve membrane induced by the action potential (Hodgkin, 1964). Membrane potential changes as a consequence of proton (and other charged species) translocation across the mitochondrial membrane are now considered a "driving force" of the energy transduction (Mitchell, 1977). Release of biogenic amines and other hormones in connection with membrane depolarization is well established in chromaffin granules of the adrenal medulla (Neumann & Rosenheck, 1972; Rosenheck et al., 1975).

Under normal conditions, the phospholipid bilayer in the cell membrane is a poor conducting medium, and it can mimic a capacitor. Species transport when it does occur is supposed to be, and often proved to be, associated with an enzymatic machinery (the so-called "pumps"). Nevertheless, when a transport system is activated, drastic changes in membrane potential are recorded with electrophysiological measurements. Under such conditions, the simplistic model of the phospholipid layer as a nonconducting capacitor may no longer be valid. In fact, electrical fields in the range of 100 kV/cm are induced (i.e., a potential of 50 mV on a membrane with a thickness of 5 nm).

When submitted to a strong external electrical field, the bilayers of closed membranous vesicles are polarized because of the movement of ions along the electric field lines. As a consequence, the electrogenicity of the cell is severely per-

† From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205. *Received August 28, 1980.* This work was supported by National Institutes of Health Grant HL18048.

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