Proton/hydroxide conductance and permeability through phospholipid bilayer membranes

(water permeability/serum albumin/fatty acid)

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ABSTRACT

Proton/hydroxide (H⁺/OH⁻) permeability of phospholipid bilayers is several orders of magnitude higher than alkali or halide ion permeabilities at pH 7. The objective of this study was to determine the mechanism(s) of H⁺/OH⁻ conductance and permeability through planar phospholipid bilayer membranes. Membranes were formed from decane solutions of bacterial phosphatidylethanolamine, diphytanoyl phosphatidylcholine, or egg phosphatidylcholine plus cholesterol. At pH 7, H⁺/OH⁻ conductance (G_H/0H) ranged from 2 to 6 nS cm⁻², corresponding to H⁺/OH⁻ “net” permeabilities of (0.4-1.6) × 10⁻³ cm sec⁻¹. G_H/0H was inhibited by serum albumin (fatty acid-free), phloretin, and low pH. G_H/0H was increased by chlorodecane, long-chain fatty acids, and voltages > 80 mV. Water permeability and G_H/0H were not correlated. The results suggest that the H⁺/OH⁻ charge carrier (i) is primarily anionic, (ii) crosses the membrane via nonpolar pathway(s), and (iii) can be removed from the membrane by “washing” with serum albumin. The simplest explanation is that the phospholipids contain weakly acidic contaminants that act as proton carriers at neutral pH. However, at low pH or in the presence of inhibitors, a “background” G_H/0H remains that may be due to other mechanisms.

Proton/hydroxide (H⁺/OH⁻) permeability of phospholipid bilayers at physiological pH is several orders of magnitude higher than alkali or halide ion permeability (1-9), but the mechanism(s) of H⁺/OH⁻ permeability is unknown. The primary purpose of this study was to determine the mechanism(s) of H⁺/OH⁻ permeability and conductance through planar phospholipid bilayers. In order to characterize the transport mechanisms(s), several inhibitors and enhancers of H⁺/OH⁻ conductance (G_H/0H) were identified. G_H/0H was inhibited by serum albumin (fatty acid-free), phloretin, and low pH. G_H/0H was increased by chlorodecane, long-chain fatty acids, and membrane voltages > 80 mV. G_H/0H was not affected by substituting ²H₂O for H₂O. Water permeability was compared with H⁺/OH⁻ conductance and found not to be correlated. The simplest explanation for these results is that most of the H⁺/OH⁻ conductance at pH 7 is due to weakly acidic contaminants, possibly long-chain fatty acids, in the phospholipids. However, at low pH or in the presence of inhibitors, a significant “background” H⁺/OH⁻ conductance remains, which may be due to other mechanisms—e.g., “water wires” (1, 2, 10, 11) or “hydrated defects” (12, 13) in the bilayer structure.

METHODS AND MATERIALS

Planar (Mueller–Rudin: ref. 14) membranes were formed from 2.5% (wt/vol) solutions of (i) bacterial phosphatidylethanolamine, (ii) diphytanoyl phosphatidylcholine, or (iii) egg phosphatidylcholine plus cholesterol (1:1 molar ratio) in either n-decane or n-decane plus 1-chlorodecane (30%, vol/vol). The membranes (2.0 mm²) were formed in a symmetrical chamber with 1.1 ml of bathing solution and a small magnetic stirrer on each side of the membrane. One side of the membrane was perfused continuously to facilitate solution changes and the measurement of tracer fluxes. The temperature was 24 ± 1°C.

The method of measuring H⁺/OH⁻ conductance (G_H/0H) is described elsewhere (7). In brief, the membranes were exposed to small (0.3–0.8 unit) pH gradients produced by mixtures of weakly acidic and weakly basic buffers (e.g., Hepes plus Tris, Mes plus Bistris). In most experiments the concentrations were adjusted so that front and rear solutions contained similar concentrations of all ions except H⁺ and OH⁻. At very high or very low pH, the solutions were “buffered” with NaOH or HCl. H⁺/OH⁻ diffusion potentials were measured by calomel–KCl electrodes and a high-impedance electrometer. H⁺/OH⁻ transference numbers (T_H/0H) were calculated from the relation T_H/0H = V_m/E_H/0H, where V_m is the diffusion potential and E_H/0H is the H⁺/OH⁻ equilibrium potential, calculated by the Nernst equation. H⁺/OH⁻ conductances were calculated from the relation G_H/0H = T_H/0H G_m, where G_m is the total membrane conductance measured by applying a small voltage step across the membrane in series with a known resistance.

The method of measuring tracer fluxes and calculating permeabilities is described elsewhere (15). Briefly, after the membrane had thinned, 10 μCi (1 Ci = 37 GBq) of tritiated water (³H₂O) was injected into the rear compartment, and the rate of tracer appearance in the front compartment was measured by continuous perfusion and collection of samples at 3-min intervals. The rear compartment was covered by a Teflon plug to prevent distillation of tracer into the front compartment.

The aqueous unstirred-layer thickness (d) in the system is 129 ± 20 μm (15, 16), similar to the value obtained by others (17). From this value the unstirred-layer permeability to H₂O was calculated—i.e., P⁺/OH⁻ = D/d, where D is the ³H₂O diffusion coefficient in water. The membrane permeability (P) was calculated from the relation 1/P = (1/P_D⁺) + (1/P_H⁻), where P_D⁺ is the total permeability—i.e., the permeability of the membrane plus unstirred layers. In this study the unstirred-layer correction ranged from 32% to 39%.

Phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL). Decane (99.9%) was obtained from Wiley Organics (Columbus, OH), and 1-chlorodecane (95%) was obtained from Aldrich. Buffers were obtained from Research Organics (Cleveland, OH). Tritiated water was obtained from ICN. Bovine serum albumin, cholesterol, deuterium oxide (³H₂O), and phloretin were obtained from Sigma.

Abbreviations: G, conductance; T, transference number; P, permeability.

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The decane and chlorodecane were passed through an aluminum oxide column to remove polar impurities. Water was deionized and then doubly distilled. Before each experiment the membrane chamber was boiled for at least 4 hr in several large volumes of 95% ethanol plus 0.1 M NaOH.

RESULTS

The effects of pH on $H^+/OH^-$ conductance are shown in Fig. 1. $G_{H/OH}$ was low (0.5-7 nS cm$^{-2}$) and moderately sensitive to pH, increasing 2- to 6-fold as pH increased from about 2 to 8. At pH > 8, $G_{H/OH}$ appeared to be fairly constant. At neutral to alkaline pH the membranes were $H^+/OH^-$ selective ($T_{H/OH} = 0.5-1.0$) in solutions of polar buffers such as Hepes plus Tris and Mes plus Bistris. At low pH, $T_{H/OH}$ ranged from 0.2 to 0.6, depending upon the specific lipid and buffer composition.

Serum albumin (0.15 ± 0.05 mg ml$^{-1}$) reduced $G_{H/OH}$ by a factor of 3 to 50, depending upon the specific lipid (Fig. 2 and unpublished data). The albumin was fatty acid-free (<0.005%) and was applied to both sides of the membrane (by injection on one side and continuous perfusion from a large reservoir on the other side). The inhibition of $G_{H/OH}$ by albumin was prevented by adding sodium octanoate (5 mM) prior to the addition of albumin (data not shown). Octanoate alone had no effect on $G_{H/OH}$. As a protein control, carbonic anhydrase (from bovine erythrocytes; 0.1 mg ml$^{-1}$) was also tested but was found not to affect $G_{H/OH}$ (data not shown).

Serum albumin is well known for its ability to reversibly bind various amphiphilic molecules, especially long-chain fatty acids (18). Thus, the inhibition of $G_{H/OH}$ by serum albumin suggests that albumin is removing from the membrane an unidentified substance that causes $G_{H/OH}$. If so, then the prevention of albumin inhibition by octanoate may reflect the saturation of binding sites by a competing ligand. The lack of octanoate-induced conductance reflects the inability of short-chain fatty acid anions to partition into the nonpolar region of the membrane. Short-chain fatty acids permeate rapidly in their protonated (nonionic) forms but do not cause significant membrane conductance at low (millimolar) concentrations (19).

Fig. 3 shows the effects of phloretin and chlorodecane on $G_{H/OH}$, as well as the relation between water permeability and $G_{H/OH}$. First, phloretin (0.1 mM) inhibited $G_{H/OH}$ by a factor >5 to >25, depending upon the lipid composition. Second, chlorodecane (30%, vol/vol) increased $G_{H/OH}$ by a factor of 3 to 9. Third, the increase in $G_{H/OH}$ caused by chlorodecane was abolished by phloretin. Fourth, there was no correlation between water permeability and $G_{H/OH}$ under several conditions that either enhance or inhibit $G_{H/OH}$.

The primary effect of phloretin on lipid bilayers is to decrease the membrane dipole potential, thus decreasing anion conductance and increasing cation conductance (20). The primary effect of chlorodecane on lipid bilayers is to increase the dielectric constant of the nonpolar region of the membrane, thus increasing the conductance of both anions and cations (21). Thus, the inhibition by phloretin and
enhancement by chlorodecane suggest that the rate-limiting step in \( H^+/OH^- \) transport is the translocation of an anion through a low-dielectric region of the membrane. As an additional control, we tested the effect of 0.1 mM phloretin on the conductance of a hydrophobic cation, tetraphenyl-

sonium, and observed a 1000-fold increase, similar to the results of others (20).

In addition to the results shown in Figs. 1–3, \( G_{OH} \) was also inhibited by glycerol, by a factor of 9 to 11 at 9.6 M (75%, wt/wt) (7, 22). The mechanism of inhibition is uncertain, because glycerol has several possible effects. For example, glycerol substitution reduces the activity of water, reduces membrane fluidity (23), and reduces the membrane dipole potential (24).

\( G_{OH} \) was also increased by membrane voltages >80 mV (7, 22). The voltage dependence of \( G_{OH} \) was similar to that expected for ion transport across a trapezoidal energy barrier (25). Similar results have been reported for unmodified phospholipid vesicles (6), mitochondrial membranes (6), and planar bilayers that contain weak-acid proton carriers (26).

Finally, \( H^+/OH^- \) conductance was compared with \( H^+/OH^- \) conductance by substitution of \( H^+/OH^- \) for \( H^+/OH^- \) in Hepes/Tris buffers (pH 7.4–8.1). Within experimental error (±20%), \( H^+/OH^- \) had no effect, similar to the results of others (8).

**DISCUSSION**

The results confirm and explain in part the previous studies of Nichols and Deamer (1, 2) and others (3–9), who found surprisingly high \( H^+/OH^- \) permeabilities and/or conductances in various types of phospholipid bilayers. The pH dependence of \( G_{OH} \) (Fig. 1) is also consistent with previous observations (1, 4, 7). For example, Nichols and Deamer (1) found an \( \approx 7 \)-fold increase in \( H^+/OH^- \) net flux as pH increased from 6 to 8. Cafiso and Hubbell (4) found an \( \approx 5 \)-fold increase in \( G_{OH} \) as pH increased from 3.5 to 8.8. In both these studies, \( G_{OH} \) tended to saturate at alkaline pH (compare Fig. 1).

At pH 7, the electrical estimates of \( G_{OH} \) reported here can be converted to \( H^+/OH^- \) “net” permeabilities by the relation \( G_{OH} = RTG_{OH}/F^2 \), where \( R, T, \) and \( F \) have their usual meanings and \( G_{OH} \) is the \( H^+/OH^- \) conductance at pH 7 (27). This conversion yields values of (0.4–1.6) \( \times 10^{-5} \) cm/sec, which fall within the range of values reported for various types of phospholipid vesicles: 10–7 to 10–3 cm/sec (1–9). Separate values of \( P_{OH} \) or \( P_{OH} \) can also be calculated at any pH, but one must assume that either \( H^+/OH^- \) or \( OH^-/H^- \) is carrying proton equivalents through the membrane (1, 7, 28, 29). This assumption cannot be verified without knowledge of the transport mechanism. Furthermore, since \( G_{OH} \) varies less than 10-fold while the \( H^+/OH^- \) concentrations vary 106-fold (Fig. 1), the calculated \( P_{OH} \) is extremely dependent upon pH and pH gradient (1, 7, 28–29).

Therefore, most investigators have made their measurements near pH 7, where “net” \( H^+/OH^- \) permeabilities can be calculated, using the minimum number of assumptions (see refs. 8 and 30 for further discussion of these problems).

During the past 7 years, \( H^/+ \) and/or \( OH^-/H^- \) permeabilities of phospholipid bilayers have been reported by about 20 different laboratories. Most permeabilities were estimated from the rate of decay of pH gradients across vesicle membranes, and permeability was calculated as the net flux divided by concentration gradient (1–3, 5, 6, 9). These pH measurements do not distinguish between ionic (conductive) and nonionic fluxes of proton equivalents. However, most investigators have minimized nonionic \( H^+/OH^- \) fluxes (e.g., diffusion of molecular HCl) by working at neutral pH and using Cl–/HCO3– free solutions. Furthermore, the “electrogenic” (conductive) nature of \( H^+/OH^- \) transport has been amply demonstrated, either by measuring \( H^+/OH^- \) current (4, 7, 8) or by using ionophores (e.g., valinomycin) to show the voltage dependence of the \( H^+/OH^- \) flux (2, 6, 9). Thus, there is reasonable agreement between values of \( P_{OH} \) obtained by electrical and pH-recording techniques, provided that both kinds of measurements are made under conditions of similar pH. However, sizable discrepancies still remain among reported values of \( P_{OH} \) (31). However, the proton carrier recycles within the membrane as an anion (e.g., \( A^- \) or \( H^+_A \) (31)). Thus, proton current (or flux) may be driven by either a voltage gradient or a pH gradient.

The results reported here conform to the predictions of the weak-acid model in the following respects. First, \( G_{OH} \) shows an “anomalous” pH dependence. \( G_{OH} \) increases as \( H^+ \) decreases (Fig. 1). Second, the inhibition by phloretin (Fig. 3) suggests that the \( H^+/OH^- \) charge carrier is primarily anionic, both in the presence and in the absence of chlorodecane. Third, the inhibition by serum albumin (Figs. 2 and 3) suggests that the weak acid can be “extracted” from the membrane. Fourth, the hypothetical weak acid, since it is present in trace amounts, has no effect on water permeability (Fig. 3). Finally, the apparent “plateau” in \( G_{OH} \) at high pH (Fig. 1) suggests a simple A’-type carrier (see ref. 26, figures 1 and 5, and ref. 31, page 832). [However, \( G_{OH} \) values at pH 10–12 must be viewed with caution because of the possibility of phospholipid hydrolysis at a high pH (29, 22)].

In recent work designed to gain additional insights into the expected behavior of bilayers containing weak acid contaminants, unmodified bilayers were compared with membranes containing small amounts of long-chain fatty acids (phthiotic and palmitic acids). In brief, the properties of \( G_{OH} \) in fatty acid-containing bilayers were qualitatively identical to the controls. For example, the \( G_{OH} \) induced by fatty acids was inhibited by low pH, phloretin, glycerol, and serum albumin, and the albumin inhibition was prevented by octanoate (ref. 29, unpublished data). Furthermore, \( G_{OH} \) was enhanced by chlorodecane and within minutes of inhibition and voltage stimulation were quantitatively similar in fatty acid-containing and unmodified membranes, i.e., \( G_{OH} \) was inhibited by a factor of 9–11 at 9.6 M glycerol and was increased by a factor of 1.4–1.7 at 160 mV (22). Also, the addition of fatty acid did not affect the water permeability (22). Finally, in fatty acid-containing membranes the \( H^+/OH^- \) and \( H^+/OH^- \) conductances were similar (±20%). Thus, eight similarities and no qualitative differences have been recorded between the characteristics of \( G_{OH} \) in modified and fatty acid-containing membranes.

The rate constants for transbilayer \( H^/+ \) transport of long-chain fatty acids can be used to calculate the expected \( H^+/OH^- \) conductance resulting from free fatty acids in the membrane. Recently, Storch and Kleinfeld (33) estimated rate constants of about (8 ± 4) \( \times 10^{-3} \) sec–1 for the ionized forms of several long-chain fatty acids labeled with fluorescent probes. From this value, I estimate that free fatty acid levels of roughly 0.1 mol % could produce \( G_{OH} \) values of 2–6 nS/cm (or pH 7 (cf. Fig. 1). Another estimate, obtained by adding phytic acid to diphytanylo phosphatidylcholine, yields an extrapolated free fatty acid level near 1% (22).

Whether the phospholipids actually contain traces of free fatty acids (or other weak acids) remains to be seen. However, numerous investigators have noticed low levels of oxidation products, anionic contaminants, and/or titratable materials in phosphatidylcholine, phosphatidylethanol-
amine, and soybean phospholipids at neutral pH (3, 9, 34–36). Thus, it seems likely that variable levels of weak acid
contaminants can explain at least part of the high values and large variation among reported H\(^+/\)OH\(^-\)
permeabilities at neutral pH, which range from about 10\(^{-7}\) to 10\(^{-3}\) cm sec\(^{-1}\) (1–9, 37).

In conclusion, the weak-acid hypothesis can explain at least 65–98% of the H\(^+/\)OH\(^-\) conductance in the system
described here, depending upon the specific phospholipid. However, a significant residual H\(^+/\)OH\(^-\) conductance
remains at low pH and in the presence of inhibitors at neutral pH (Figs. 1–3). Possible pathways for the “background
G\(_{\text{H}^+/\text{OH}^-}\) include hydrogen-bonded “water wires” through the
nopolar region (1, 2, 10, 11) and/or “hydrated defects” in
the bilayer structure (12, 13). Unfortunately, the background
G\(_{\text{H}^+/\text{OH}^-}\) is too low to study in conventional planar bilayers, and
a more sensitive technique, such as that developed by Cafiso and Hubbell (4), must be employed.

Whether weak acids are important in biological H\(^+/\)OH\(^-\)
transport remains to be seen. However, most estimates of
biological membrane H\(^+/\)OH\(^-\) permeability fall near or
above the high end of the range for unmodified phospholipid
bilayers (2, 6, 37). For example, the inner mitochondrial mem-
brane G\(_{\text{H}^+/\text{OH}^-}\) is about 400 nS cm\(^{-1}\) (6, 38), which
converts to a P\(_{\text{H}^+/\text{OH}^-}\) of about 10\(^{-3}\) cm sec\(^{-1}\) (1, 6). In renal
(proximal tubule) brush-border membranes, P\(_{\text{H}^+/\text{OH}^-}\) is 5 \(
\times\) 10\(^{-3}\) cm sec\(^{-1}\), and H\(^+/\)OH\(^-\) diffusion utilizes a lipid path-
way that is independent of the pathway for water (39). To the
extent that biological membranes contain weak acid “con-
taminants,” such as free fatty acids and bile acids, the bilayer
results I have described may help explain these high H\(^+/\)
OH\(^-\) permeabilities. For example, in hepatocyte plasma membranes and intestinal brush-border membranes, free
fatty acids comprise 6–15% of the total lipids (40–42). In
brush-border membranes of small intestine, proximal tubule,
and choroid plexus, P\(_{\text{H}^+/\text{OH}^-}\)/P\(_{\text{Na}^+}\) ranges from 10\(^{4}\) to 10\(^{6}\) (43).
The presence of free fatty acids could help to explain these
high ratios, as well as the fact that the ratios increase \(\approx\)6-fold
as pH increases from 5.5 to 8.5 (43). If this suggestion is
correct, then exposure of biological membranes to serum
albumin should reduce the H\(^+/\)OH\(^-\) conductance and per-
meability.

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