

# Fatty acid flip-flop and proton transport determined by short-circuit current in planar bilayers

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**Abstract** The effect of palmitic acid (PA) and oleic acid (OA) on electrical parameters of planar membranes was studied. We found a substantial difference between the effects of PA and OA on proton transfer. PA induced a small increase in conductance, requiring a new technique for estimating proton-mediated currents across low-conductance planar bilayers in which an electrometer is used to measure the transmembrane current under virtual short circuit (SCC). Open-circuit voltage and SCC were used to determine proton and leak conductances. OA caused a marked increase in membrane conductance, allowing the use of a voltage-clamp technique. From SCC data, we were able to estimate the flip-flop rate constants for palmitate ( $1 \times 10^{-6} \text{ s}^{-1}$ ) and oleate ( $49 \times 10^{-6} \text{ s}^{-1}$ ) anions. Cholesterol, included in the membrane-forming solution, decreased importantly the leak conductance both in membranes unmodified by FA and in membranes modified by PA added to the bath.—Brunaldi, K., M. A. Miranda, F. Abdulkader, R. Curi, and J. Procopio. Fatty acid flip-flop and proton transport determined by short-circuit current in planar bilayers. *J. Lipid Res.* 2005. 46: 245–251.

**Supplementary key words** lipid membranes • cholesterol • electrometer

Long-chain FA transport across the cell membranes is still a controversial subject. There are two lines of evidence pointing to either a simple diffusional mechanism (1) or a protein-mediated process (2). Through the diffusional mechanism, FA transport is described as being coupled to proton transport, as the protonated (uncharged) form of the FA diffuses better in the hydrophobic interior of the lipid bilayer (3). In this way, FA flip-flop would act as a proton shuttle.

On the other hand, the presence of FA in the membrane may enhance proton transport independently of this shuttle mechanism, mainly in the case of unsaturated fatty acids, which reduce membrane order (4), and so could increase proton transport through aqueous defects. Cho-

lesterol, through its membrane-ordering effect (5), could modulate this shuttle-independent proton pathway.

The issue of long-chain FA translocation across cell membranes has been dealt with using many approaches (6–8). In biomimetic systems, indirect evidence of FA-mediated proton transfer has been obtained mainly from unilamellar vesicles by acidification studies using fluorescent pH-sensitive probes (1).

Despite being very sensitive regarding time resolution of the acidification process, studies using vesicles lack information concerning electrical parameters such as transmembrane potential difference, proton-associated electrical currents, and electrical conductance. On the other hand, electrical determinations of FA effects on proton transfer are scant and have been based on membrane conductance measurements (9), suggesting that FAs increase proton transport across the lipid bilayer. In such studies, the membrane-unspecific or leak conductance contributes importantly to the measured conductance and constitutes a major source of indeterminacy.

The problem in measuring total membrane conductance arises when the membrane is subjected to an externally applied field, which drives protons, through a proton-selective pathway, and other ions, through a nonspecific (leak) pathway. Identifying separately the proton and leak conductances is not trivial and requires short-circuit or tracer techniques under a transmembrane proton gradient.

A natural procedure to determine proton transport would be to short circuit a membrane under an imposed pH gradient. However, the usual voltage-clamp (VC) technique is not adequate when membrane conductances are very low, on the order of a few picosiemens. Preliminary experiments have shown that doping membranes with saturated FAs, such as palmitic acid (PA), induces subtle electrical modifications that are not detectable with the conventional patch-clamp amplifier in the VC mode (our unpublished results).

In this work, a new approach to studying FA-mediated

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charge transfer in very low-conductance membranes has been used, in which the voltage and current generated by proton transport are the sole driving forces for current and voltage generation. In this way, we circumvent electrical effects from externally applied fields.

## MATERIALS AND METHODS

Soybean L- $\alpha$ -phosphatidylcholine (asolectin), PA, oleic acid (OA), and Tris were obtained from Sigma (St. Louis, MO). Cholesterol was obtained from Serva Feinbiochemica (Heidelberg, Germany), and *n*-decane was from ICN Pharmaceuticals (Plainview, NY).

Planar bilayers were either formed from asolectin or from a mixture of asolectin and cholesterol in an 83:17 proportion, in all cases the solvent being *n*-decane. The membrane-forming solution was spread across a hole (0.4–2.4 mm diameter) (10) in the wall of a polypropylene vial and inserted onto an acrylic chamber, defining a *trans* compartment (inside the vial) and a *cis* compartment (outside the vial).

Bathing solutions were symmetric at the time of membrane formation (5 mM KCl, 5 mM KH<sub>2</sub>PO<sub>4</sub>, and 5 mM Tris, pH 7.4). Sulfuric acid was added to the *cis* side to create a pH difference ( $\Delta$ pH) across the membrane (from 0.4 to 2.0 pH units). Aliquots of PA or OA (ethanolic solutions) were added to both *cis* and *trans* sides at a final aqueous concentration of 40–65  $\mu$ M. Final ethanol concentration was less than 0.1% and was not found, per se, to affect the electrical parameters of membranes (data not shown).

Electrical contact to the bathing solutions was established with Ag|AgCl electrodes. Access resistance, which includes solutions and interfaces, was negligible. Experiments were carried out at controlled room temperature (22–25°C).

Figure 1A depicts the relevant elements in our study and indicates that, under a transmembrane  $\Delta$ pH, a proton electromotive force (EMF =  $E_H$ ) is generated across the membrane.  $E_H$  is associated with a resistance ( $R_H$ ) and is given by

$$E_H = 0.059 \Delta \text{pH} \quad (\text{Eq. 1})$$

In parallel with the proton pathway, we consider a leak pathway, present in all types of bilayers, that is considered unspecific and thus has no EMF.

The membrane open-circuit voltage ( $E_m$ ), membrane conductance ( $G_m$ ), and membrane resistance ( $R_m$ ) are derived from the elements of Fig. 1A and given (Fig. 1B) by equation 2:

$$(a) \quad E_m = \frac{E_H G_H}{G_H + G_{\text{leak}}} \quad (\text{Eq. 2})$$

$$(b) \quad G_m = G_H + G_{\text{leak}}$$

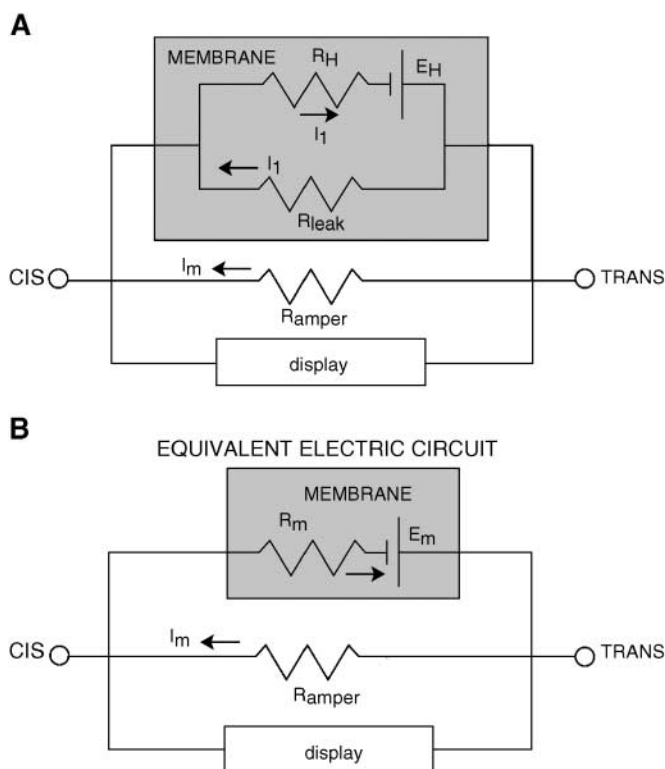
$$(c) \quad R_m = \frac{1}{G_m}$$

where  $G_H$  is the proton conductance and  $G_{\text{leak}}$  is the leak conductance. In this way, the circuit of Fig. 1A can be reduced to that of Fig. 1B, to which we have direct experimental access. Fig. 1B constitutes a closed circuit in which the current is given by equation 3:

$$I_m = \frac{E_m}{R_m + R_{\text{amper}}} \quad (\text{Eq. 3})$$

### Electrical monitoring

*Membranes modified by PA.* Low-conductance membranes were monitored by a high-impedance and very sensitive electrometer



**Fig. 1.** Equivalent electric circuit of a lipid bilayer connected to a digital electrometer. A: The membrane conductive pathway is subdivided into unspecific and proton-related routes with resistance  $R_{\text{leak}}$  and  $R_H$ , respectively. The proton-permeable pathway has an electromotive force (EMF) given by  $E_H = 0.059 \Delta \text{pH}$  (equation 1), where  $\Delta \text{pH}$  is the difference between pH in the *trans* and *cis* compartments. The membrane is connected to the electrometer, the internal resistance of which,  $R_{\text{amper}}$ , is variable.  $I_1$  is a nonmeasurable current that corresponds to the current leaking through unspecific routes of the membrane. B: The membrane circuit is reduced to a single EMF ( $E_m$ ) in series with a resistance,  $R_m$ .  $i_m$  is the total current through the membrane, which can be calculated from equation 3. "Display" refers to the electrometer digital display, which always shows the transmembrane voltage in volts ( $V_{\text{display}}$ ).

(Keithley 616 Digital Electrometer). The instrument was used alternately in three operation modes. In voltmeter mode, the electrometer has a very high input impedance ( $2 \times 10^{14} \Omega$ ) and was used to determine the membrane open-circuit voltage ( $E_m$ ) or spontaneous voltage. In ohmmeter mode, the electrometer was used to measure the membrane resistance and imposes a fixed and precise current of 1.0 pA across the membrane. The resulting transmembrane voltage ( $V_m$ ) was then read in the display according to equation 4, and the membrane resistance ( $R_m$ ) was obtained from the numerical display of the electrometer ( $V_{\text{display}}$ ), which, in all operation modes, is identical to  $V_m$ .

$$V_{\text{display}} = (1.0 \times 10^{-12} \text{ Amp}) \times R_m + E_m \quad \therefore \quad (\text{Eq. 4})$$

$$R_m = \frac{V_{\text{display}} - E_m}{(1.0 \times 10^{-12} \text{ Amp})}$$

The measurement of the membrane conductance ( $G_m = 1/R_m$ ) requires, however, the application of an external driving force that, in the case exemplified in equation 4, usually imposes more than 50 mV across the membrane.

Finally, in amperemeter mode, the electrometer was used as a

shunt-type picoammeter. In this mode, the instrument connects a shunt resistance (that we call  $R_{\text{amper}}$ ) directly across the input terminals. The current ( $I_m$ ) through the membrane (which is identical to that across the shunt resistance) is then obtained as

$$I_m = \frac{V_m}{R_{\text{amper}}} = \frac{V_{\text{display}}}{R_{\text{amper}}} \quad (\text{Eq. 5})$$

While on amperemeter mode, and by decreasing sufficiently the shunt resistance ( $R_{\text{amper}}$ ), we can make the transmembrane voltage ( $V_m = V_{\text{display}}$ ) decrease below 1 mV, and this was considered a virtual short-circuit because there is no true voltage clamping at zero voltage. In this condition,  $R_{\text{amper}} \ll R_{\text{leak}}$ , with more than 95% of the current generated by the battery  $E_H$  flowing through  $R_{\text{amper}}$ . The resulting membrane current is then referred to as  $\text{SCC}_{\text{virtual}}$ . In this condition, the current across  $R_{\text{leak}}$  in Fig. 1A becomes negligible compared with the current through  $R_{\text{amper}}$  and  $\text{SCC}_{\text{virtual}}$  is approximated to the true short-circuit current (SCC) given by

$$\text{SCC}_{\text{true}} = E_H G_H \equiv \text{SCC}_{\text{virtual}} = \frac{V_{\text{display}} (\text{when smaller than 1 mV})}{R_{\text{amper}}} \quad (\text{Eq. 6})$$

whence we obtain  $G_H$  as

$$G_H = \frac{\text{SCC}_{\text{virtual}}}{E_H} \quad (\text{Eq. 7})$$

In this way,  $\text{SCC}_{\text{virtual}}$  can be determined with an accuracy of  $\sim 0.1$  pA.

$G_{\text{leak}}$  was determined from equation 8, which is derived from equation 2a by substituting for the  $E_m$  values (measured) and  $G_H$  (calculated from equation 7):

$$G_{\text{leak}} = \frac{E_H G_H - E_m G_H}{E_m} \quad (\text{Eq. 8})$$

In this way,  $G_H$  and  $G_{\text{leak}}$  are determined independently of the total membrane conductance ( $G_m$ ), whose determination requires the application of an external driving voltage and was thus avoided. Values of  $G_m$  obtained from equation 4 were used only as an estimate of membrane quality.

The permeability of the membrane to protons ( $P_{H^+}$ ) is obtained from the value of the  $\text{SCC}_{\text{virtual}}$ , which is converted into proton flux ( $J_{H^+}$ ) using equation 9:

$$J_{H^+} = \frac{\text{SCC}_{\text{virtual}}}{F} = P_{H^+} \Delta[H^+] \quad (\text{Eq. 9})$$

so that

$$P_{H^+} = \frac{\text{SCC}_{\text{virtual}}}{F \Delta[H^+]} \quad (\text{Eq. 10})$$

where  $F$  is Faraday's constant and  $\Delta[H^+]$  is the proton concentration difference.

**Membranes modified by OA.** Treatment with OA induced high membrane conductivities, allowing the use of the patch-clamp amplifier (Dagan 8900). The instrument was set to the VC mode using a 10 G $\Omega$  probe.

Current-to-voltage (I-V) relations were determined by clamping the membrane potential from  $-120$  to  $+120$  mV and recording the corresponding membrane currents by means of an A/D converter using the software Axotape.

Reversal potential ( $V_{\text{rev}}$ ), SCC, and membrane conductance ( $G_m$ ) were derived from I-V curves.  $G_m$  was determined as the

slope of the I-V relation around zero current.  $V_{\text{rev}}$  corresponds to the open-circuit voltage ( $E_m$ ) defined in equation 2a. SCC corresponds to the current measured with 0 mV applied voltage. Proton permeability ( $P_{H^+}$ ) and proton conductance ( $G_H$ ) were calculated from equations 10 and 7, respectively, with  $\text{SCC}_{\text{virtual}}$  substituted by the  $\text{SCC}_{\text{true}}$ .  $G_{\text{leak}}$  was calculated from  $G_m$  and  $G_H$  through equation 2b.

## Data analysis

Results are expressed as means  $\pm$  SEM. Statistical significance was determined by Student's  $t$ -test using GraphPad Prism 3.0 software.

## RESULTS AND DISCUSSION

### Membranes without FA in the bath

In unmodified membranes of pure asolectin, a transmembrane  $\Delta\text{pH}$  (*cis* pH < *trans* pH) generates a spontaneous potential difference (open-circuit potential difference =  $E_m$ ), with the *trans* side invariably positive in relation to the *cis* side.  $E_m$  was found to be substantially smaller than the proton equilibrium potential ( $E_H$ ). The ratio  $E_m/E_H$ , taken as a measure of the proton transference number, averages 0.06 (Fig. 2A), indicating that a leak pathway dominates the membrane conductance. The corresponding SCC directed from the *cis* to the *trans* side was found to be typically in the few picoampere range (Table 1).

Membranes formed by a mixture of 83% asolectin and 17% cholesterol had substantially higher values of  $E_m/E_H$  than those of pure asolectin under a  $\Delta\text{pH}$  (Fig. 2A). In these membranes, cholesterol almost doubled the proton conductance ( $G_H$ ) (Fig. 2B) and decreased 3.6 times the leak conductance ( $G_{\text{leak}}$ ) (Fig. 2C). Both the decrease in  $G_{\text{leak}}$  and the increase in  $G_H$  can explain the effect of cholesterol in increasing the proton transference number (Fig. 2A).

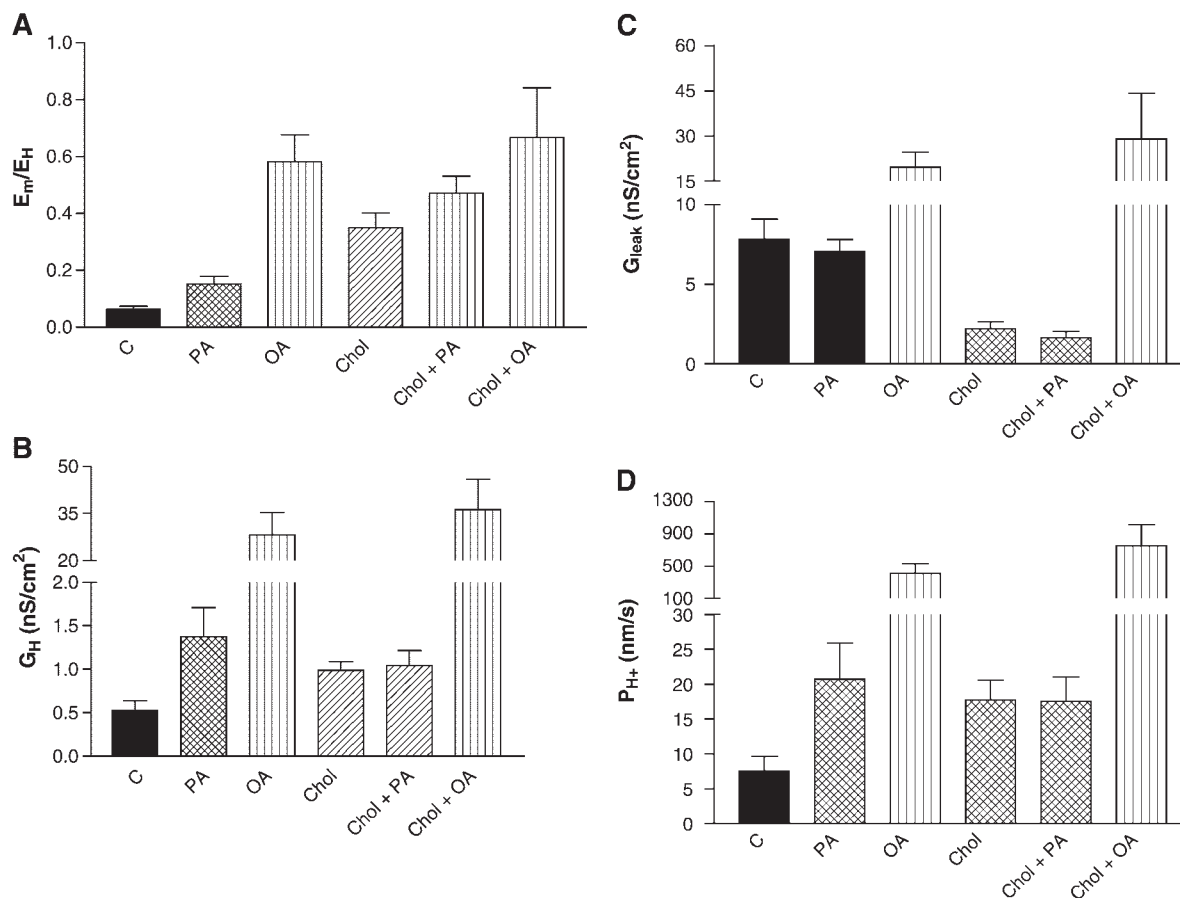
### Membranes modified by FA in the bath

The effect of PA added to the bath was studied in membranes of pure asolectin and of asolectin plus cholesterol. In pure asolectin membranes, PA importantly increases  $E_m/E_H$  and more than doubles  $G_H$  and SCC (Fig. 2A, B, Table 1, respectively) under a transmembrane  $\Delta\text{pH}$ .  $G_{\text{leak}}$  is not changed by PA (Fig. 2C). These findings suggest an effect of PA enhancing a proton translocation mechanism.

In membranes formed by a mixture of asolectin and cholesterol, PA added to the bath increases  $E_m/E_H$  only slightly and does not change the SCC or  $G_H$  (Fig. 2A, B, Table 1, respectively). As observed in pure asolectin membranes, PA did not significantly change  $G_{\text{leak}}$  (Fig. 2C).

As shown in Fig. 2D, the proton permeability calculated in equation 10 was within the range of reported values for bilayer systems (from  $10^{-2}$  to  $10^{-7}$  cm/s) (11).

The existence of a substantial proton permeability across planar bilayers has been recognized in earlier studies using this system (12, 13). Despite a large number of studies and many interesting theories, the mechanism of proton movement across the bilayer matrix remains the subject of debate and is plagued with an undeniable dose of mystery.



**Fig. 2.** Effects of oleic and palmitic acids on electrical parameters of asolectin bilayers. A:  $E_m/E_H$  ratio.  $E_m$  is the membrane potential in open-circuit mode and is given by equation 2a.  $E_H$  is the EMF generated by the membrane proton-permeable pathway and is given by equation 1. B: Proton-selective conductance ( $G_H$ ). C: Unspecific leak conductance ( $G_{leak}$ ). D: Proton permeability ( $P_{H^+}$ ). C, control; Chol, cholesterol-containing membranes; nS, nanosiemens; OA, oleic acid; PA, palmitic acid. Bars filled with different patterns are statistically different ( $P < 0.05$ ). Error bars correspond to SEM.

Almost as obscure is the mechanism of the unspecific current leak, which is present in all bilayers in smaller or larger proportions. The presence of this leak introduces a bias in any attempt to electrically characterize a given ion pathway. Complicating matters still further is the fact that

the mechanism of proton translocation per se in aqueous solution extends in part to the membrane interior, because there is an appreciable amount of water between the phospholipid acyl chains. The membrane water is considered to be highly structured, with the proton move-

**TABLE 1.** Paired typical values (not averages) for electrical parameters derived from SCC and  $E_m$  measurements

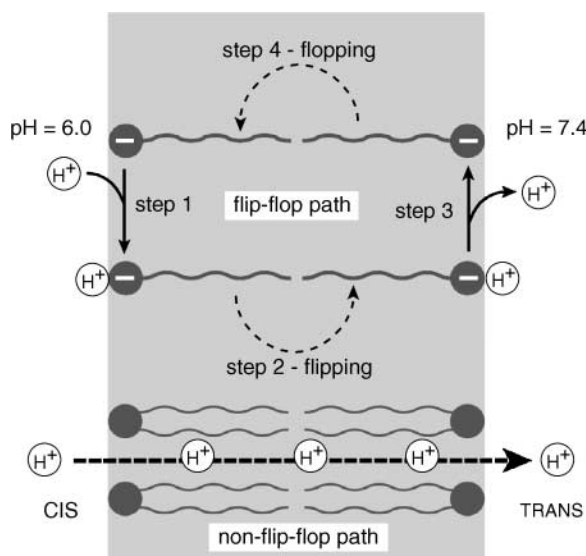
Membrane Composition	Modifier	$\Delta pH$	$E_H$	$E_m$	$E_m/E_H$	$G_H$	$G_{leak}$	SCC	$P_{H^+}$
			<i>mV</i>			<i>nS cm<sup>-2</sup></i>		<i>pA cm<sup>-2</sup></i>	<i>nm s<sup>-1</sup></i>
Low-conductance membranes (electrometer measurements)									
Pure asolectin	None	0.77	45.4	3.0	0.066	0.34	4.70	14.0	7.0
	Palmitic acid	0.77	45.4	6.0	0.132	0.80	5.15	33.0	16.0
Asolectin plus cholesterol	None	0.68	40.1	12.8	0.319	0.51	1.14	18.6	9.0
	Palmitic acid	0.68	40.1	17.0	0.423	0.51	0.66	19.7	10.0
High-conductance membranes (patch-clamp amplifier under voltage-clamp)									
Pure asolectin	None	0.76	45.0	<i>a</i>	<i>a</i>	<10 <sup><i>b</i></sup>	<10 <sup><i>b</i></sup>	<i>a</i>	<i>a</i>
	Oleic acid	0.65	38.0	31.0	0.815	55.00	13.00	2125.0	877.0
Asolectin plus cholesterol	None	0.65	38.0	<i>a</i>	<i>a</i>	<7 <sup><i>b</i></sup>	<7 <sup><i>b</i></sup>	<i>a</i>	<i>a</i>
	Oleic acid	0.65	38.0	31.5	0.828	63.0	14.00	2,429.0	1,000.0

$E_H$ , proton equilibrium potential;  $E_m$ , reversal potential;  $G_H$ , proton conductance;  $G_{leak}$ , leak conductance; nS, nanosiemens;  $P_{H^+}$ , membrane proton permeability; SCC, short-circuit current. For each membrane composition, nonmodified membrane was used as a control for fatty acid effect. Low-conductance membranes were studied using the electrometer, and high-conductance membranes were studied with a patch-clamp amplifier.

<sup>a</sup> Current versus voltage relation not determined.

<sup>b</sup> Only total membrane conductance was available.





**Fig. 3.** Proton translocation pathways across a bilayer membrane treated with fatty acids in the presence of a pH gradient ( $\text{pH}_{\text{cis}} < \text{pH}_{\text{trans}}$ ). The upper panel shows the flip-flop shuttling pathway, indicating the four steps: 1) association of the proton with the fatty acid anion; 2) flipping of the protonated fatty acid; 3) dissociation; and 4) flopping of the fatty acid anion. The lower panel shows the classical proton route, which includes many mechanisms, such as proton wires, through the phospholipid molecules.

ment occurring through a combination of proton wires (14), hydrogen-bonded water chains (15), aqueous cluster contacts (5), and possibly other mechanisms as well.

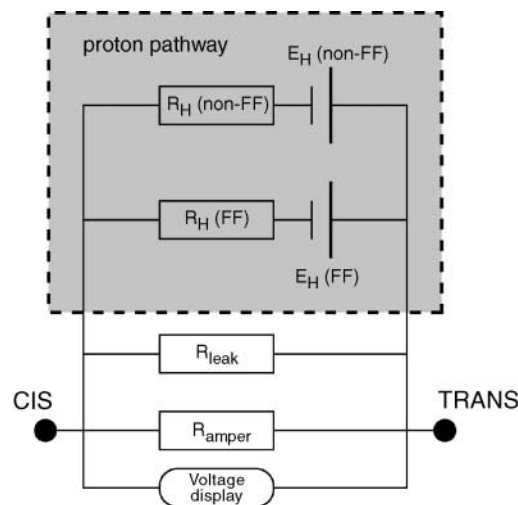
Recent studies of vesicular acidification using fluorescent probes have raised new hopes of solving some of these problems. Particularly interesting are the studies by Hamilton and Kamp (1) and Jesek, Modriansky, and Garlid (16) on the role of FAs in vesicular acidification. A proton shuttle mechanism was put forward to explain the experimental results and provided a new way of understanding the interactions between protons and FAs in the membrane interior.

Our results can be explained on the basis of a proton-selective pathway in parallel with a nonspecific leak route. The proton pathway can be divided on its own into two routes (Figs. 3, 4): one in which the proton is coupled to the FA anion (shuttle mechanism), and another in which the proton crosses the membrane through other diffusional mechanisms. In this way, the shuttle mechanism can be viewed as both a proton and a FA transporter, since the flip-flop of the protonated fatty acid is more favored than that of the anion.

From our experimental values of SCC, an estimative can be made of the flip-flop rate of PA and OA anions by assuming that the proton flux in a membrane modified by FA is limited by the FA anion ( $\text{FA}^-$ ) flip-flop (Fig. 3, step 4), as made explicit by equation 11:

$$\text{proton flux}(J_{\text{H}^+}) = \text{FA}^- \text{ flip-flop rate} \quad (\text{Eq. 11})$$

The proton flux ( $J_{\text{H}^+}$ ) is given by equation 9. The  $\text{FA}^-$  flip-flop rate is given by equation 12:



**Fig. 4.** Equivalent electrical circuit of a lipid bilayer indicating the proton pathway depicted in Fig. 3. Both proton pathways, in parallel, constitute an EMF in series with a resistance. Other elements of the circuit are described in Fig. 1. FF, flip-flop; non-FF, non-flip-flop.

$$\text{flip-flop rate} = k_{\text{ff}}([\text{FA}^-]_{\text{leaflet}}) \quad (\text{Eq. 12})$$

where  $k_{\text{ff}}$  is the  $\text{FA}^-$  flip-flop rate constant and  $[\text{FA}^-]_{\text{cis}}$  is the  $\text{FA}^-$  bidimensional concentration ( $\text{mol cm}^{-2}$ ) in either membrane leaflet. From equations 11 and 12, we have

$$J_{\text{H}^+} = k_{\text{ff}}[\text{FA}^-]_{\text{leaflet}} \quad (\text{Eq. 13})$$

hence:

$$k_{\text{ff}} = \frac{J_{\text{H}^+}}{[\text{FA}^-]_{\text{leaflet}}} \quad (\text{Eq. 14})$$

$[\text{FA}^-]_{\text{leaflet}}$  is given by equation 15:

$$[\text{FA}^-]_{\text{leaflet}} = (1/2)[\text{FA}]_{\text{sol}}\beta(d/2) \quad (\text{Eq. 15})$$

where  $[\text{FA}]_{\text{sol}}$  is the total concentration of fatty acid (both anion and acid forms) in the bath solution,  $d$  is the membrane thickness, and  $\beta$  is the partition coefficient of the fatty acid. The term  $1/2$  describes the fatty acid dissociation of 50% at pH 7.4. Finally, multiplying both terms of equation 13 by the Faraday's constant,  $F$ , and combining with equation 15, we obtain equation 16:

$$\text{SCC} = Fk_{\text{ff}}[\text{FA}]_{\text{sol}}(1/2)\beta(d/2) \quad (\text{Eq. 16})$$

where SCC is the area-normalized SCC.

**TABLE 2.** Estimation of  $k_{\text{ff}}$  from experimental SCC values in planar bilayers (from Table 1) and physicochemical constants for palmitic and oleic acids from the literature

Fatty Acid	$[\text{FA}]_{\text{sol}}^a$	$\beta^a$	SCC	$k_{\text{ff}}$
	$\text{mol cm}^{-3}$		$\text{pA cm}^{-2}$	$\text{s}^{-1}$
Palmitic	$4 \times 10^{-9}$	$6 \times 10^5$	33	$1 \times 10^{-6}$
Oleic	$6 \times 10^{-9}$	$5 \times 10^5$	2,125	$4.9 \times 10^{-5}$

Flip-flop rate constant ( $k_{\text{ff}}$ ) values were obtained from equation 16.  $\beta$ , partition coefficient;  $[\text{FA}]_{\text{sol}}$ , fatty acid aqueous solubility limit.

<sup>a</sup> Compiled by Hamilton and Kamp (1).

Using data of FA aqueous solubility limit ( $[FA]_{sol}$ ) and partition coefficient ( $\beta$ ) compiled by Hamilton and Kamp (1) in vesicles and our SCC values from Table 1, estimations of  $k_{ff}$  were made using equation 16 (Table 2).

The steady-state flip-flop rates in Table 2 refer to the limiting step of the translocation cycle, which is the return of the FA anion (Fig. 3, step 4). As such, they are considerably lower than the values of  $k_{ff}$  obtained from acidification studies in vesicles [small unilamellar vesicle (SUV)s  $\sim 220\text{ s}^{-1}$  (17), large unilamellar vesicle (LUV)s  $\sim 15\text{ s}^{-1}$  (17), and giant unilamellar vesicle (GUV)s  $\sim 0.1\text{ s}^{-1}$  (18)], where the acidification corresponds only to the flipping of the protonated FA (Fig. 3, step 2).

Unlike PA, OA added to the bath of asolectin bilayers promoted a dramatic increase in membrane conductance, allowing current-voltage relations to be determined. OA substantially increased proton selectivity, as seen by the ratio  $E_m/E_H$  approaching 1 (Fig. 2A, Table 1). Cholesterol included in the bilayer composition (17%) did not change the above response to OA, as depicted in Fig. 2 and Table 1. This may be attributable to the fact that as OA greatly increases membrane conductance, cholesterol effects are masked in the overall conductance increase.

Bilayers modified by OA display a substantially larger proton conductance compared with those modified by PA (Fig. 2B). In effect, the  $k_{ff}$  calculated for oleate was almost 50 times higher than that calculated for palmitate. This can be attributable to two possible effects: 1) the flip-flop rate of oleate is indeed much faster than that of palmitate; 2) OA also increases the conductance of the non-flip-flop-related proton pathway.


Probably the presence of a *cis* unsaturation in the OA molecule disorganizes the lipid bilayer structure (4), which would lead to an increased formation of aqueous defects in the bilayer and consequently increased proton permeability through water wires or cluster contact. Moreover, this disorganization could also facilitate the flopping of the FA anion. The increase in leak conductance observed with OA could be taken as further electrical evidence of the matrix lipid disorganization brought by the OA introduction between acyl phospholipid chains.

The reduction of the leak conductance of asolectin membranes induced by cholesterol is consistent with findings that cholesterol greatly reduces the permeability of lipid membranes to different substances (19–22), which is generally attributed to the ability of cholesterol to promote order in lipid chains. However, we observed that cholesterol incorporation into asolectin membranes essentially eliminated the PA-induced increase in proton selectivity observed in pure asolectin membranes.

The energy of activation for fatty acid flip-flop must compare with the energy needed to create a void that extends across the leaflet to which the fatty acid diffuses (23). The cholesterol order-inducing effects on acyl chains, which constrain the motion of acyl chains, probably increase this energy, reducing PA flip-flop.

The effect of cholesterol on the proton permeability of lipid bilayers may be affected by hydrogen bonding between the 3-OH group of cholesterol and other compo-

nents of the hydrophobic core, such as ester carbonyl groups of phospholipids and the amide group of sphingolipids (24). These interactions could provide a suitable early docking site for protons close to the aqueous/lipid headgroup interface, reducing the free energy required for protons to partition into the initial hydrophobic region of the membrane. The possible formation of cholesterol domains interfering with the available area for charge movements across the bilayer cannot be ruled out. Our results indicate that this might affect  $G_{leak}$ , which is decreased in the presence of cholesterol. However, the observed increase in  $G_H$  induced by cholesterol in membranes lacking FA suggests that these domains would not affect proton transport.

Thus, this study presents an alternative method to determine subtle electrical changes brought about by the movement and distribution of protons across lipid bilayers modified by fatty acids. Such results bring new evidence in favor of a proton-shuttle mechanism for fatty acid translocation across the lipid bilayer, which can be modulated by other lipids present in the membrane, such as cholesterol. 

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