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MINI-REVIEW

Characterization of H^+/OH^- Currents in Phospholipid Vesicles

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Abstract

In pure phospholipid vesicles, the conductivity of H^+/OH^- ions exceeds that for other simple inorganic ions. Protons achieve electrochemical equilibrium across egg phosphatidylcholine vesicles within tens of minutes. When pH gradients are established across vesicles, transmembrane potentials develop. Conversely, the establishment of transmembrane potentials leads to the formation of pH gradients. When the phenomenological permeability of H^+/OH^- ions in vesicles is estimated, values are obtained that are much greater (six orders of magnitude larger) than those for Na^+ or K^+. A wide range in the values for this permeability has been reported; however, much of the discrepancy can be attributed to differences in the vesicle systems and experimental conditions. The H^+/OH^- current appears to be modulated by changes in membrane dielectric constant. However, the dependence of this current on the pH gradient and on the membrane voltage argues against simple diffusion mechanisms as the source of the H^+/OH^- current. In addition, in vesicle systems the H^+/OH^- current shows a surprising invariance to changes in the membrane dipole potential, an observation that argues against the role of simple carriers for H^+ and OH^- ions.

Key Words: Proton permeability; lipid vesicles; membrane potential; spin labels.

Introduction

Phospholipid membranes present a large energy barrier to the transmembrane movement of ions. This energy barrier is primarily the result of the Born–Image energy experienced by ions when crossing the low-dielectric

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interior of the membrane. As a result of this energy, the background ion conductances found in pure lipid bilayer systems are typically very low. The permeabilities of inorganic ions such as K^+ and Na^+ are in the range of 10^{-12} to 10^{-14} cm/s, and gradients of these ions can be maintained across model membrane systems for many weeks (see, for example, Johnson and Bangham, 1969; Hauser *et al.*, 1972). Hydrophobic ions, such as tetraphenylphosphonium, have much higher permeabilities in membranes because this energy barrier is lower. This is due to the large size of the ion (which reduces the Born–Image energy) and the negative free energy gain experienced by placing hydrophobic groups in the membrane interior (see, for example, Ketterer *et al.*, 1971).

When the background conductance of phospholipid bilayers is examined, it is found that the largest current-carrying species is due to proton and/or hydroxide ions. At present, the mechanisms giving rise to this net proton current are not known. The magnitude of the conductance measured for protons is not high, but because of the low aqueous concentrations of H^+/OH^- ions, the phenomenological membrane permeability that is calculated is much larger than that for simple inorganic ions. There is a wide variation in the reported values for the permeability of H^+/OH^- ions; however, the values have been obtained in a wide variety of systems using very different experimental conditions.

In vesicle systems, the conduction of H^+/OH^- ions has been characterized using several different approaches. For example, pH electrodes have been used to monitor net proton flows across large vesicles under conditions where the vesicle membrane remains electrically neutral (see Nichols and Deamer, 1980; Deamer and Nichols, 1983). Fluorescent pH probes have also been used to monitor the internal vesicle pH (Nichols *et al.*, 1980; Clement and Gould, 1981; Rossignol *et al.*, 1982; Elamrani and Blume, 1983). In addition, the flux of H^+/OH^- ions has been measured in planar bilayers (Gutknecht and Walter, 1981; Gutknecht, 1984, 1987). Measurements made using these techniques are reviewed elsewhere in this volume. In this review we will discuss the use of a set of paramagnetic probe techniques to characterize H^+/OH^- currents in both small and large unilammellar vesicles. Some unique observations are obtained using this approach. Conditions can be established so that the flow of H^+/OH^- ions charges the vesicle membrane capacitance. Using complementary probe techniques for $\Delta\psi$ and ΔpH, it is possible to distinguish between H^+/OH^- flow that is electrically neutral and flow that is "electrogenic." Thus, the H^+/OH^- flows we measure are well characterized. This methodology is also well suited for measuring the small membrane currents observed here. We present recent findings regarding the properties of this current in vesicles and discuss the implications of these findings for several proposed mechanisms.

Materials and Methods

Determination of Membrane Potentials

In the experiments described here, H^+/OH^- currents are obtained using EPR spectroscopy. We estimate transmembrane electrical potentials and pH gradients by measuring the phase partitioning of spin-labeled phosphonium and secondary amines, respectively. Procedures to quantitate the potentials from phase partitioning data are described in detail elsewhere (Cafiso and Hubbell, 1981). In this method, the amplitude of the high-field nitroxide resonance is calibrated in terms of the concentration of aqueous spin. The amplitude of this signal, which may be recorded as a function of time, can then be used to calculate the probe partitioning. The time-dependent partitioning of the probe is directly related to either $\Delta\psi$ or ΔpH using simple thermodynamic models (see Cafiso and Hubbell, 1978a, b for a more detailed description of these models).

Calculations of P_{net}

From the derivative of the potential immediately after creating a pH gradient $(\partial\psi/\partial t)_{t=0}$ the initial H^+/OH^- current can be calculated. Here we assume a specific membrane capacitance c of $0.9\,\mu F/cm^2$ (Montal and Mueller, 1972). For the purposes of comparison with data in the literature, we calculate the sum of the proton and hydroxide permeabilities, which has been termed the net permeability P_{net} (Nichols and Deamer, 1980). Under conditions where $[H^+]_{in} \cdot [H^+]_{out} = 10^{-14}$, the initial current i_0 and the pH gradient are related to P_{net} by

$$i_0 = (\partial\psi/\partial t)_{\psi=0} \cdot c = F \cdot P_{net} \cdot ([H^+]_{in} - [H^+]_{out}) \quad (1)$$

Here, F is the faraday constant and $P_{net} \equiv P_{H+} + P_{OH-}$ (Cafiso and Hubbell, 1983). We can also obtain an independent estimate of P_{net} from the initial rate of change in the internal vesicle pH. When a pH gradient is established with a weak internal buffer, the expression for P_{net} is

$$P_{net} = \frac{r_i^2 B \cdot (\partial \mathrm{pH}/\partial t)_{\Delta\psi=0}}{3 \cdot r_o([H^+]_{in} - [H^+]_{out})} \quad (2)$$

Here, B is the buffer capacity and r_i and r_o are the internal and external vesicle radii, respectively (Cafiso and Hubbell, 1983).

Observing Electrogenic H^+/OH^- Flows across Vesicles

In sonicated vesicle systems and vesicles formed by reverse phase evaporation, ether injection, or detergent dialysis, the quantitation of H^+/OH^-

flows can be made using both $\Delta\psi$ and ΔpH-dependent probes. Establishing a pH gradient in these systems, under well-buffered conditions, leads to the development of a transmembrane potential. Conversely, the establishment of a transmembrane potential results in the development of a pH gradient. In both cases, protons come to electrochemical equilibrium across the membrane.

The permeability of H^+/OH^- ions has been determined in vesicle systems from time-dependent changes in the membrane potential that follow the establishment of a pH gradient. Data for this time-dependent potential are shown in Fig. 1A. The potential is measured using the spin-labeled hydrophobic ion indicated in the figure (Cafiso and Hubbell, 1983). Here, a membrane potential of 65 $\pm$ 3 mV is established which compares favorably with a potential of 62 mV expected from the proton equilibrium. From the rate of voltage change and the vesicle capacitance, the magnitude of the H^+/OH^- current can be determined. In absolute terms, the magnitude of the initial current ($\approx$25 pA/cm^{-2}) is small and can be accounted for by the transmembrane migration of $\approx$10 H^+/OH^- ions per hour per vesicle.

Under the conditions of this experiment, it can be demonstrated that the net proton current observed in Fig. 1A is due solely to an electrogenic movement of H^+/OH^- ions. That is, there is no electroneutral flow of protons. Figure 1B shows the time dependence of the pH gradient, measured using a spin-labeled secondary amine probe, following the establishment of a pH gradient. In this figure, the time dependence of ΔpH is shown for proton gradients formed in the presence of both 10 and 100 mM buffer. In the presence of 100 mM buffer (the same concentration used in Fig. 1A) the gradient remains stable during the time-dependent voltage buildup. If the buffer concentration is reduced to 10 mM, the pH gradient no longer remains stable but decays with time. From the rate of change of ΔpH, the total number of protons that move can be calculated. This number is consistent with the number of ions required to charge the vesicle capacitance to the observed membrane voltage (Cafiso and Hubbell, 1983).

The dependence of the H^+/OH^- current upon the proton gradient and ΔpH indicate that simple diffusion mechanisms are not the source of this current. The H^+/OH^- current that is measured in vesicle systems is surprisingly insensitive to the bulk concentration of H^+. When the size of the H^+ gradient is varied over five orders of magnitude, the value of the H^+/OH^- current changes by approximately a factor of 10. This nonideal behavior was observed previously by Nichols and Deamer (1980) and was characterized in planar bilayers by Gutknecht (1984). As a result of this behavior, the permeabilities that are estimated for H^+/OH^- ions are highly dependent upon the experimental conditions. For the conditions shown in Fig. 1 we calculate a value for P_{net} of 5 $\pm$ 2 $\times$ 10^{-7} cm/s. This net permeability for

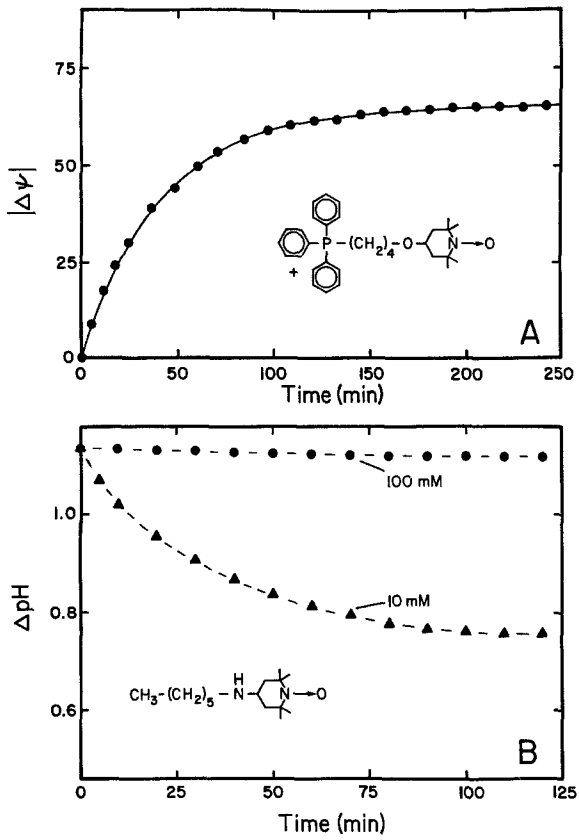

Fig. 1. (A) Time-dependent transmembrane potential following the establishment of a pH gradient across sonicated egg PC vesicles. The pH gradient was formed by mixing 52 μl of a vesicle suspension prepared in 100 mM MES, pH 6.51, with 48 μl of 100 mM MOPS, pH = 8.50. The final external pH was 7.55, yielding a ΔpH of $\approx$ 1.1. The values of $\Delta\psi$ (●) were calculated from the phase partitioning of the spin-labeled phosphonium as indicated in the text. An exponential fit to the data is indicated by the solid line, $|\Delta\psi(t)| = 65(1 - e^{-0.024t})$, and represents an ohmic conductance. Values of P_{net} are estimated from the initial slope of these data (see text). (B) Time-dependence of ΔpH following the establishment of a pH gradient across sonicated egg PC vesicles. The conditions are identical to those in (A), except that data at buffer concentrations of both 100 mM (●) and 10 mM (▲) are shown. Values of ΔpH are obtained using the alkyl secondary amine shown. From the time dependence of the pH gradient in weakly buffered solutions, P_{net} can also be calculated. This value is consistent with that obtained from the data in (A). (For more details see Cafiso and Hubbell, 1983.)

H^+/OH^- ions, which is defined by Eq. (1), is a constant of the pH gradient only for simple diffusion mechanisms (Cafiso and Hubbell, 1983).

There is a wide range in the reported values of P_{net} [see Eq. (1)]; however, much of the discrepancy between these values is likely due to differences in the lipid composition and experimental conditions. When values are compared,

taking into account the effects of vesicle size and the magnitude of the proton gradients ($[H^+]_{in} - [H^+]_{out}$), much of the discrepancy in the reported values of P_{net} can be reconciled (Perkins and Cafiso, 1986).

Effect of Vesicle Morphology and Lipid Composition upon H^+/OH^- Currents

Vesicle Morphology

The H^+/OH^- currents and values of P_{net} are dependent upon the type of vesicle system examined. Under identical experimental conditions, the lowest currents and values of P_{net} are obtained in small sonicated vesicle systems. The values of P_{net} are usually an order of magnitude smaller in sonicated vesicles than those found for vesicles prepared by reverse phase evaporation, extrusion, or ether injection. Vesicles prepared by detergent dialysis techniques usually exhibited the highest values for the H^+/OH^- current (Perkins and Cafiso, 1986). The reasons for the permeability differences between these vesicle types are not known, but may be related to the strong packing asymmetry present in the sonicated systems. In sonicated vesicles, which are the smallest vesicles and the least permeable, there is a well-documented asymmetry in the packing density of the lipids on the inner and outer monolayers (see, for example, Huang and Mason, 1978).

The higher H^+/OH^- currents in vesicles formed from ether injection or reverse-phase evaporation do not appear to be the result of oxidation or other damage to the membrane lipid. Extraction of the lipid from large vesicles, and formation of sonicated vesicles from this lipid, yields permeabilities that are typical of sonicated systems.

Effects of Lipid Saturation and Oxidation

The egg PC used in our studies was chromatographically purified on an aluminum oxide column (Singleton *et al.*, 1965). When vesicles were formed from early column fractions of PC, approximately fivefold greater H^+/OH^- permeabilities were found compared to vesicles formed from later column fractions. This difference was found to be due to small differences in the level of polyunsaturates between early and late fractions. In homogeneous lipids it was found that the H^+/OH^- permeability increased as the number of double bonds on the lipid alkyl chains was increased (Perkins and Cafiso, 1986). There are several ways that greater unsaturation could be associated with higher H^+/OH^- currents. The H^+/OH^- current appears to be dependent

upon membrane dielectric constant (see below), and the additional double bonds may simply act by increasing the membrane dielectric constant. Since unsaturated lipids are susceptible to oxidation, oxidation products present in these lipids may promote the H^+/OH^- current. In fact, allowing these unsaturated lipids to oxidize increases the conductivity of the membrane to H^+/OH^- ions. In egg PC, when about 4% of the double bonds are oxidized, an increase in the H^+/OH^- current of approximately 15-fold is observed (Cafiso and Hubbell, 1983). Toyoshima and Thompson (1975) observed titratable binding sites in egg PC following lipid oxidation. Indeed, by acting as carriers or providing a transmembrane pathway for protons, such titratable sites might facilitate an H^+/OH^- current.

Effects of Halogenated Hydrocarbons

Halogenated hydrocarbons, such as chloroform and halothane, increase the H^+/OH^- permeability of sonicated vesicle systems (Cafiso and Hubbell, 1983). When vesicles are prepared from stock lipid solutions containing chloroform, it is important to remove completely the chloroform from the lipid. Failing to do so leads to enhanced proton permeabilities. It has been proposed that the effects of these agents on the rates of H^+/OH^- permeation might play a role in their action as anesthetics (Bangham *et al.*, 1980). The H^+/OH^- permeability in the presence of chloroform and halothane is increased along with the permeability of potassium ions (Barchfeld and Deamer, 1985). This result argues that anesthetics act to change a general property of lipid bilayers, such as the membrane dielectric constant, which can affect the transport of all ions. As described below, changes in the H^+/OH^- permeability with chlorodecane addition are likely the result of changes in the membrane dielectric constant.

Sources of the Variability in Reported Values for H^+/OH^- Permeability

It is readily apparent from the above discussion that a wide range of conditions and factors can significantly alter the net proton current. In our experience, when care is taken to reduce lipid oxidation and remove contaminants, the lowest H^+/OH^- permeabilities are measured. As discussed above, a constant value of P_{net} with the pH gradient is implied only for simple diffusion mechanisms. Since these simple mechanisms are apparently not operating here, no valid comparison of P_{net} values can be made unless the magnitudes of the H^+ gradient and ΔpH are identical (Perkins and Cafiso, 1986). Thus, the wide range in reported values for the permeability of H^+/OH^- ions is not surprising.

Electrical Characterization of the H^+/OH^- Current in Vesicles

Current–Voltage Relationship

When the derivative of the time–voltage data is multiplied by the vesicle capacitance, the net membrane current is obtained. In this way, time–voltage data can be used to construct a current–voltage curve. The slope of this curve yields the integral membrane resistance. For the data shown in Fig. 1, a linear current–voltage curve is obtained with an integral membrane resistance of approximately $3 \times 10^{-9}\,\Omega\,cm^2$ (Cafiso and Hubbell, 1983). This surprising linearity is maintained up to 100 mV. We have not studied this current–voltage relationship at higher voltage ranges, primarily because the uncertainty in the values of $\Delta\psi$ grows rapidly at high values for this spin probe technique.

This linearity provides another indication that simple diffusion mechanisms are not the source of the H^+/OH^- current we measure; that is, it indicates that the movement of H^+OH^- ions is not controlled by the thermally activated diffusion of a charged species across a well-defined central energy barrier. The linear curve that we obtain could be accounted for by a more complex energy curve having multiple barriers, but the solution is not unique.

Dependence of H^+/OH^- *Currents on the Membrane Dipole Potential*

In PC bilayers, positive and negative ions experience different energies in the hydrocarbon interior due to the presence of oriented dipoles at the membrane–solution interface (Haydon and Hladky, 1972; Szabo *et al.*, 1972). The internal membrane dipole potential that results from these groups is large, about 240 mV in egg PC vesicles, and results in greater permeation rates for negative versus positive ions of similar structures (see Flewelling and Hubbell, 1986). Recently, we developed a procedure to modify and measure dipole potential changes induced by phloretin in vesicle systems (Perkins and Cafiso, 1986, 1987). The procedure involves monitoring both positive and negative currents in vesicles as a function of phloretin concentration. Shown in Fig. 2 are currents that result from the transmembrane migration of a spin-labeled phosphonium ion and from the movement of the CCCP anion in egg PC vesicles. The addition of phloretin dramatically enhances the rate of movement of the phosphonium ion while decreasing the migration rate for the CCCP anion. These changes in current could be modeled using a simple point-dipole model for the adsorption of dipoles to the membrane interface. Both the relative magnitudes of the currents and the asymmetry between changes in the positive and negative currents are predicted using this model (Perkins and Cafiso, 1987).

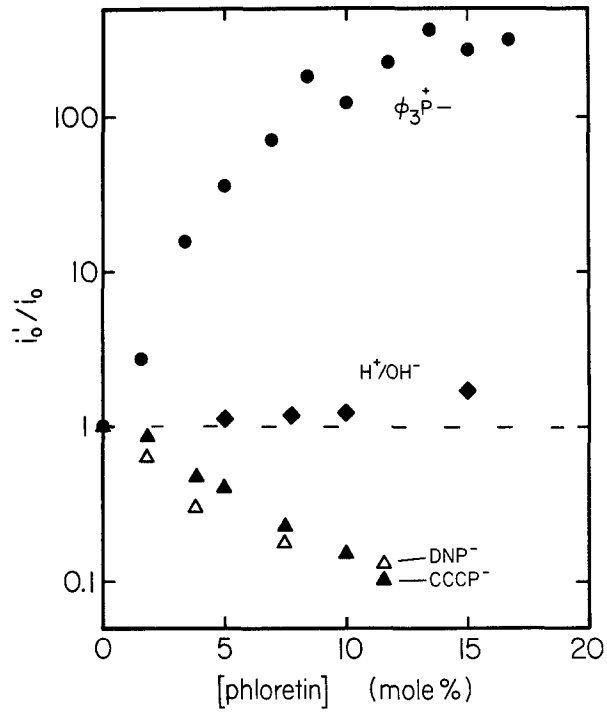

Fig. 2. The effect of phloretin in egg PC vesicles on the transmembrane currents of hydrophobic ions and H^+/OH^- ions. The ratios, i_0'/i_0, are plotted versus phloretin concentration (i_0' and i_0 are the currents in the presence and absence of phloretin, respectively). Transmembrane currents increase for the spin-labeled phosphonium (●), and decrease for the $CCCP^-$ (▲) and DNP^- (△) anions. The H^+/OH^- current (◆) is not strongly affected by the addition of phloretin. The effect of phloretin on phosphonium and $CCCP^-$ currents can be accounted for by a simple point dipole model for phloretin addition to the membrane (see Perkins and Cafiso, 1987).

To determine the sign of the charge-carrying species, we tested the effect of phloretin on the H^+/OH^- current. If a positive species dominated the H^+/OH^- current, the rates should increase. If a negative species carried the current, the rate would be expected to decrease. For example, if weak acid contaminants were the source of the H^+/OH^- current, the movement of an anionic carrier would be expected to be rate limiting (see, for example, McLaughlin and Dilger, 1980). In this case, phloretin addition should diminish the H^+/OH^- current. As seen in Fig. 2, neither of these cases is observed. High levels of phloretin have little effect on the H^+/OH^- current. Again, these result provide an indication that simple diffusion mechanisms are not the source of the H^+/OH^- current measured here. These data may provide an indication of the position and delocalization of the membrane-bound charge important for H^+/OH^- conduction.

We also examined the effect of phloretin on hydrophobic ion and H^+/OH^- conduction in large vesicles formed by reverse-phase evaporation. The results are qualitatively similar to those we see in sonicated vesicles. Within our experimental error, there was no significant effect of dipole field changes on the H^+/OH^- current. Measurements made in planar bilayer systems produce dramatically different results than those observed here. Phloretin addition in planar bilayer systems has been shown to dramatically reduce the measured H^+/OH^- current, providing evidence that weak acid carriers may be functioning in planar systems (Gutknecht, 1987). These differences are discussed below.

Effect of Chlorodecane and Decane

The effects of halothane or lipid unsaturation on H^+/OH^- currents could be the result of changes in the membrane dielectric constant. Small

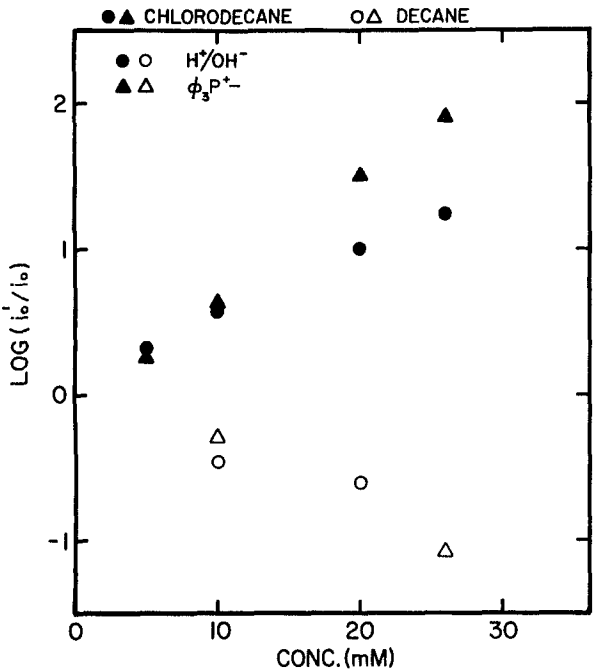

Fig. 3. The effect of chlorodecane and decane addition on the currents for a the spin-labeled phosphonium (▲, △) and H^+/OH^- ions (●, ○). The log (i'_0/i_0) is plotted as a function of the added concentration of decane (△, ○) or chlorodecane (▲, ●) (here, i'_0 and i_0 are the currents in the presence and absence of decane or chlorodecane). The lipid concentration is 26 mM with maximum concentrations of added decane or chlorodecane near 50 mol.%. Decane and chlorodecane were added to the lipid suspensions during the sonication procedure. The experimental errors for the measured currents are estimated to be $\leqslant 20\%$ for chlorodecane and $\leqslant 50\%$ for decane-containing samples.

changes in the membrane dielectric constant will affect the energies of ions in the membrane interior. As a result, transmembrane charge movement can be significantly altered. It was previously demonstrated that the effect of chlorodecane on ion conductances in bilayers is primarily due to its effect on the membrane dielectric constant. Changes in ion conductance due to changes in bilayer structure and dynamics are minimal (Dilger *et al.*, 1979). Assuming that its effect in vesicles is similar, we measured currents in the presence of varied levels of chlorodecane. Similar increases are seen in both the permeability of the phosphonium ion and the H^+/OH^- ion conduction with increasing chlorodecane. These results are shown in Fig. 3. The addition of decane decreases the conductances of both the phosphonium ion and H^+/OH^- ion conduction (Perkins and Cafiso, unpublished). Thus, while the H^+/OH^- currents do not appear to be rate limited by thermally activated diffusion over a single central energy barrier, the preliminary data in Fig. 3 are consistent with the idea that these currents are affected by changes in membrane dielectric constant.

Possible Mechanisms for H^+/OH^- Flow in Phospholipid Vesicles

Several features of the H^+/OH^- flow in vesicles are unusual and likely provide important clues about the mechanisms by which net proton permeation occurs in lipid membranes. Mechanisms that account for the H^+/OH^- permeability must be able to account for the relative insensitivity of the flux to the magnitude of the H^+ gradient. However, they must allow the H^+/OH^- current to be coupled to the chemical potential of protons. As stated above, these features rule out simple diffusion of H^+ and/or OH^- ions as the source for this conduction.

Oxidation byproducts that bind protons could act as carriers or provide conductive pathways for transmembrane H^+/OH^- ion movement. In fact we measure the lowest conductivities for H^+/OH^- ions in sonicated diphytanoyl PC vesicles, a lipid that contains no oxidizable centers (Cafiso and Hubbell, 1983). Saturable carriers for protons could yield an H^+ conductance that was independent of the H^+ gradient, over a range of pH's. Free fatty acids are likely contaminants, and we examined their ability to act as carriers. When present in vesicles, they do not significantly increase the electrogenic H^+/OH^- permeability, although they do increase electrically neutral proton flow and deplete the pH gradient (Cafiso and Hubbell, unpublished). Other acid contaminants or byproducts of lipid oxidation are also potential H^+ carriers; however, the lack of a dipole potential dependence for the H^+/OH^- current argues against these simple carrier mechanisms. In planar bilayer systems, the behavior appears to be quite different from that observed here for vesicles.

Gutknecht (1987) has reported large decreases in the H^+/OH^- conductivity in planar bilayers with phloretin addition. Also he observes a modest dependence of the H^+/OH^- current on pH. The results in bilayers suggest a weak acid carrier as the source of the H^+/OH^- current. The reasons for the differences between his results and those found here for vesicles are not clear. The results suggest the possibility that fundamentally different mechanisms are being observed in the two systems.

One possible mechanism that has drawn considerable attention involves conduction via linear aggregates of water (Nichols and Deamer, 1980). These models are attractive because they can apparently explain the insensitivity of the H^+/OH^- current to the size of the H^+ gradient (see Nagle, 1987) and could account for the linear current–voltage behavior we see here. Unfortunately, unlike carriers, experimental models to test the properties of a water chain dissolved into a hydrocarbon are not easy to find. It has been suggested that conduction pathways involving water may not involve single linear chains, and that quite large aggregates or membrane defects containing water might be expected to preferentially translocate protons. A more detailed analysis and discussion of water chain models can be found elsewhere in this volume (see, for example, Deamer, 1987; Nagle, 1987).

One of the more fascinating aspects of H^+/OH^- conduction in vesicles is that it appears to be an intrinsic property of the lipid bilayer. If the mechanisms for this conduction can be elucidated, fundamental information regarding lipid bilayers will be obtained. The direct biological significance of this conduction is less clear. The H^+/OH^- current is expected to be small compared to the currents generated by active transport systems. Therefore, it is not likely to be a problem for the maintenance of proton potentials in living systems. However, the electrogenic H^+/OH^- conduction must be an important consideration in certain transport experiments. For example, if we are interested in quantitating transmembrane proton currents in mitochondria, the background H^+/OH^- current will represent a portion of the total current. In experiments where transmembrane potentials or pH gradients are established, in the absence of active pumping systems, protons will come to electrochemical equilibrium across membranes. Thus, establishing transmembrane potentials or pH gradients will result in pH gradients or transmembrane potentials, respectively.

Acknowledgments

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