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Proton Conductance Caused by Long-Chain Fatty Acids in Phospholipid Bilayer Membranes

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Summary. Mechanisms of proton conductance (G_H) were investigated in phospholipid bilayer membranes containing long-chain fatty acids (lauric, myristic, palmitic, oleic or phytanic). Membranes were formed from diphytanoyl phosphatidylcholine in decane plus chlorodecane (usually 30% vol/vol). Fatty acids were added either to the aqueous phase or to the membrane-forming solution. Proton conductance was calculated from the steady-state total conductance and the H^+ diffusion potential produced by a transmembrane pH gradient. Fatty acids caused G_H to increase in proportion to the first power of the fatty acid concentration. The G_H induced by fatty acids was inhibited by phloretin, low pH and serum albumin. G_H was increased by chlorodecane, and the voltage dependence of G_H was superlinear. The results suggest that fatty acids act as simple (A^- type) proton carriers. The membrane : water partition coefficient (K_p) and adsorption coefficient (β) were estimated by finding the membrane and aqueous fatty acid concentrations which gave identical values of G_H. For palmitic and oleic acids K_p was about 10^5 and β was about 10^{-2} cm. The A^- translocation or "flip-flop" rate (k_a) was estimated from the value of G_H and the fatty acid concentration in the membrane, assuming that A^- translocation was the rate limiting step in H^+ transport. The k_A's were about 10^{-4} sec^{-1}, slower than classical weak-acid uncouplers by a factor of 10^5. Although long-chain fatty acids are relatively inefficient H^+ carriers, they may cause significant biological H^+ conductance when present in the membrane at high concentrations, e.g., in ischemia, hypoxia, hormonally induced lipolysis, or certain hereditary disorders, e.g., Refsum's (phytanic acid storage) disease.

Key Words proton conductance · fatty acid · phospholipid bilayer membrane · serum albumin · proton permeability

Introduction

Nonesterified (free) fatty acids are normal constituents of most biological membranes. Normal membrane levels of free fatty acids range widely from <1 mol% to approx. 40 mol% of total lipids, depending on the specific type of cell membrane and metabolic conditions [7, 13, 18, 27, 42, 56, 78, 83]. Under abnormal conditions, free fatty acid levels may increase dramatically. For example, ischemia or anoxia causes large and rapid increases in free fatty acid concentrations in brain [29, 67, 84], heart [12, 14, 78], kidney [39, 70] and liver [7, 19]. Increased membrane or tissue concentrations of free fatty acids may also be associated with hypothermia [29], thermogenesis [50], ethanol abuse [38], hormonally induced lipolysis [1, 75] and several hereditary disorders [4, 72].

Long-chain fatty acids are moderately hydrophobic and strongly amphiphilic molecules which adsorb to lipid bilayers and biological membranes [20, 52, 58, 61, 63, 65]. Since fatty acids are weak acids, it would not be surprising if they also increased the membrane conductance to protons [45]. In this regard, fatty acids have long been known to uncouple oxidative phosphorylation [8, 43, 59, 81]. However, the mechanisms by which fatty acids alter proton conductance and/or other membrane properties are poorly understood. Recent reviews and research reports have identified at least ten different ways that fatty acids may alter biological membrane function [14, 29, 39, 55, 57, 64, 72, 75].

Planar phospholipid bilayers provide a convenient system for studying fatty acid transport and the effects of fatty acids on membrane permeability. Fatty acids can be added to either the aqueous phase or the membrane forming solution, and fluxes and conductances can be easily measured over a wide range of pH and other environmental conditions [22, 79]. Previous studies have shown that phospholipid bilayers can accommodate long-chain fatty acids at mole fractions up to about 0.67 (2 : 1 mole ratio) [36, 66]. However, little is known about the effects of free fatty acids on bilayer permeability. Recently we found that phytanic acid (a 20-

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carbon, branched-chain fatty acid) increases the proton conductance of planar bilayers [22]. Others have shown that long-chain fatty acids increase the permeability of phospholipid bilayers to various drugs [30, 48].

The present study describes the effects of some long-chain fatty acids (lauric, myristic, palmitic, oleic and phytanic) on proton conductance (G_H) through planar phospholipid bilayers. The results suggest that long-chain fatty acids act as simple (A^- type) proton carriers at physiological pH. This conclusion is based on the stoichiometry of fatty acid-induced G_H, as well as the effects of G_H inhibitors (phloretin, serum albumin and low pH) and G_H enhancers (chlorodecane and membrane voltage). Although long-chain fatty acids adsorb strongly to membrane, they are relatively inefficient H^+ carriers, due primarily to the slow translocation (flip-flop) rates of the anionic species. Nevertheless, comparison with data from biological membranes suggests that fatty acid-induced proton conductances may be physiologically important under a variety of conditions associated with high membrane levels of free fatty acids. A preliminary account of this work has been published [24].

Materials and Methods

Materials

Diphytanoyl phosphatidylcholine (PC) was obtained from Avanti (Birmingham, AL). Decane (99.9%) was obtained from Wiley Organics (Columbus, OH), and 1-chlorodecane (95%) was obtained from Aldrich (Milwaukee, WI). Buffers were obtained from Research Organics (Cleveland, OH). Phytanic acid (3,7,11, 15-tetramethylhexadecanoic acid) (99+%) was obtained from Foxboro/Analabs (North Haven, CT). All other fatty acids, bovine serum albumin, egg albumin, phloretin and tetraphenylarsonium chloride were obtained from Sigma (St. Louis, MO). The serum albumin was fatty acid and globulin free (Sigma No. A0281).

The decane and chlorodecane were passed through an aluminum oxide column to remove polar impurities. Water was deionized and then doubly distilled. In some experiments, the water was HPLC grade obtained from Burdick and Jackson (Muskegon, MI).

Methods

Planar (Mueller-Rudin) [47] membranes (1.4–2.0 mm^2) were formed from diphytanoyl PC (approx. 31 mg/ml or 37 mM) dissolved in *n*-decane or *n*-decane plus 1-chlorodecane (usually 30%, vol/vol). Membranes were formed in an open Plexiglas chamber designed so that the front compartment could be perfused continuously at a rate of 1–2 ml/min and the rear compartment volume could be adjusted by means of a microsyringe [79, 80]. The front and rear solution volumes were 1.1 ml each, and both solutions were stirred magnetically. The temperature was 24 ± 2°C.

Fatty acids were added either to the aqueous phase or to the membrane forming solution. Before addition to the aqueous phase, fatty acids (as sodium salts) were dissolved in methanol and then injected into the bath after the membrane had thinned. Before addition to the membrane-forming solution, the fatty acids (as free acids) were first dissolved in chloroform and then added to aliquots of the PC-chloroform stock solution. Then the chloroform was evaporated under a stream of argon, and decane and chlorodecane were added to give the desired lipid concentration. Adding fatty acids to the membrane-forming solution gave more reproducible results, so this method was used in most experiments.

The method of measuring proton conductance is described elsewhere [21–23]. In brief, the membranes were exposed to small (0.3–0.7 unit) pH gradients produced by mixtures of weakly acidic and weakly basic buffers, e.g., HEPES plus Tris, MES plus Bis-tris, TAPS plus Bis-Tris propane, etc. The buffer concentrations and pH's were chosen so that the front and rear solutions contained similar concentrations of all ions except H^+ and OH^-. The H^+/OH^- diffusion potentials produced by the pH gradient were measured with a high impedance electrometer and calomel-KCl electrodes.

The transference number for H^+/OH^- was calculated from the relation, $T_{H/OH} = V_m/E_{H/OH}$, where V_m is the measured diffusion potential and $E_{H/OH}$ is the calculated Nernst equilibrium potential for H^+/OH^-. The H^+/OH^- conductance was calculated from the relation, $G_{H/OH} = T_{H/OH}\, G_m$, where G_m is the total steady-state conductance. G_m was measured by applying a small voltage pulse (usually 20 mV) across the membrane.

Since $E_H = E_{OH}$, it is impossible to distinguish between H^+ and OH^- currents without a prior knowledge of the transport mechanism. However, in this study we will be dealing with weak acids which are expected to act as H^+, not OH^-, carriers. For this reason, as well as for convenience, I will use the term "proton conductance" (G_H) rather than "proton/hydroxide conductance."

In previous studies [21, 22] I used a perfusion method for creating transmembrane pH gradients. In the present study I usually used an injection technique, which was more convenient for most experiments. In the injection method the membrane was formed in a solution containing equal concentrations of a weak acid and a weak base, usually 30 mM HEPES plus 30 mM Tris, pH 7.75. After the membrane had thinned, small volumes of concentrated buffer were injected on each side. In most experiments the amounts added were 20 mM HEPES to the front and 20 mM Tris to the rear, which produced final pH's of 7.4 ad 8.1 (E_H = 41 mV).

As a result of the buffer injections, both front and rear solutions increased slightly in osmolarity (usually from 0.06 to 0.08 osmoles · $liter^{-1}$) and in ionic strength (usually from 0.021 to 0.025). However, the front and rear solutions remained ionically "balanced," i.e., the buffer ion equilibrium potentials were approx. zero (<1 mV). Thus, T_H and G_H could be easily calculated from the relations, $T_H = V_m/E_H$, and $G_H = T_H G_m$.

Results

H^+ Conductance in Unmodified Membranes

Unmodified phospholipid bilayers display a small "intrinsic" H^+ conductance (G_H) which increases two- to sevenfold as pH increases from 3–9 [11, 21, 51] (*see* Fig. 5). At pH 7 diphytanoyl PC-decane

membranes have a G_H of 1 to 2 nS cm^{-2} (Figs. 1 and 5). Other lipids, e.g., bacterial phosphatidylethanolamine, have a somewhat higher intrinsic G_H [22]. The origin of the intrinsic G_H has been the object of considerable study, but the mechanism(s) remain unclear [for recent reviews *see* Refs. 16, 23, 49, 54]. In some of the experiments described below (indicated in the figure legends), the intrinsic G_H was subtracted from the G_H induced by fatty acids.

Effects of Chlorodecane on Conductance to H^+, SCN^- and TPA^+

A difficulty encountered in our previous work [22] was that inhibitors of G_H, e.g., serum albumin and phloretin, often reduced G_H below the limit of measurement (approx. 0.1 nS cm^{-2}). Therefore, a method of amplifying G_H without altering the H^+ transport mechanism(s) was needed. A simple way to enhance ionic conductance through a lipid bilayer is to increase the dielectric constant (ε) of the nonpolar region [17]. For example, substitution of 1-chlorodecane ($\varepsilon = 4.5$) for decane ($\varepsilon = 2.0$) as the lipid solvent increases the ionic conductance by 10- to 1000-fold, depending upon the size of the permeant ion [17]. However, a difficulty encountered with chlorodecane is that it reduces membrane stability, especially during experimental procedures involving perfusion and/or injections. Therefore, I tested partial substitutions of chlorodecane for decane in order to find a mixture that would substantially increase conductance without decreasing mechanical stability.

Figure 1 shows the effects of chlorodecane on the membrane conductances of tetraphenylarsonium (TPA^+), thiocyanate (SCN^-), and H^+. G_H was measured in both unmodified and fatty acid-containing membranes. TPA^+ and SCN^- were included for comparison, i.e., to see whether G_H, G_{TPA} and G_{SCN} responded similarly to changes in dielectric constant. Figure 1 shows that all the ionic conductances increased about one order of magnitude as the chlorodecane concentration increased from 0 to 50%. SCN^- appeared to be somewhat more sensitive to chlorodecane, possibly due to the smaller size of SCN^- [17].

For most subsequent experiments with fatty acids, I used decane/chlorodecane (70:30, vol/vol) as the lipid solvent. This provided a 7- to 10-fold enhancement of G_H without loss of membrane stability. An additional advantage of chlorodecane is that the intrinsic ionic conductance of chlorodecane-containing membranes may be more like that of biological membranes, which contain integral membrane proteins [17, 45]. However, a disadvantage of chlorodecane is that it complicates the interpretation of experiments with some inhibitors, e.g., serum albumin, because the extraction of chlorodecane from the membrane is a possible mechanism of inhibition of conductance.

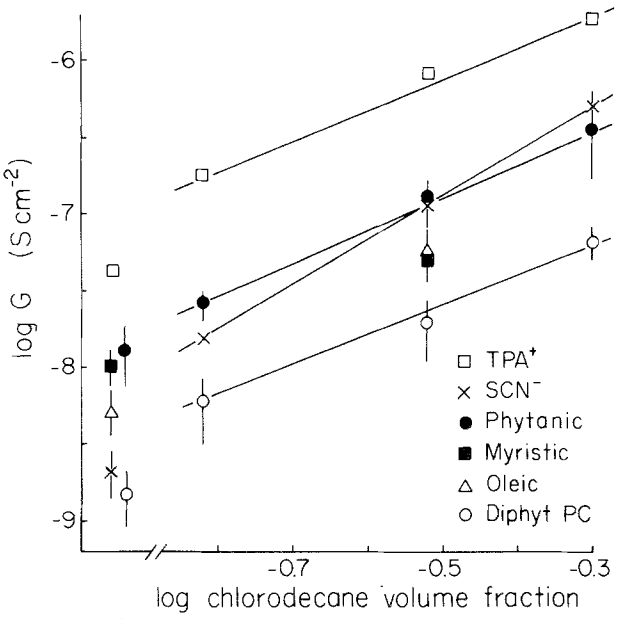

Fig. 1. Effects of 1-chlorodecane on membrane conductance to tetraphenylarsonium (TPA^+) (1.0 mM), thiocyanate (SCN^-) (10 mM) and H^+ (8–40 nM). G_H was measured in unmodified diphytanoyl PC membranes and in membranes containing either phytanic acid (21 mol%), myristic acid (28 mol%) or oleic acid (15 mol%). Chlorodecane (0–50%, vol/vol) was substituted for decane in the membrane-forming solution. TPA^+ (Cl^- salt) and SCN^- (Na^+ salt) were added either before or after membrane formation. The aqueous solutions were HEPES-Tris mixtures, usually pH 7.4 and 8.1, as described in Methods. Vertical bars indicate SD. If no SD is shown, then the data point represents a single membrane

Effects of Fatty acids on H^+ Conductance

Figures 2 and 3 show the effects of several long-chain fatty acids on G_H at pH 7.4–8.1. The fatty acids were added either to the membrane-forming solution (Fig. 2) or to the aqueous phase (Fig. 3). Fatty acids produced proton conductances which were approximately proportional to the first power of the fatty acid mole fraction or concentration. However, rather high levels of fatty acids were required to increase G_H. For example, in Fig. 2 the mole fraction of fatty acid relative to phospholipid ranged from 0.05 to 0.6, and in Fig. 3 the aqueous concentration of fatty acids ranged from 3 to 400 μM. Addition of long-chain alcohols (*n*-dodecanol or *n*-hexadecanol) to the membrane-forming solutions had no effect on G_H at mole fractions up to 0.6 (relative to PC).

Fatty acids also increased H^+ selectivity. In unmodified diphytanoyl PC membranes at pH 7–8, T_H

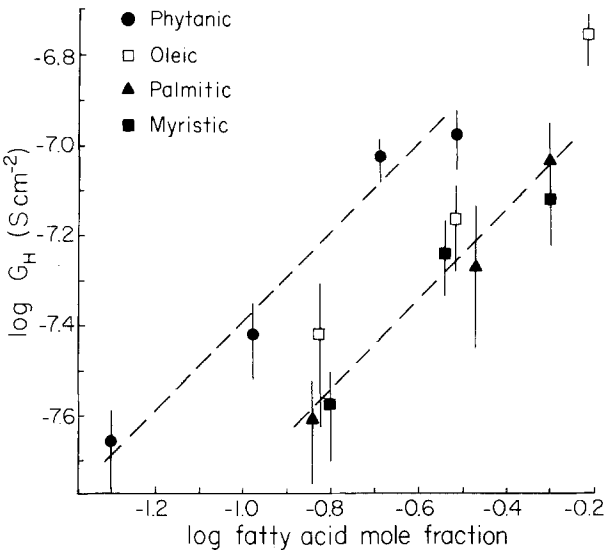

Fig. 2. Effects of fatty acids on H^+ conductance (G_H) at pH 7.4–8.1. Fatty acids were added to the membrane-forming solution as described in Methods. The fatty acid mole fraction, relative to PC, ranged from 0.05 to 0.6, and the solvent was decane plus chlorodecane (70 : 30, vol/vol). The aqueous solutions were buffered with HEPES plus Tris, as described in Methods. The diphytanoyl PC (control) G_H of 15 ± 6 nS cm^{-2} was subtracted from the fatty acid-induced G_H. Error bars indicate SD of 2–4 membranes. The dashed lines have slopes of 1.0

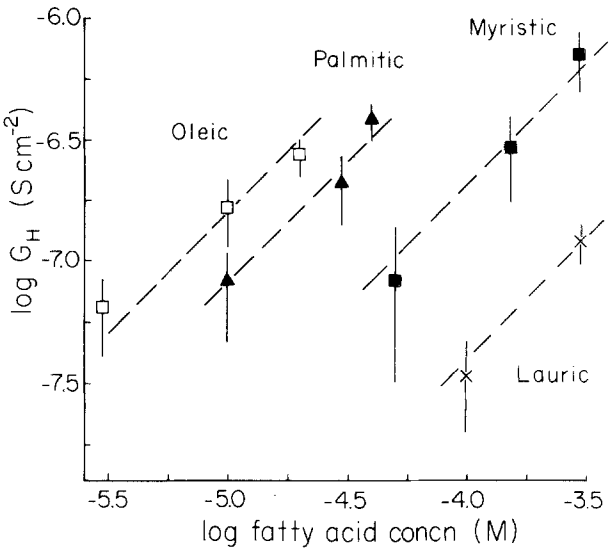

Fig. 3. Effects of aqueous fatty acids on G_H at pH 7.4–8.1. After membrane formation, the fatty acids (as Na^+ salts) were added to the aqueous phase at concentrations ranging from 3 to 400 μM. The lipid solvent was decane plus chlorodecane (70 : 30, vol/vol). Aqueous solutions were buffered with HEPES plus Tris, as described in Methods. The diphytanoyl PC (control) G_H of 15 ± 6 nS cm^{-2} was substracted from the fatty acid-induced G_H. Error bars indicate SD of 2–4 membranes. Dashed lines have slopes of 1.0

ranged from 0.4 to 0.8, i.e., 25–47 mV/pH unit [22]. Addition of fatty acids increased T_H to 0.8–1.0, i.e., 47–59 mV/pH unit [22 and *unpublished data*]. With myristic and lauric acids the H^+ selectivity was somewhat lower and less reproducible than with longer chain fatty acids. Octanoate (5 mM, aqueous phase) caused no detectable increase in either T_H or G_H.

When fatty acids were added to the aqueous phase, their effectiveness increased with chain length in the order laurate < myristate < palmitate < oleate (Fig. 3). However, when fatty acids were added to the lipids, their effectiveness was nearly independent of chain length, at least for myristic, palmitic and oleic acids (Fig. 2). Addition of lauric and decanoic acids to the lipid solution also caused increases in G_H, but G_H decreased with time, probably due to the higher water solubility of these fatty acids. In contrast, "washing out" of C_{14}–C_{20} fatty acids was not apparent during the 20–25 min required to complete an experiment. Apparently, the bulk lipid tours around the membrane acts as a source for the replacement of long-chain fatty acids, which slowly diffuse out of the membrane. Similar behavior has been observed for other hydrophobic weak acids in planar bilayers [69].

Effects of Phloretin on H^+, SCN^- and TPA^+ Conductances

Figure 4 shows that phloretin (4–100 μM) inhibited the G_H produced by fatty acids. For comparison, thiocyanate (SCN^- and tetraphenylarsonium (TPA^+) were also tested. The inhibitory effects of phloretin were similar for SCN^- and H^+. For example, a phloretin concentration of 20 μM reduced both G_{SCN} and G_H by a factor of about 10. In contrast, 4 μM phloretin caused a 10-fold increase in G_{TPA}. Thus, the enhancement of cation conductance was more pronounced than the inhibition of anion conductance, a difference noted also by others [2, 53, 62]. A possible explanation has been proposed by Perkins and Cafiso [53].

The primary effect of phloretin on bilayers is to decrease the membrane dipole potential [2]. Thus, the inhibition of G_H in fatty acid-containing bilayers suggests that the rate-limiting step in H^+ transport is the translocation of the fatty acid anion (A^-) or acid-anion complex (HA_2^-). In unmodified bilayers, the H^+ (or OH^-) charge carrier is unknown. However, the inhibition of G_H by phloretin (Fig. 4) suggests that the charge carrier is primarily anionic.

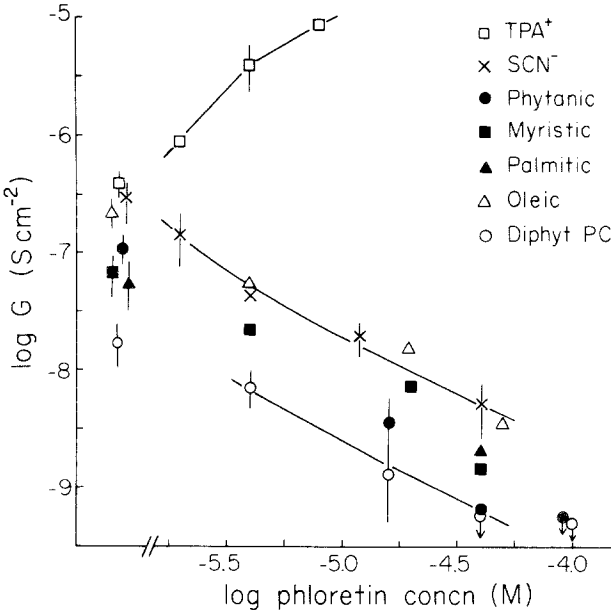

Fig. 4. Effects of phloretin on membrane conductance to tetraphenylarsonium (TPA^+) (0.2 mM), thiocyanate (SCN^-) (20 mM) and H^+ (8–40 nM). G_H was measured in PC controls and in membranes containing either phytanic acid (21 mol%), myristic acid (28 mol%), palmitic acid (33 mol%) or oleic acid (60 mol%). The solvent was decane/chlorodecane (70:30, vol/vol). Phloretin (in ethanol) was added either before or after membrane formation. Tetraphenylarsonium chloride and sodium thiocyanate were added after membrane formation. The aqueous solutions contained HEPES plus Tris, pH 7.4–8.1, as described in Methods. However, the TPA^+ experiments were done in unbuffered NaCl (pH ≈ 5.6), because TPA^+-phloretin mixtures precipitate at alkaline pH [2]. Error bars indicate SD. Single points represent single measurements. A downward arrow indicates that the data point is an upper limit

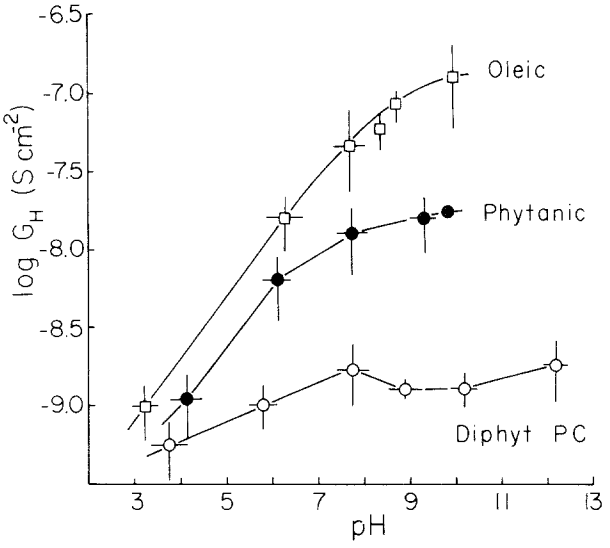

Fig. 5. Effects of pH on H^+ conductance through fatty acid-containing and unmodified membranes. The oleic acid and phytanic acid mole fractions were 0.15 and 0.21, respectively. The oleic acid-containing membranes contained 30% chlorodecane solvent. The phytanic acid-containing membranes and the PC controls contained only decane. Aqueous solutions were buffered as described in Methods and in Refs. 21 and 23. Buffer mixtures were β-alanine plus HCl (pH 3.6–4.2), MES plus Bis-Tris (pH 5.6–6.7), HEPES plus Tris (pH 7.4–8.1), Tris plus HCl (pH 8.1–8.7), TAPS plus Bis-Tris propane (pH 8.4–9.5) and β-alanine plus NaOH (pH 9.8–10.6). Horizontal bars indicate that measurements were averaged over a range of pH. Vertical bars indicate SD of, in most cases, 3–4 membranes

Effects of pH on H^+ Conductance and Selectivity

Figure 5 shows the effects of pH on G_H in unmodified PC membranes and in membranes containing either phytanic or oleic acids. At pH 3–4, G_H was low and there was little difference between controls and fatty acid-containing membranes. However, G_H increased with pH, especially in fatty acid-containing membranes (Fig. 5 and *unpublished data*). At high pH, G_H appeared to saturate. Unfortunately, the fatty acid-containing membranes were unstable at pH > 10, so the complete pH dependence was not determined.

Proton selectivity was also pH dependent in both the controls and the fatty acid-containing membranes. As mentioned above, at neutral to alkaline pH the unmodified PC membranes were moderately H^+ selective, and fatty acid-containing membranes were highly H^+ selective. However, at pH 3–4, both controls and fatty acid-containing membranes were relatively nonselective (T_H = 0.1–0.4, or 6–24 mV/pH unit).

Proton selectivity, but not proton conductance, varied somewhat with ionic strength and buffer composition, especially at low values of G_H (low pH). Therefore, in most experiments, I used fairly low ionic strengths (0.025–0.05) in order to minimize the "background" conductances of ions other than H^+ and OH^+. In a few experiments with oleic and phytanic acids at pH 7.4–9.8, I added 50–100 mM NaCl or KCl to the standard buffer mixtures and observed no effect on T_H or G_H.

Conductance-Voltage Relationships

Figure 6 shows the normalized steady-state conductance as a function of voltage for several types of membranes. The conductances were normalized by taking the ratio of the conductance at voltage, V, to the conductance at 40 mV. Then G_V/G_{40} was plotted against membrane voltage. The results were com-

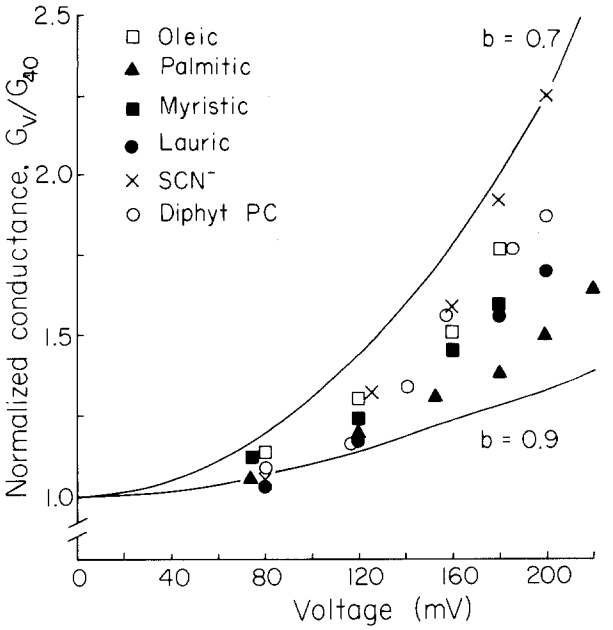

Fig. 6. Voltage dependence of the steady-state conductance of several types of membranes. Oleic acid (15 mol%), palmitic acid (14 mol%), myristic acid (50 mol%), phytanic acid (21 mol%) were added to the membrane-forming solutions. SCN^- (10–30 mM) and laurate (600 μM) were added to the aqueous phase. In most experiments the pH was 7.7, but in the oleic acid experiments pH ranged from 7.7–9.8. The membrane-forming solutions contained either 15 or 30% chlorodecane. The solid lines are calculated from Eq. (1), where b is the fraction of the membrane spanned by the minor base of the trapezoidal energy barrier. Each point is the average of measurements from at least two membranes. Some of the data points at low voltages are shifted slightly to prevent overlap

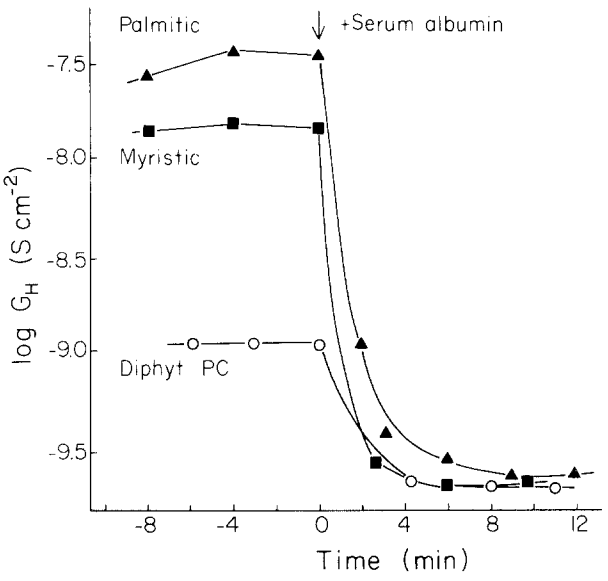

Fig. 7. Inhibition of H^+ conductance by serum albumin. Serum albumin (fatty acid free) was added to both sides of the membrane 10–20 min after membrane formation. The albumin concentration was 0.15 ± 0.05 mg ml^{-1} (about 2 μM). Aqueous solutions contained HEPES plus Tris, pH 8.1 (rear) and 7.4 (front). The palmitic and myristic acid concentrations were 14 and 28 mol%, respectively, in the membrane-forming solutions. Chlorodecane (30%, vol/vol) was included in the palmitic acid-containing membrane, but not in the myristic acid-containing membrane or in the PC control. The time scale was normalized so that the three experiments could be plotted together. The exact rate of inhibition is uncertain because 2–3 min are required to inject albumin into the rear solution and to replace the front solution with albumin-containing solution

pared to the predictions of a trapezoidal energy barrier model [25, 28], i.e.,

$$G_V/G_{40} = b \sinh(u/2)/\sinh(bu/2) \quad (1)$$

where b is the fraction of the membrane spanned by the minor base of the trapezoid, $u = FV/RT$, and R, T and F have their usual meanings.

The conductance-voltage curves were moderately superlinear for all types of membranes, the data points falling within the range of $b = 0.7$–0.9. However, membranes frequently ruptured at voltages > 160 mV, so the exact voltage dependencies are uncertain. Nevertheless, the voltage dependence of G_H was apparently less than that observed with classical proton ionophores, where b ranges from 0.5 to 0.65 [6, 33, 34].

Effects of Serum Albumin on H^+ Conductance

Serum albumin is well known for its ability to reversibly bind amphiphilic molecules and ions, especially long-chain fatty acids [9, 71]. Figure 7 shows that serum albumin (fatty acid free) inhibits G_H in fatty acid-containing and, to a less extent, in unmodified membranes. Similar results were obtained with palmitic and myristic acids (Fig. 7), phytanic acid [22] and oleic acid (*data not shown*). Qualitatively similar results were obtained either with or without 30% chlorodecane in the membrane-forming solution (Fig. 7). Thus, inhibition of G_H is not due to a selective extraction of chlorodecane by albumin.

The serum albumin was applied by injection into the rear solution and continuous perfusion from a large reservoir through the front compartment. When albumin was applied by injection alone, the inhibition of G_H was less pronounced and rather unpredictable. The probable reason is that the total amount of albumin injected was about 5 nmol, whereas the total fatty acid ranged from about 1 to 50 nmol, depending upon the experimental concentration and the amount of excess lipid solution on the partition. Also, the albumin may absorb some alkane solvent [71], which is the predominant component of the bulk lipid solution.

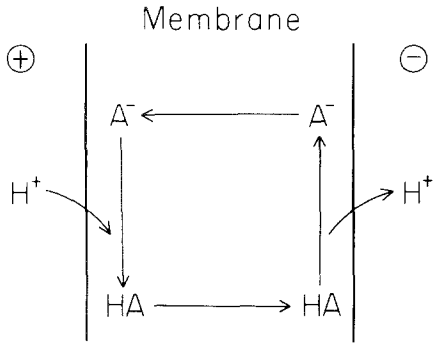

Fig. 8. Simple (A^--type) carrier model of proton transport through bilayers containing long-chain fatty acids. Proton transport may be driven by either a voltage gradient, as shown here, or by a pH gradient. The model is similar to the A^- type proton carrier described by LeBlanc [40] and reviewed by McLaughlin and Dilger [45]

Discussion

Mechanism of H^+ Conductance Caused by Fatty Acids

Addition of fatty acids to phospholipid bilayers caused an increase in the membrane H^+ conductance (G_H), which was proportional to either the mole fraction of fatty acids in the lipid solution (Fig. 2) or to the concentration of fatty acids in the aqueous phase (Fig. 3). The fatty acid-induced G_H was increased by chlorodecane (Fig. 1) and membrane voltage (Fig. 6) but was inhibited by phloretin (Fig. 4), low pH (Fig. 5) and serum albumin (Fig. 7).

The inhibition of G_H by phloretin and low pH suggests that translocation of either the fatty acid anion (A^-) or acid-anion complex (HA_2^-) is the rate-limiting step in H^+ transport. The enhancement of G_H by chlorodecane and membrane voltage is consistent with this hypothesis. Furthermore, the first-power dependence of G_H on fatty acid concentration (Figs. 2 and 3) suggests that A^-, rather than HA_2^-, is the primary charge carrier in the membrane [45]. The pH dependence of G_H (Fig. 5) is consistent with the A^- model, because G_H seems to "plateau" on the alkaline side of the pK (*cf.* Fig. 5 in Ref. 34)[1]. The A^- carrier model is shown in Fig. 8.

[1] Fatty acids in dilute aqueous solutions have pK's near 4.8. However, fatty acids in bilayers have surface pK's which are much higher. For example, oleic and stearic acids (1.2–5.0 mol%) in PC vesicles have pK's in the range of 7.0–7.5 [63, 68, 77]. The increase in fatty acid pK is due in part to the lower dielectric constant in the membrane surface (*see* Ref. 74 for discussion and references). Also, ionization of fatty acids in the membrane produces a negative surface charge which broadens the titration curve and contributes to the increase in pK [77]. Thus, the exact relationship between G_H and degree of ionization is difficult to determine, especially at high fatty acid mole fractions.

The ability of serum albumin to inhibit fatty acid-induced G_H (Fig. 7) probably reflects the removal of fatty acids from the membrane. For example, serum albumin reverses the uncoupling effect of free fatty acids on mitochondria [7, 8, 50]. Serum albumin binds a wide variety of fatty acids [9, 71], including phytanic acid [3]. A reversible transfer of oleic acid between albumin and phospholipid vesicles has been described recently by Hamilton and Cistola [26]. However, the detailed mechanism of transfer of fatty acid between albumin and phospholipid bilayers is not known.

Previous studies of carrier-mediated H^+ transport have focused mainly on the properties of weak acid uncouplers of oxidative phosphorylation [6, 33, 34, 45]. Compared to classical "protonophores," fatty acids are very inefficient H^+ carriers. For example, in order to produce a G_H of 100 nS cm^{-2}, the required concentration of oleic acid is about 6 μM (Fig. 3), 3–4 orders of magnitude higher than the equivalent concentration of FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone) [45]. The ability of classical protonophores to transport H^+ is due in part to the presence of π electrons, which delocalize the charge and increase the solubility of the weak acid anion in the low dielectric region of the membrane [45]. Thus, fatty acids are neither expected nor observed to be efficient H^+ carriers in lipid bilayer membranes.

Partition Coefficients and Translocation Rate Constants

The membrane/water distribution coefficients (K_p and β) can be estimated by finding the membrane mole fraction and aqueous concentration which produce the same G_H. Oleic acid, for example, produces a G_H of about 80 nS cm^{-2} when the membrane mole fraction is about 0.3 (Fig. 2) or when the aqueous concentration is about 5 μM (Fig. 3). The membrane mole fraction can be converted to a molar concentration by assuming molecular volumes of 480 Å^3 for long-chain fatty acids and 1,430 Å^3 for diphytanoyl PC [10, 41, 63]. For oleic acid this calculation yields a membrane concentration of about 0.5 M when the aqueous concentration is 5 μM. Alternatively, an adsorption coefficient (β) can be calculated, assuming molecular areas of 25 Å^2 for fatty acids and 70 Å^2 for diphytanoyl PC [10, 31, 41]. For oleic acid this yields a surface concentration of 1×10^{-10} mol cm^{-2} when the aqueous concentration is 5 μM (5×10^{-9} mol cm^{-3}).

Estimates of K_p and β for myristic, palmitic and oleic acids are shown in the Table. The major assumption is that the mole fraction of fatty acid relative to PC in the bilayer is the same as that in the bulk lipid solution. Given this possibly major source is systematic error, plus the approx. 50% experi-

Table. Membrane/water distribution coefficients (K_p and β) for fatty acids and their anions (HA + A$^-$) and translocation (flip-flop) rate constants (k_A) for fatty acid anions[a]

Fatty acid	Partition coeff. (K_p)[b]	Adsorption coeff. (β) (cm)[c]	Rate constant (k_A)[d] (sec^{-1})
Myristic acid	2×10^4	3×10^{-3}	2×10^{-4}
Palmitic acid	7×10^4	1×10^{-2}	2×10^{-4}
Oleic acid	1×10^5	2×10^{-2}	4×10^{-4}

[a] Membrane forming solution contained diphytanoyl PC (37 mM) in decane/chlorodecane (70:30). Fatty acids were added to either the membrane forming solution or to the aqueous phase (pH 7.4–8.1), as described in Methods.

[b] K_p is the molar concentration of fatty acid in the membrane divided by the molar concentration in water. *See* text for details.

[c] β is the surface concentration of fatty acid divided by the molar concentration in water. *See* text for details.

[d] k_A is the A$^-$ translocation rate divided by the surface concentration, estimated as described in the text.

mental errors in Figs. 2 and 3, the values shown in the Table represent, at best, the correct orders of magnitude.

The translocation (flip-flop) rate constant (k_A) for the ionized fatty acid can be estimated from G_H and the surface concentration of A$^-$, assuming that A$^-$ translocation is the rate-limiting step in H$^+$ transport. For example, for oleic acid the surface concentration of HA plus A$^-$ was about 1×10^{-10} mole cm^{-2} when the mole fraction was 0.3, assuming once again that the mole fraction in the bilayer is similar to that in the bulk lipid solution. The surface concentration of A$^-$ was estimated by assuming 50% ionization at pH 7.4 (Fig. 5) [63, 68]. The A$^-$ flux at small voltages was estimated by the relation, $J_A = RTG_H/F^2$. Thus, when $G_H = 80$ nS cm^{-1}, $J_A = 2 \times 10^{-14}$ mol cm^{-2} sec^{-1}. The translocation rate constant, k_A, is equal to flux/surface concentration. Thus, for oleic acid, $k_A = 4 \times 10^{-4}$ sec^{-1}. Given the several major sources of error, the estimates of k_A yield, at best, the correct orders of magnitude.

The values of k_A in the Table reflect the presence of 30% chlorodecane in the membrane-forming solution. When chlorodecane was omitted, the estimates of k_A were reduced by a factor of 7–10 (*cf.* Fig. 1). If 100% chlorodecane were used as solvent, the k_A's would probably be about one order of magnitude higher than the values shown in the Table and two orders of magnitude higher than the values obtained with decane [6, 17, 45]. Finally, if the bilayers were "solvent free," then the values of k_A might be close to the values in the Table, i.e., intermediate between decane and chlorodecane containing membranes [17, 54; *also cf.* data in Ref. 2 with Ref. 62]. The effect of chlorodecane on G_H is important because bilayers containing chlorodecane apparently have "intrinsic" ionic conductances similar to inner mitochondrial membranes [17, 45].

The fatty acid k_A's in the Table are 4–6 orders of magnitude lower than the measured k_A's for classical protonophores. For example, the value of k_A for FCCP in diphytanoyl PC bilayers is 7–700 sec^{-1}, depending upon whether the bilayer contains decane or chlorodecane [6]. However, the membrane/water partition coefficients for long-chain fatty acids are roughly similar to the values obtained for FCCP and other protonophores [6, 33, 34, 45]. Thus, both fatty acids and protonophores adsorb strongly to membranes. However, due to their slow A$^-$ flip-flop rates, the fatty acids are much less effective H$^+$ carriers.

Do Fatty Acids Cause Biological H$^+$ Conductance?

Free fatty acids are normal constituents of most biological membranes. Normal membrane levels of free fatty acids range from <1 mol% to approx. 40 mol% of total lipids, depending on the specific membrane and metabolic conditions [5, 7, 13, 18, 27, 42, 56, 78, 83]. Under abnormal or pathological conditions, free fatty acid levels may increase dramatically. For example, ischemia or hypoxia causes large and rapid increases in membrane levels of free fatty acids [7, 12, 14, 29, 39, 67, 70, 78, 84]. Increased membrane concentrations of free fatty acids may also be associated with hypothermia [29], thermogenesis [50], ethanol abuse [38], hormonally induced lipolysis [1, 75] and several hereditary disorders, e.g., Refsum's (phytanic acid storage) disease [4, 72].

Biological membrane "intrinsic" G_H is difficult to define and measure because multiple pathways exist for H$^+$ and/or OH$^-$ diffusion [32, 76]. However, most published estimates of biological G_H fall between 10 and 2,000 nS cm^{-2}, generally higher than the G_H's reported for unmodified phospholipid bilayers. For example, the inner mitochondrial membrane G_H is 350–450 nS cm^{-2} [37, 46], the mammalian erythrocyte membrane G_H is 10–100 nS cm^{-2} [35, 73], and certain bacterial membranes and chloroplast thylakoid membranes display G_H's ranging from 400–1,600 nS cm^{-2} [44, 60].

In fatty acid-containing bilayers (Figs. 2 and 3), the range of G_H partially overlaps the range of G_H in biological membranes. However, the similarity may be partly fortuitous because biological G_H may be increased by the presence of membrane proteins. For example, the intrinsic G_H of mitochondrial membranes is about 10-fold higher than the G_H of bilayers made from mitochondrial lipids [37]. Ap-

parently, the presence of integral membrane proteins alone can increase the intrinsic conductance at least an order of magnitude above the normal lipid bilayer value [17, 45]. This might explain, for example, why the inner mitochondrial membrane is especially sensitive to free fatty acids [7, 15, 43, 57].

Collectively, my data suggest that free fatty acid levels of at least several mole percent would be required to cause a significant G_H in most biological membranes. However, due to their large partition coefficients, low aqueous concentrations of fatty acids can produce significant membrane mole fractions (Table) [52, 58, 63, 65]. For example, cytosolic concentrations of unbound fatty acids in the range of 10^{-7}–10^{-6} M are potentially toxic to mitochondria [15, 57]. Outside the cell, unbound fatty acids in the plasma become highly toxic at concentrations of about 10^{-5} M [71]. For comparison, the normal unbound fatty acid concentrations are $<10^{-7}$ M in the cytosol [13, 57] and $<10^{-6}$ M in the plasma [71].

The mechanisms of fatty acid toxicity are complex and poorly understood [14, 39, 55, 57]. However, my results suggest that fatty acid toxicity is due at least in part to increased G_H and consequent dissipation of electrochemical H^+ gradients across cell and organelle membranes [8, 43, 59]. In ischemia, for example, free fatty acids in the inner mitochondrial membrane exceed 10 mol% within 2–3 hr, at which time the mitochondria are swollen and uncoupled [7, 70].

In some biological membranes even the normal levels of free fatty acids exceed 5 mol% of total lipids [5, 18, 42, 56], and values as high as 40 mol% have been reported in brush border membranes from small intestine [27, 83]. Thus, it would not be surprising to find that free fatty acids sometimes contribute to, or even dominate, the normal H^+ conductance pathways. For example, in adipocytes free fatty acids released during hormonally induced lipolysis apparently act as metabolic regulators by depolarizing the mitochondrial membrane potential [75]. In brush-border membrane vesicles from small intestine, proximal tubule and chloroid plexus the H^+/Na^+ permeability ratios are very high, i.e., 10^4 to 10^7 [82]. The presence of free fatty acids [5, 18, 27, 56, 83] might explain these high ratios, as well as the fact that the H^+/Na^+ permeability ratios increase about sixfold as pH increases from 5.5 to 8.5 [82] (*cf.* Fig. 5). If this suggestion is correct, then "washing" brush border vesicles with defatted serum albumin should reduce H^+ permeability and conductance.

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