J. Membrane Biol. 96, 165–173 (1987)

The Journal of
Membrane Biology

Procedure Using Voltage-Sensitive Spin-Labels to Monitor Dipole Potential Changes in Phospholipid Vesicles: The Estimation of Phloretin-Induced Conductance Changes in Vesicles

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Summary. Voltage-sensitive membrane potential probes were used to monitor currents resulting from positive or negative charge movement across small and large unilamellar phosphatidylcholine (PC) vesicles. Positive currents were measured for the paramagnetic phosphonium ion or for K^+-valinomycin. Negative currents were indirectly measured for the anionic proton carriers CCCP and DNP by monitoring transmembrane proton currents. Phloretin, a compound that is believed to decrease dipole fields in planar bilayers, increases positive currents and decreases negative currents when added to egg PC vesicles. In these vesicles, positive currents are increased by phloretin addition to a much larger degree than CCCP currents are reduced. This asymmetry, with respect to the sign of the charge carrier, is apparently not the result of changes in the membrane dielectric constant. It is most easily explained by deeper binding minima at the membrane-solution interface for the CCCP anion, when compared to the phosphonium. The measured asymmetry and the magnitudes of the current changes are consistent with the predictions of a point dipole model. The use of potential-sensitive probes to estimate positive and negative currents, provides a methodology to monitor changes in the membrane dipole potential in vesicle systems.

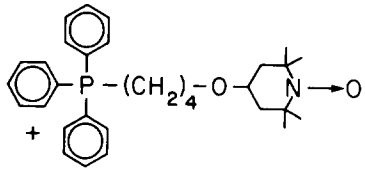
Key Words dipole potential · phospholipid vesicles · spin labels · CCCP · membrane potentials · phloretin

Introduction

Electrical forces are of fundamental importance for process such as ion transport and membrane protein conformation. The largest electrical forces experienced by ions in membranes are those resulting from the electrostatic charging energy for ions when placed into the low dielectric membrane interior; this is known as the Born charging energy (Neumcke & Lauger, 1969). The next largest electrical forces for ions in membranes are those resulting from the membrane dipole potential. The magnitude of the dipole potential is on the order of 200 to 300 mV, interior positive, in phosphatidylcholine vesicles and is therefore much larger than the transmembrane or surface potentials typically found in biological systems. The existence of this dipole potential in membranes has been known for some time, and it accounts for the dramatic permeability difference seen between organic anions and cations (Leblanc, 1969; Liberman & Topaly, 1969; Hladky, 1974; Andersen & Fuchs, 1975; Pickar & Benz, 1978; Hladky & Haydon, 1983; Flewelling & Hubbell, 1986). While its effect on proteins is not known, the magnitude of this potential suggests that it is likely to be an important element in the determination of protein conformation and folding in membranes. The molecular origin of the dipole potential is presently unknown, but it is believed to result from a net orientation of groups located near the membrane-solution interface. The most likely molecular sources for the dipole potential appear to be oriented water in the interface or the lipid carbonyls.

The membrane dipole potential can be modified by agents such as phloretin, which appears to reduce the magnitude of this potential (Andersen et al., 1976; Melnik et al., 1977; Reyes et al., 1983). Phloretin, the aglycone of phlorizin is also an inhibitor of hexose transport in the red blood cell. While reductions of the dipole potential can be monitored in planar bilayer systems, procedures to monitor dipole potential changes have not been developed for membrane vesicle systems. There are a number of important reasons to quantitate these electrical changes in vesicle systems. In planar systems, the phase partitioning of phloretin cannot be easily determined. This makes a quantitative evaluation of the action of agents such as phloretin difficult. In addition, because of the similarity of vesicles to biological membranes and the ability to define their composition, vesicles are ideal systems with which to study the effects of dipole fields on protein conformation and function.

In the present article, we describe a procedure using potential-sensitive paramagnetic amphiphiles to monitor dipole potential changes in membrane vesicles using EPR[1] spectroscopy. We previously used this approach to characterize the effects of dipole potential changes on H^+/OH^- currents in lipid vesicles (Perkins & Cafiso, 1986). The experiments described here investigate this approach in more detail and establish methodologies that can be used to quantitate ion currents in vesicles systems. We directly measure positive phosphonium currents and K^+-valinomycin currents using the paramagnetic phosphonium I, shown below. We estimate negative currents due to CCCP and DNP anions by measuring proton currents with the secondary amine probe II, shown below. We measure



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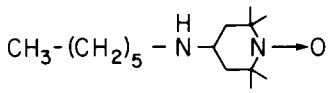

II

the binding of phloretin and characterize the current changes it induces in sonicated phospholipid vesicles. These current changes are compared with the predictions of a simple point dipole model used previously to describe hydrophobic ion energies in bilayers.

Materials and Methods

Materials

Egg phosphatidylcholine was purified from fresh hen eggs according to the procedure of Singleton and co-workers (1965) and stored in chloroform under an argon atmosphere at −20°C. All other lipids were obtained from Avanti Biochemicals (Birmingham, Ala.). Phloretin was obtained from Sigma Chemical Co. (St. Louis, Mo.) Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was obtained from Calbiochem (La Jolla, Calif). Dinitrophenol (DNP) was obtained from Aldrich Chemical Co. (Milwaukee, Wis.). The ether-linked alkylphosphonium nitroxide I was synthesized, as previously described, by producing a bromoalkylether nitroxide (from dibrombutane and TEMPOL). This bromoalkylether was then reacted with triphenylphosphine (Flewelling & Hubbell, 1986*a*). Label II was synthesized by a procedure used previously (Cafiso & Hubbell, 1978*b*) as was the labeled phospholipid, tempophosphatidylcholine, TEMPO PC (Kornberg & McConnell, 1971).

Procedures

Preparation and Characterization of Vesicles

Small unilamellar phospholipid vesicles were prepared by drying aliquots of lipid under vacuum for periods of at least 15 hr, dissolving the lipid in the appropriate buffer solution and ultrasonically irradiating the lipid dispersion as previously described (Castle & Hubbell, 1976). Large unilamellar vesicles were formed by reverse phase evaporation as previously described and sized through a nucleopore filter (Szoka & Papahadjopoulos, 1980). The phospholipid concentrations in these samples were determined using a phosphate assay similar to that described by Bartlett (1959). For small sonicated vesicles, the ratio of internal-to-external aqueous volumes V_i/V_o was determined from this concentration (Cafiso & Hubbell, 1978*a*). For large unilamellar vesicles, this volume ratio was determined by adding *ca.* 200 μM of the permanent spin probe TEMPOL to the lipid suspension after vesicle formation. The fraction of the total spin signal that could be broadened by $K_3Fe(CN)_6$ (an impermeant broadening agent) provided a measure of this volume ratio.

Phloretin was added to vesicle suspensions in one of two ways. In the first method, phloretin was added from a concentrated ethanolic stock solution to the vesicle suspension, followed by brief (*ca.* 15 sec) sonication. In the second method, the appropriate quantity of phloretin was added to the chloroform-lipid solution. The lipid solution was then dried under vacuum and sonicated as described above. Either method was found to give identical results.

Determination of Phloretin Phase Partitioning

To determine the phase partitioning of phloretin in egg PC vesicle suspensions, the aqueous concentration of phloretin was determined by filtering a small fraction of the vesicle suspension through a centricon ultrafiltration device (Amicon Corp., Lexington, Mass.). At the lowest lipid concentrations used here, greater than 95% of phloretin was bound to the vesicle membrane.

EPR Spectra and the Estimation of Ion Currents

EPR spectra were recorded on a Varian E-109 or modified V-4500 spectrometer fitted with a pneumatic rapid mixing device. We measured the phase partitioning of probes I and II from their EPR spectra using a procedure discussed previously (Cafiso & Hubbell, 1982). Briefly, the phase partitioning is determined from the amplitude of the high-field resonance ($m_I = -1$) due to aqueous spin A_f. In the present case, we determined A_f from A, the high-field resonance amplitude, according to:

$$A_f = (A - (\beta/\alpha)A_f^0)/(1 - \beta/\alpha). \quad (1)$$

[1] Abbreviations used: EPR, electron paramagnetic resonance; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DNP, dinitrophenol; TEMPO, 2,2,6,6,-tetramethylpiperidine-1-oxyl; TEMPOL, 4-hydroxy-TEMPO; PC, phosphatidylcholine; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(morpholino)propanesulfonic acid; TPP^+, tetraphenylphosphonium; TPB^-, tetraphenylboron.

Here, A_f^0 is the amplitude of the high-field resonance in the absence of vesicles. The ratio β/α is determined by measuring the ratio of amplitudes for equal moles of totally membrane bound to aqueous probe.

Transmembrane currents for the spin-labeled phosphonium were measured by mixing solutions of label I (final concentration 20 μM) with a suspension of vesicles as described previously (Cafiso & Hubbell, 1982). Following mixing, a time-dependent decrease in the aqueous signal of I is observed as a result of its transmembrane migration. A_f can be directly related to the membrane current density i of the phosphonium I by:

$$i = -\frac{\beta Z F}{S_o}\left[\frac{K_o V_{mo}/V_o}{1 - \varepsilon_o/\varepsilon_i}\right] \cdot \left[\frac{dA_f}{dt}\right] \tag{2}$$

where $\varepsilon_o = (1 + K_o V_{mo}/V_o)$, $\varepsilon_i = (1 + K_i V_{mi}/V_i)$, S_o is the external vesicle surface area, K_o and K_i are the external and internal probe binding constants, respectively, and V_{mo} and V_{mi} are the volumes of the external and internal binding regions occupied by probe I, respectively. In Eq. (2) the derivative of the free signal amplitude with time dA_f/dt can be used to calculate the membrane current density at any time. In the experiments carried out here, we estimate the initial current i_o using Eq. (2) and $(dA_f/dt)_{t=0}$. The initial current for phosphonium i_o can be related to the external aqueous concentration of label $[I_o]$ and the inward rate constant for label movement k_f in the absence of a transmembrane potential by:

$$i_o = F \cdot k_f \cdot [I_o] \cdot K_o V_{mo}/S_o. \tag{3}$$

The permeability of the phosphonium P is defined as $P = k_f K_o V_{mo}/S_o$. We assume that the ratio V_{mo}/S_o remains constant with phloretin addition and that changes in i_o can be related either to changes in k_f or changes in K_o. K_o is determined from a measure of the aqueous spin signal at $t = 0$, $A_f(0)$, as given by: $[A_f^0 - A_f(0)]/A_f(0) = K_o V_{mo}/V_o$.

We also used the spin-labeled phosphonium I to estimate currents that resulted from valinomycin-mediated K^+ conductances in vesicles. In this case, transmembrane potentials were calculated from the free signal amplitude A_f as described previously (Cafiso & Hubbell, 1978*a*). Vesicles with K^+ ion gradients ($[K^+]_{in} > [K^+]_{out}$ were formed as previously described. Time-dependent changes in the potential across these vesicles occur with valinomycin addition. The initial K^+ currents were determined simply from the derivative of the time-voltage data as given by:

$$i_o = (\partial\psi/\partial t)_{\psi=0} \cdot c \tag{4}$$

where c is the specific membrane capacitance. We used a value for c of 0.9 μF/cm^2 that was found previously for solvent free planar bilayers (Montal & Mueller, 1972). The hydrophobic anion TPB^- is added to these vesicle suspensions at very low concentrations ($\approx$1 ion/2000 lipids). TPB^- selectively accelerates the transmembrane equilibration of the phosphonium at these concentrations and ensures that label movement is not rate limiting (Cafiso & Hubbell, 1982).

Proton currents, due to CCCP or DNP, were estimated using label II. This secondary amine nitroxide was used to measure transmembrane pH gradients as previously described (Cafiso & Hubbell, 1983). From a measure of the time rate of change of the internal vesicle pH, the proton current i_o was estimated from:

$$i_o = F \cdot B(\partial \mathrm{pH}/\partial t)_{\Delta\psi=0} \cdot r_i^2/3r_o \tag{5}$$

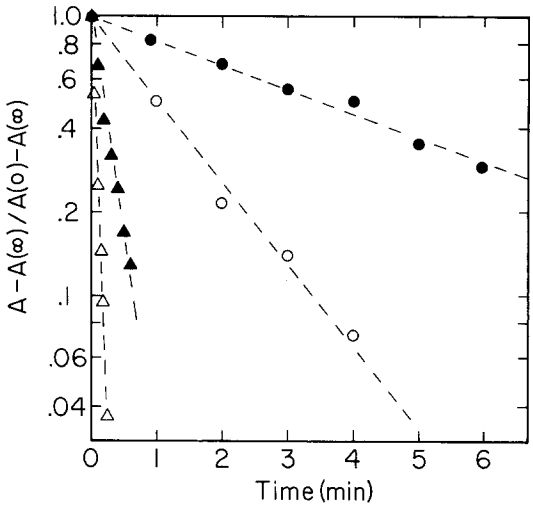

Fig. 1. A plot of the fractional change in the free signal intensity with time following the mixing of the labeled phosphonium I with phospholipid vesicles: without (●), and with 0.5 mM (○), 1 mM (▲) and 2 mM (△) phloretin added. The final phospholipid concentration is approximately 23 mg/ml and the label concentration is 20 μM. Vesicle suspensions were made in a 125-mM Na_2SO_4 solution containing 50 mM MOPS, pH = 7.0. From the initial rate of change in the high-field resonance intensity A the transmembrane electrical current for this labeled ion is calculated

where B is the buffer capacity and r_i and r_o are the internal and external vesicle radii, respectively. Due to the small internal volumes of the vesicle suspensions used here, changes in Δph are essentially the result of changes in the internal vesicle pH alone.

The effects of point dipoles added to the lipid interface on the transport of positive and negative hydrophobic ions were modeled using a total potential model for hydrophobic ions previously described. Except where noted, all parameters in this model are those found for TPB^- and TPP^+ (*see* Flewelling & Hubbell, 1986*b*).

Results

Transmembrane phosphonium currents are increased by phloretin

As shown previously, the addition of the phosphonium probe I to vesicle suspensions results in a time-dependent increase in the binding of I to vesicles. This change is a result of the transmembrane migration of I (Cafiso & Hubbell, 1982). Shown in Fig. 1 are plots of the fractional change in the high-field resonance amplitude for the phosphonium probe I, when added to phospholipid vesicles containing varied levels of phloretin. As was found previously, the curves appear to be first order, within experimental error. From the initial rate of change in this signal amplitude, we calculated the transmembrane currents for the phosphonium using Eqs.

Table 1. Phosphonium currents[a]

[Phloretin]	i_o(pA/cm²)	K_o	$k_f \times 10^4$ (sec^{-1})
0	2.55	135 ± 20	3.5 (±1.5)
0.5 mM	6.93	131 ± 20	9.8 (±3)
1.0 mM	40.1	125 ± 19	60 (±30)
2.0 mM	182	125 ± 19	270 (±70)
3.0 mM	321	148 ± 23	420 (±170)
4.0 mM	930	162 ± 25	1,100 (±300)

[a] Currents were obtained using Eq. (2) by measuring the change in the high-field resonance amplitude at $t = 0$ following the mixing of I with egg PC vesicles. The final concentration of I was 20 μM in 23 mg/ml egg PC. Values of K_o were obtained directly from the phase partitioning of probe I at $t = 0$ and k_f was calculated using Eq. (3). The errors given for K_o and k_f were estimated from the uncertainty in EPR signal amplitude and lipid concentrations.

(1) and (2). Shown in Table 1 are some representative currents obtained for the phosphonium alone and at several concentrations of phloretin. As seen in Fig. 1 or Table 1, the addition of phloretin results in a dramatic increase in the phosphonium currents. Changes in the initial current i_o can result either from changes in the binding constant of I, K_o, or the forward rate constant k_f. Values we obtain for K_o and k_f are given in Table 1 for several phloretin concentrations. K_o remains relatively constant with phloretin addition until *ca.* 10 mol/100 mol, where an increase in the binding of I to PC vesicles is observed.

CCCP-Induced Proton Currents in Vesicles

Shown in Fig. 2 are tracings of the high-field resonance amplitude for the spin-labeled secondary amine II when weakly buffered pH gradients are created across phospholipid vesicles both in the presence and absence of 1 μM CCCP. The change in the high-field resonance amplitude of II represents a change in the vesicle pH gradient. As shown previously, an electrically active movement of protons, due to the intrinsic H^+/OH^- permeability of the vesicle membrane, accounts for this process in the absence of CCCP (Cafiso & Hubbell, 1983). This net proton movement ceases when the pH gradient comes to electrochemical equilibrium across the vesicle. As expected, the addition of CCCP changes only the time-course of the decay in Fig. 2 and has no effect on the equilibrium value of ΔpH. Under the conditions used here the number of protons that cross the membrane is consistent with the measured equilibrium potential (measured using I) and the expected bilayer capacitance (Cafiso & Hubbell,

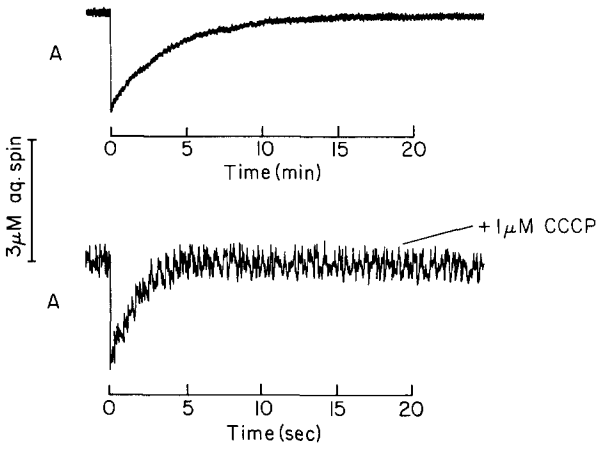

Fig. 2. Tracings of the high-field resonance of the secondary amine nitroxide II following creation of a pH gradient across sonicated egg PC vesicles (*ca.* 15 mg/ml). The probe is at a concentration of 30 μM and the vesicles contain 5 mM MES buffer with 125 mM Na_2SO_4. The increase in signal intensity with time is due to the decay of a transmembrane pH gradient. Simultaneous development of a transmembrane potential also occurs (Cafiso & Hubbell, 1983). At $t = 0$, a pH gradient is created: ΔpH is 1.4 with $pH_{in} = 6.17$ and $pH_{out} = 7.58$. The equilibrium ($t = \infty$) value of ΔpH is 1.2. The upper and lower curves show the time course without and with the addition of 1 μM CCCP, respectively

1983). Increasing the buffer concentration (eg. to 100 mM) dramatically reduces the magnitude of the time-dependent ΔpH changes. From the data in Fig. 2, we calculated values for ΔpH as a function of time and determined the initial proton current i_o from the initial change in ΔpH using Eq. (5). The proton currents estimated in each case are shown in Table 2. The proton current in the presence of 1 μM CCCP exceeds the background H^+/OH^- current by *ca.* two orders of magnitude.

In planar bilayer systems, the mechanism of action of CCCP has been well characterized (Kasianowicz, Benz & McLaughlin, 1984). The CCCP-induced proton conductivity, at neutral pH's, should be rate limited by the movement of the CCCP anion. This appears to be the case here. Intervesicle movement of CCCP is not rate limiting. We examined the concentration dependence of the CCCP-induced proton current in vesicles and found it to be linear from 0.25 to 2 μM CCCP (from ≈ 0.12 to 1.0 CCCP's/vesicle). Under the conditions of our experiment, we can estimate the CCCP anion current from the current found from Eq. (5). Assuming that protonated (uncharged) CCCP equilibrates rapidly across the vesicle membrane, the apparent outward proton current should equal the inward CCCP anion current. With this assumption, we estimated the rate constant for movement of the CCCP anion using the following expression:

Table 2. Proton currents

Additions	i_o(nA/cm^2)
None	0.02 ± 0.01
1 μM CCCP[a]	3.5 ± 0.4
1 μM CCCP + 1 mM Phloretin	3.0 ± 0.4
1 μM CCCP + 2 mM Phloretin	1.7 ± 0.3
1 μM CCCP + 3 mM Phloretin	1.1 ± 0.2
1 μM CCCP + 4 mM Phloretin	0.30 ± 0.1
40 μM DNP[b]	5.2 ± 0.4

[a] CCCP-induced proton currents were estimated from $(\partial pH/\partial t)_{t=0}$ using Eq. (5) with 20 μM II in egg PC vesicles at a concentration of approx. 20 mg/ml. The vesicles contained 10 mM citrate buffer, pH 5.14 and the external pH was shifted to a value of 6.55 by the addition of a 10 mM MOPS buffer (pH = 8.0). All buffer solutions contained 125 mM Na_2SO_4.

[b] DNP-induced proton currents were estimated in egg PC vesicles at 20 mg/ml PC with internal and external pH's of 5.13 and 6.50, respectively.

$$i_o = (kV_{mo}/S_o) \cdot (N_o/V_{mo} - N_i/V_{mi}). \quad (6)$$

Here, k is the rate constant for anion movement and N_o and N_i are the moles of CCCP anion on the external and internal vesicle interfaces, respectively (in Eq. (6) we assume that the inward and outward rate constants for CCCP movement are equal). Using the binding constants and pK determined previously for CCCP in egg PC vesicles (Kasianowicz et al., 1984), we estimate N_o and N_i and determine k from the measured values of i_o.[2] At the lipid concentrations used here, virtually all CCCP is membrane bound; less than 0.5% of the CCCP is present in the aqueous phase. As expected, the CCCP-induced proton currents vary dramatically with pH, decreasing at lower pH's. However, the rate constant for CCCP anion movement remains relatively constant. In a range of pH's from 7.5 to 4.5, the value of k is approximately 6 sec^{-1}. This is considerably less than the value found in bilayers formed from chlorodecane (175 sec^{-1}), but only slightly more than the value expected in decane-containing bilayers (Kasianowicz et al., 1984). At higher pH's, where the movement of protonated CCCP is expected to become rate limiting, the initial proton current i_o decreases. This data provides evidence that within a range of neutral and more acidic pH's, the CCCP-induced proton current in vesicles is rate-limited by the transmembrane movement of the CCCP anion.

[2] We estimated the moles of the CCCP anion associated with the external and internal interface, N_o and N_i, respectively, using the expressions given below:

$$N_i = N_t\{1 + ([H^+]_{in}/10^{-6}) \cdot [1 + (V_{mo}/V_{mi}) \cdot (1 + 10^{-6}/[H^+]_{out})]\}^{-1}$$

$$N_o = N_t\{1 + ([H^+]_{out}/10^{-6}) \cdot [1 + (V_{mi}/V_{mo}) \cdot (1 + 10^{-6}/[H^+]_{in})]\}^{-1}$$

where N_t is the total number of moles of CCCP present, and the pK of CCCP associated with the membrane is taken to be approximately 6 (Kasianowicz et al., 1984).

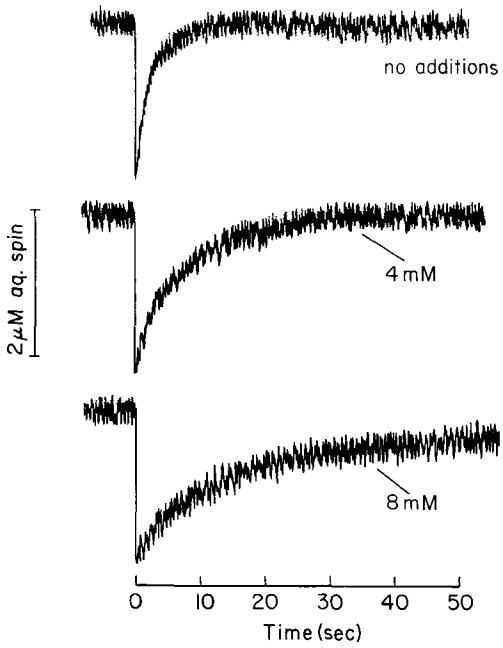

Fig. 3. Tracings of the high-field resonance of the secondary amine nitroxide II following the establishment of a pH gradient across sonicated egg PC vesicles containing 2 μM CCCP (40 mg/ml lipid). The probe is at a concentration of 40 μM and the internal vesicle buffer is 5 mM MES with 125 mM Na_2SO_4. At $t = 0$, the internal pH is 6.5 and the external pH is 7.8. The three tracings are shown for vesicles that contain 0, 4, and 8 mM phloretin. From the initial slope of this curve, the initial current for protons is calculated as described in the text

CCCP- and DNP-Induced Proton Currents are Reduced by Phloretin

Shown in Fig. 3 are tracings of the high-field resonance amplitude for label II following the creation of a pH gradient in vesicles with 1 μM CCCP present. From this data we calculated values for ΔpH versus time and used Eq. (5) to calculate the proton currents. From this current we subtracted the background H^+/OH^- current measured in the absence of CCCP. The background current is usually insignificant, except at the highest phloretin concentrations. Some of these CCCP-induced proton currents are listed in Table 2. As expected, the CCCP-induced proton movement decreases with phloretin addition. Shown in Fig. 4 is a plot of the

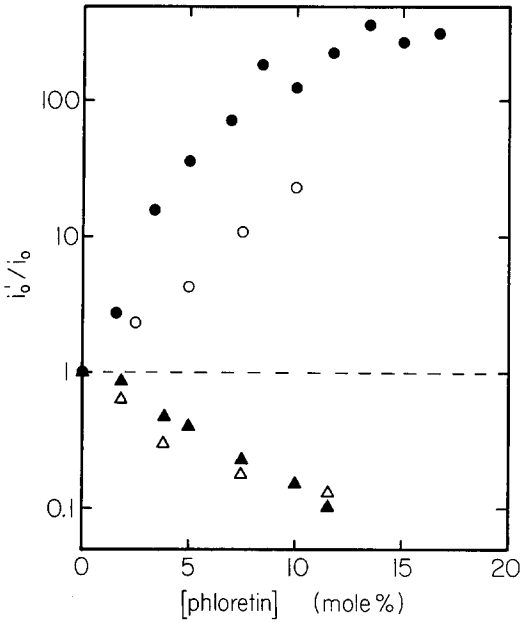

Fig. 4. A plot of the ratio of currents i_o'/i_o, as a function of the added concentration of phloretin (i_o' and i_o are the currents in the presence and absence of phloretin). Phosphonium currents (●) and K^+-valinomycin currents (○) increase as a function of phloretin concentration. K^+-valinomycin currents are measured using 30 μM I for a 10 : 1 (K_{in}^+/K_{out}^+) K^+ gradient with the addition of 1.5 μM valinomycin and 5 μM TPB^- (in 10 mg/ml egg PC). CCCP (▲) and DNP (△) currents are also shown and decrease with added phloretin. CCCP and DNP currents are average ratios for three and two sets of data, respectively

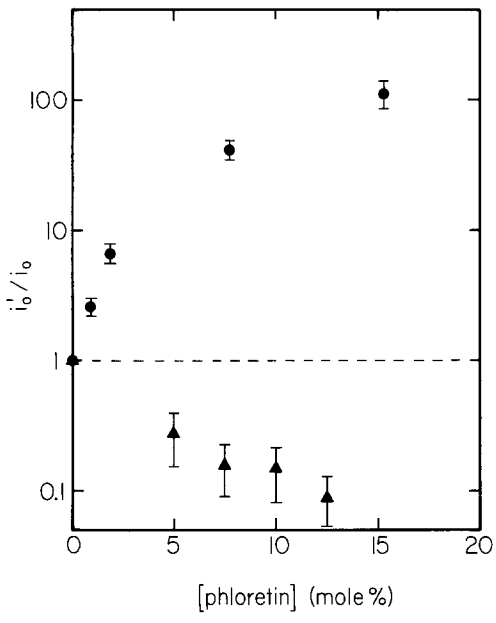

Fig. 5. A plot of the ratio of currents i_o'/i_o, as a function of the added concentration of phloretin in large unilamellar vesicles formed by reverse phase evaporation. Vesicle suspensions are at a lipid concentration of *ca.* 15 mg/ml. Ratios are shown for currents of the phosphonium I (●) and CCCP-induced proton currents (▲). Error bars represent cumulative errors of the measurements due to uncertainty in the lipid concentrations and EPR measurements

log of i_o'/i_o as a function of phloretin concentration, where i_o' is the membrane current in the presence of phloretin. Proton currents measured in a range of pH's from 7.5 to 4.5 show the same dependence on phloretin concentration (within our experimental error). Also shown in Fig. 4 are the phosphonium currents, K^+-valinomycin currents and DNP-induced proton currents. DNP-induced proton currents are also expected to be rate limited by a hydrophobic anion, in this case the anion A_2H^- (*see* McLaughlin & Dilger, 1980). We measured proton currents induced by DNP in an identical fashion to those measured for CCCP, except that the concentration of DNP used was 40 μM. As with CCCP, the values of i_o'/i_o for DNP were found to be invariant in the pH range 4.5 to 7.5. Currents due to the K^+-valinomycin conductance were estimated as described above (*see* Materials and Methods). From the data shown in Fig. 4, it can be seen that phloretin has a greater effect upon cation currents than proton currents induced by CCCP or DNP.

The behavior seen in Fig. 4 could be influenced by the unique lipid packing environment in small sonicated vesicles. To test this possibility, we investigated the effect that phloretin had on phosphonium and CCCP-induced proton currents in larger unilamellar vesicles formed by reverse phase evaporation. These currents are plotted in Fig. 5 as a function of phloretin concentration. The phloretin-induced changes seen in larger vesicles are identical, within experimental error, to those seen in sonicated vesicles.

The asymmetry seen between the positive and negative currents shown in Figs. 4 and 5 could be due to several factors. For example, an increase in membrane dielectric constant with phloretin addition could account for the greater effect of phloretin on positive compared to negative currents. To test this possibility, we examined the effect phloretin had on the transmembrane movement of the zwitterionic headgroup labeled TEMPO-PC. The transmembrane movement of this species was measured as previously described using ascorbate as a reducing agent (Kornberg & McConnell, 1971). With the addition of up to 8 mol/100 mol phloretin, transmembrane migration rates for this label remain unaffected; thus, significant changes in the dielectric constant of the membrane interior with phloretin addition seem unlikely. Structural or dynamic changes in the bilayer that affect phosphonium and

CCCP movement would also be expected to affect TEMPO-PC migration. As described previously, H^+/OH^- currents are essentially unchanged by phloretin addition (Perkins & Cafiso, 1986). However, H^+/OH^- currents are dramatically increased by the addition of chlorodecane to sonicated vesicle systems. Phosphonium currents are also increased to the same extent by chlorodecane addition. In these cases, chlorodecane is expected to act simply by increasing the membrane dielectric constant. The fact that H^+/OH^- movement appears to be affected by changes in membrane dielectric, but not phloretin addition, also argues that the asymmetry seen between positive and negative species in large or small egg PC vesicles is not the result of changes in the membrane dielectric constant. Several other possible sources for this asymmetry are discussed below.

Estimating Conductance Changes Using a Model for Hydrophobic Ion Energies

If a uniform dipole model for phloretin in bilayers is assumed, the change in the membrane dipole potential $\Delta\phi_d$ can be estimated from a simple capacitor model. For a density of dipoles of 1/700 Å^2, with a dipole moment of ≈5 D and a dielectric constant of 10, we estimate that the absolute change in potential $|\Delta\phi_d|$ will be approximately 30 mV. Experimentally, under these conditions, we estimate values for $|\Delta\phi_d|$ of *ca.* 45 and 120 mV using CCCP and the phosphonium I, respectively. Clearly, the uniform dipole model does not account for the asymmetric behavior of anion and cation conductances and underestimates the magnitudes of the changes in ion conductance. To obtain a more realistic estimate of the expected changes in currents for the phosphonium and $CCCP^-$ we calculated the changes in the free energy barrier for the movement of positive and negative hydrophobic ions using a point dipole model previously described (Flewelling & Hubbell, 1986*b*). We placed point dipoles due to phloretin on a square lattice at the level of the groups giving rise to the membrane dipole field, and oriented them antiparallel to the intrinsic dipole field. We calculated the total energy profile for a positive or negative hydrophobic ion taking a path normal to the bilayer surface in the center of the square lattice.[3] We used the energy difference between the hydrophobic ion binding minima, at the membrane-solution interface, and the central energy barrier to determine the energy barrier for transport. Using a simple Eyring analysis we then calculated the expected changes in the rate constant for movement of the positive and negative species.

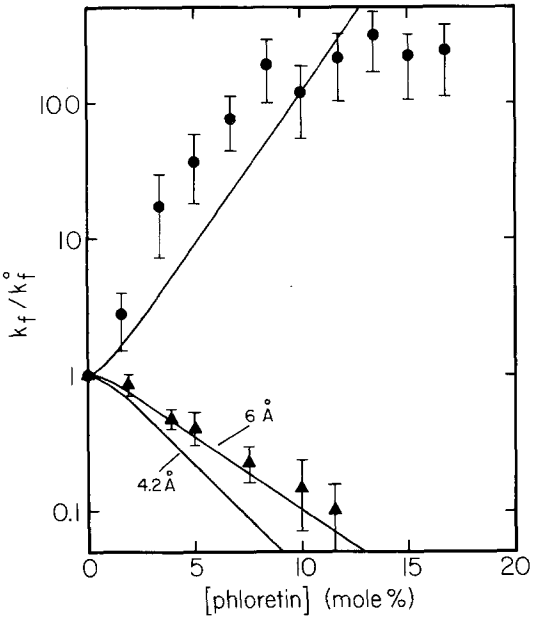

Fig. 6. A plot showing the ratio of the inward rate constants k_f/k_f^0, as a function of the added concentration of phloretin (k_f and k_f^0 are the forward rate constants in the presence and absence of phloretin). Data is shown for phosphonium I (●) and the CCCP-induced proton current (▲). The error bars for the phosphonium represent cumulative errors of the measurements due to uncertainty in the lipid concentrations and error in the EPR measurements. For the CCCP data, error bars represent standard deviations. The solid lines represent the ratio of rate constants calculated using a model for hydrophobic ion energies in membranes described previously (Flewelling & Hubbell, 1986*b*). The phloretin dipole (5.6 D) and the intrinsic membrane dipoles are aligned antiparallel and are placed 22 Å from the bilayer center. The phosphonium current was calculated for an effective ionic radius of 4.2 Å. Negative currents are calculated for effective ionic radii of 4.2 and 6 Å. Other parameters are identical to those used previously for TPP^+ and TPB^-. The neutral hydrophobic binding energy for the ion had little effect on the slopes of these curves

The results of this calculation are shown in Fig. 6 (solid lines). Also shown are experimental rate constant ratios for the phosphonium and the CCCP anion. The values of k_f/k_f^0 differ from $i_o'/_o$ for the phosphonium at higher concentrations of phloretin. At these higher concentrations, increases in current are in part due to an increase in the binding constant K_o. In spite of the approximations made in this model, the general trends in the data are predicted. For hydrophobic anions and cations of identical size, an asymmetry between cation and anion currents is expected with phloretin addition; in the model we use, this is the result of the deeper bind-

[3] For the model we examined, changes in the free energy barrier for hydrophobic ion transport $\Delta G^{\ddagger}$, with phloretin addition were not significantly altered by the position of the ion path in the dipole lattice.

ing minima for the anion. In effect, the anion "senses" a smaller fraction of the phloretin potential than does the cation. We could fit the data for the CCCP anion better by increasing the effective radius of the anion, thus lowering the energy contribution due to the born changing energy. In this case, the free-energy minimum for the ion drops deeper into the bilayer and the asymmetry becomes larger (*see* Fig. 6). Given the uncertainties in the data and the approximations in the calculation, attempts to fit this radius may not be realistic. Moving the position of the phloretin dipole ± 2Å from the position of the membrane dipole layer (at 22Å from the bilayer center) made relatively small differences in the predicted currents. However, if phloretin was placed deep into the hydrocarbon region of the bilayer (e.g. 15 to 16Å from the bilayer center) the asymmetry seen for positive and negative currents was lost.

Discussion

The results presented above indicate that voltage-sensitive paramagnetic probes can be used to monitor changes in the dipole potential within bilayers. Positive currents are monitored directly by measuring the currents of labeled phosphoniums. An attractive feature of this approach is that current changes resulting from binding constant changes can be distinguished from changes in the rate constant. We indirectly measured negative currents by monitoring CCCP- or DNP-mediated proton fluxes in vesicles. This approach is reasonable since proton currents, under the conditions used here, are apparently rate limited by the movement of the CCCP or DNP anions. A more elegant approach would have been to measure negative currents directly, by quantitating the transmembrane migration rate of a labeled hydrophobic anion. Unfortunately, the energy barrier for hydrophobic anion movement is lower by about 6 kcal/mole than the barrier for hydrophobic cation movement, yielding transmembrane migration rates in the msec time scale (time scales which are currently just beyond the time resolution of our equipment).

A striking feature of the data shown here is the asymmetry seen for changes in positive versus negative currents with phloretin addition. An asymmetry of this type was previously observed in planar bilayer systems containing cholesterol and PC, but did not appear in PE-containing bilayers (Andersen et al., 1976). As described above, major changes in membrane dielectric constant with phloretin addition do not appear to account for this asymmetry. Another possible source for the asymmetry could be a discrete dipole effect. Discreteness-of-charge effects were observed previously for the binding of hydrophobic ions to membranes (Andersen et al., 1978; Wang & Brunner, 1978). The dipole field due to phloretin is clearly not uniformly smeared in the interface and the asymmetry we see could be the result of differences in the distribution of $CCCP^-$ or the phosphonium in the plane of the membrane. While discreteness may play some role in the asymmetry seen here, a model used previously to describe hydrophobic ion interactions with membranes (Flewelling & Hubbell, 1986*b*) can account for our observations. In this model, an asymmetry is expected simply because of the different binding locations for cations and anions in the membrane interface. Factors such as the hydrophobic binding energy and the ion radius are expected to affect the magnitude of the current changes, since they affect the positions of hydrophobic ions in the interface. These factors may well account for the differences seen between the phosphonium and K^+-valinomycin currents seen in Fig. 4. Presently, the reasons for the symmetrical behavior of cations and anions seen previously in planar PE bilayers are not clear. This could be the result differences in the interaction of phloretin or hydrophobic ions with PE.

The changes in phosphonium current with phloretin are clearly not as linear (on a log plot) as expected from a simple point dipole calculation. Given the approximations in the model we use, this is not surprising and several factors could account for the discrepancies. For example, the effective orientation or position of the phloretin dipole may change with phloretin concentration. The model we use also assumes that the profile for the dielectric constant in the interfacial region remains unchanged as phloretin is added.

In conclusion, we have characterized methodologies that can be used to monitor both positive and negative ion currents in vesicle systems. This permits an estimation of dipole potential changes in vesicle membranes. The effect of phloretin on conductances in vesicles appears to be consistent with the expected current changes based on a point dipole calculation. The ability to make these measurements in vesicle systems allows the quantitative investigation of agents that modify dipole fields. It also facilitates studies aimed at examining the effects of these fields on more complex membrane protein systems.

This research was supported by grant BNS 8604101 from the National Science Foundation, grant J-61 from the Jeffress Trust, and an award from the Camile and Henry Dreyfus Foundation for New Faculty in Chemistry (to DSC).

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Received 7 October 1986; revised 18 December 1986