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Effects of palmitic acid and cholesterol on proton transport across black lipid membranes☆

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Abstract

We studied the effect of palmitic acid (PA) and cholesterol (~ 17 wt.%) on proton translocation across asolectin (charged) and diphytanoylphosphatidylcholine (DPhPC, neutral) black lipid membranes (BLMs). Potential difference (PD), short circuit current (SCC), and conductance (G_{total}) were measured with a digital electrometer. Membranes were exposed to pH gradients (0.4–2.0 units), followed by PA addition to bath (symmetrically, 40–65 μM). The membrane conductive pathway was subdivided into an unspecific and a proton-related routes. A computer program estimated the conductances (G_{un} and G_H) of the two pathways from the measured parameters. No significant differences in proton selectivity were found between DPhPC membranes and DPhPC/cholesterol membranes. By contrast, cholesterol incorporation into asolectin increases membranes selectivity to proton. Cholesterol dramatically reduced G_{un} reflecting, probably, its ability of inducing order in lipid chains. In asolectin membranes, PA increases proton selectivity, probably by acting as a proton shuttle according to the model proposed by Kamp and et al. [Biochemistry 34 (1995) 11928]. Cholesterol incorporation into asolectin membranes eliminates the PA-induced increase in proton selectivity. In DPhPC and DPhPC/cholesterol membranes, PA does not affect proton selectivity. These results are discussed in terms of the presence of cardiolipin (CL) in asolectin, cholesterol/PA interactions, and cholesterol order-inducing effects on acyl-chains.

Keywords: Black lipid membrane; Palmitic acid; Cholesterol; Proton; Conductance; Permeability

1. Introduction

Evidence of effects of long fatty acids (FA) on proton translocation across lipid membranes has been provided mainly by the studies of acidification transients in unilamellar vesicles (for review see Ref. [2]),in which acidification is greatly enhanced by FA added to the external medium. The hypothesis put forward to explain such effect was that FA functions as a shuttle for H^+, while being mostly restricted to the intramembrane ambient.

The plasma membranes of mammalian cells are rich in cholesterol (e.g., larger than 20 wt.% in plasma membranes). Cholesterol affects the physical and structural properties of bilayers, modifying the barrier role of membranes. At high proportion, cholesterol could decrease membrane permeability by introducing ordering of the lipid chains [3], whereas in low concentration, the membrane permeability increases dramatically in the phase transition temperature range [4]. Therefore, it seems possible that small variations in membrane cholesterol content can provide for an effective control of membrane permeability.

The aim of the present study was to use electrical monitoring techniques to investigate possible interactions among protons, long-chain fatty acids, and cholesterol as influencing both fatty acid and proton translocation across planar bilayers.

Abbreviations: FA, long-chain fatty acids; BLM, black lipid membrane; DPhPC, diphytanoylphosphatidylcholine; CL, cardiolipin; PA, palmitic acid; Chol, cholesterol; PD, potential difference; SCC, short circuit current; R, resistance; V_m, transmembrane voltage; EMF, electromotive force; G_H, proton conductance; G_{un}, unspecific conductance; G_{total}, total conductance; R_{amper}, electrometer equivalent resistance; J_{H^+}, proton flux; P_{H^+}, proton permeability; F, Faraday's constant.

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2. Experimental

2.1. Materials

Diphytanoylphosphatidylcholine (DPhPC) was obtained from Avanti Polar Lipids (Birmingham, AL, USA). Soybean L-α-phosphatidylcholine (asolectin), bovine heart cardiolipin (CL), palmitic acid (PA) and Tris-hydroxy-amino-methane (Tris) were obtained from Sigma (St. Louis, MO, USA). Cholesterol was obtained from Serva Feinbiochemica (Heidelberg, GER) and *n*-decane, from ICN Pharmaceuticals (Plainview, NY, USA).

2.2. Methods

Black lipid membranes (BLMs) were formed according to Mueller et al. [5], across a hole (0.4- to 2.4-mm diameter) in the wall of a polypropilene vial, coupled with an acrylic chamber defining two compartments: *trans* and *cis*. Bathing solutions were symmetric in composition (KCl 5 mM + KH_2PO_4 5 mM + Tris 5 mM, pH 7.4). Electrically neutral BLMs were formed from DPhPC, and negatively charged BLMs were formed from asolectin (cardiolipin 8.8% [6]). Membranes without cholesterol were formed from a phospholipid solution 2.5% in *n*-decane. Membranes with cholesterol or with cardiolipin were formed by adding cholesterol or cardiolipin to the phospholipid solution (0.5% cholesterol—cholesterol content ~ 17 wt.% of total lipids; 0.66% cardiolipin—cardiolipin content = 21 wt.% of total lipids). Potential difference (PD), short circuit current (SCC) and resistance (*R*), were measured with a Keithley 616 digital electrometer (input impedance: 2×10^{14} Ω), by means of Ag/AgCl electrodes. Initially, the membranes were exposed to pH differences (0.4–2.0 unit), adding sulfuric acid to *cis* side, and then the PD, SCC and *R* were determined. Subsequently, an aliquot of palmitic acid (alcoholic solution—final ethanol concentration of 140 μM) was added bilaterally, at a final concentration of 40 to 65 μM, and the same parameters were measured again. Palmitic acid was added bilaterally in order to provide a more uniform entry of this fatty acid into the membrane phase as well as to avoid gradients of fatty acids across the

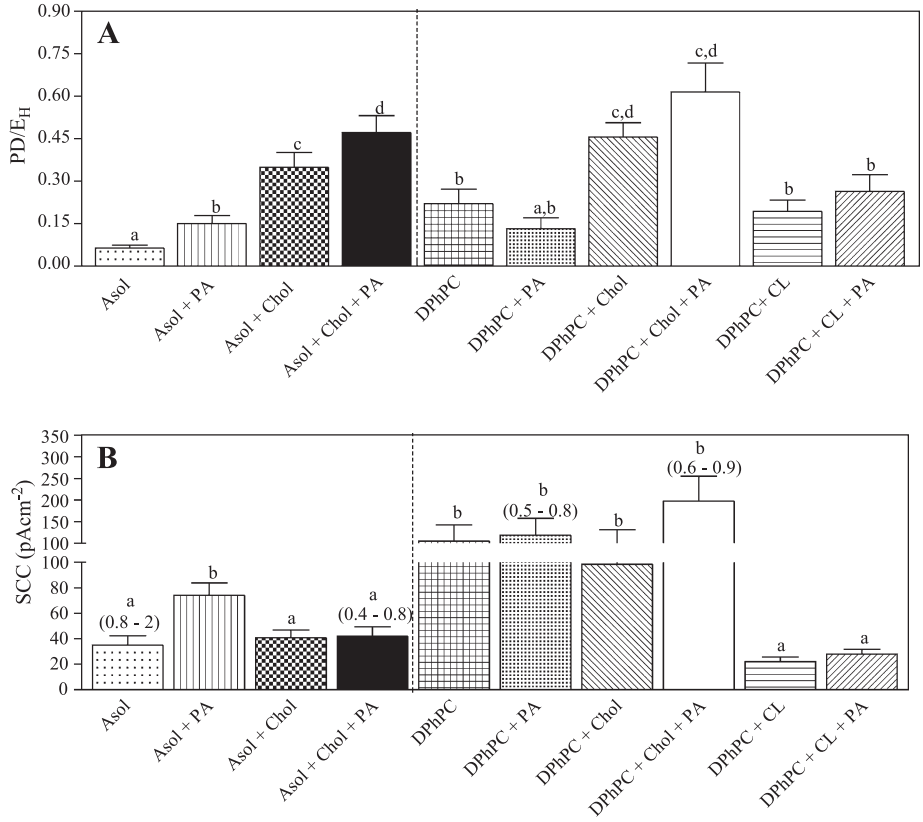

Fig. 1. Effect of palmitic acid and cholesterol on potential difference (PD) and short circuit current (SCC). PD is expressed as a function of E_H (Eq. (3)). PD and SCC were measured on the same membrane under two experimental conditions: (1) after a pH gradient (addition of sulfuric acid to *cis* side); and (2) after subsequent PA addition to *cis* and *trans* side. (A) PD of asolectin, asolectin plus cholesterol, DPhPC, DPhPC plus cholesterol, and DPhPC plus cardiolipin membranes before (Asol, Asol + Chol, DPhPC, DPhPC + Chol, DPhPC + CL) and after (Asol + PA, Asol + Chol + PA, DPhPC + PA, DPhPC + Chol + PA, DPhPC + CL + PA) PA addition, respectively. (B) SCC of asolectin, asolectin plus cholesterol, DPhPC, DPhPC plus cholesterol, and DPhPC plus cardiolipin membranes before (Asol, Asol + Chol, DPhPC, DPhPC + Chol, DPhPC + CL) and after (Asol + PA, Asol + Chol + PA, DPhPC + PA, DPhPC + Chol + PA, DPhPC + CL + PA) PA addition, respectively. Results shown in (A) and (B) are mean ± S.E.M. The numbers in parentheses are pH gradient ranges (unit of pH). Means with different letters are statistically different.

membrane. Moreover, we tested the effect of ethanol per se and found no effect. The experiments were performed at room temperature (23 ± 2 °C).

2.3. Data analysis

Results are expressed as mean ± standard error of the mean (S.E.M.). Statistical significance, P, was determined by Student's t-test.

A membrane inserted in the circuit containing the electrometer–amperimeter can be reduced to a electromotive force (EMF) E_m in series with a resistance R_m. The current I_m flowing through the membrane is given by:

$$I_m = E_m/(R_m + R_{amper}) \quad (1)$$

where R_{amper} is the electrometer equivalent internal resistance. Reducing R_{amper}, the transmembrane voltage, V_m, can be made to decrease to below 1 mV (what we consider a reasonable approach to a short-circuit condition). The "SCC" is then obtained by:

$$SCC = V_m/R_{amper} \quad (2)$$

A program written in BASIC was used to obtain the derived electrical parameters from the measured ones. This program divides the membrane conductive pathway into an unspecific and a proton-selective route. The unspecific current route has no associated EMF. It comprises all leakage routes, border sealing included. The proton-permeable pathway has an EMF given by:

$$E_H = 0.059\Delta pH \quad (3)$$

The proton-related SCC is then given by:

$$SCC = E_H G_H \quad (4)$$

where G_H is the proton conductance. The membrane potential in open-circuit mode is given by:

$$V_m = (E_H G_H + E_{un} G_{un})/(G_H + G_{un}) \quad (5)$$

where E_{un} and G_{un} are the unspecific EMF and conductance, respectively. We assume $E_{un} = 0$ also

$$(G_H + G_{un}) = G_{total} \quad (6)$$

where G_{total} is the measured membrane conductance. The computer program is based on the fact that for a given E_H, there is only one set of G_H and G_{un} values that satisfies the measured PD and SCC and simulates all experimental conditions under a given pH difference (before and after adding PA to the bath). The proton permeability, P_{H^+}, was obtained from SCC, which was converted into proton flux, J_{H^+}, given by:

$$J_{H^+} = SCC/F = P_{H^+}\Delta[H^+], \quad (7)$$

thus

$$P_{H^+} = SCC/F\Delta[H^+] \quad (8)$$

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

3. Results and discussion

The effect of PA on electrical properties of lipid bilayers was studied in four groups of BLMs: asolectin, DPhPC, asolectin plus cholesterol, and DPhPC plus cholesterol. All groups of membranes, under a *cis*-to-*trans* pH difference (pH *cis* < pH *trans*), presented a potential difference (PD) always positive on the side *trans* (Fig. 1A) and a SCC directed from *cis* to *trans* (Fig. 1B), evidencing a significant proton permeability.

In asolectin membranes, PA added to the bath promoted a substantial increase in proton selectivity. This effect mani-

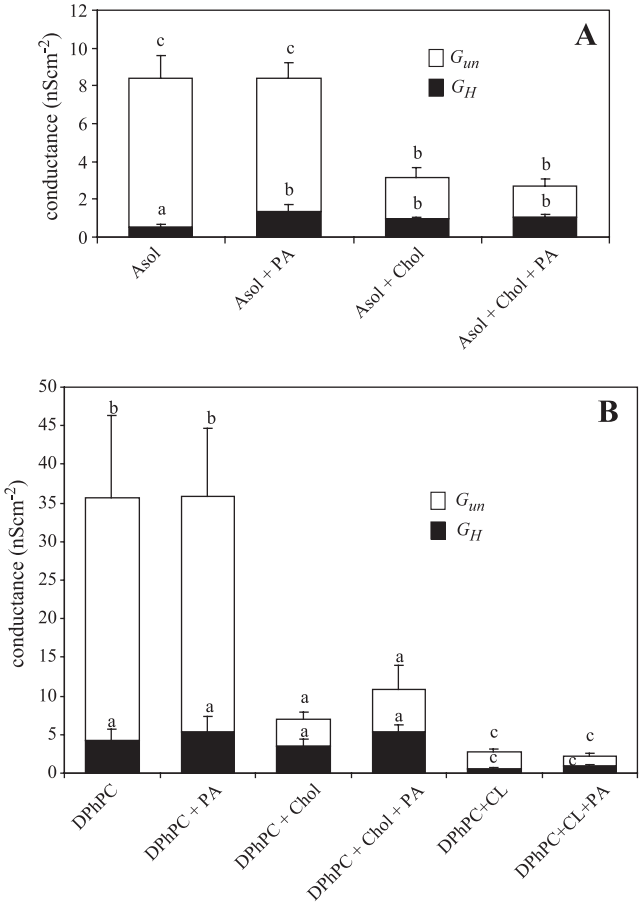

Fig. 2. Effect of palmitic acid and cholesterol on proton conductance (G_H) and unspecific conductance (G_{un}). Using the computer program, G_H and G_{un} were estimated on the same membrane under the two experimental conditions described in Fig. 1. (A) G_H and G_{un} of asolectin and asolectin plus cholesterol membranes before (Asol, Asol + Chol) and after (Asol + PA, Asol + Chol + PA) PA addition, respectively. (B) G_H and G_{un} of DPhPC, DPhPC plus cholesterol, and DPhPC plus cardiolipin membranes before (DPhPC, DPhPC + Chol, DPhPC + CL) and after (DPhPC + PA, DPhPC + Chol + PA, DPhPC + CL + PA) PA addition, respectively. Results shown in (A) and (B) are mean ± S.E.M. Means with different letters are statistically different.

fests as an increase in PD (Fig. 1A) and SCC (Fig. 1B), which reflects an increase of G_H (Fig. 2A) (i.e., enhancement of a proton-selective pathway) after treatment with PA. The P_{H^+} was $7.6 \pm 5.5 \times 10^{-7}$ cm s^{-1} and $2.1 \pm 1.4 \times 10^{-6}$ cm s^{-1} before and after PA addition, respectively (Fig. 3).

Our results with asolectin substantiate reports on fatty acids enhancement of proton transfer in unilamellar vesicles, in which the fatty acid molecule functions as a proton shuttle, its protonated form having a higher flip-flop rate [1]. A yet unsolved issue concerns the fate of the fatty acid anion in the back-flipping leg of the cycle. In natural membranes, such as in mitochondria, coadjuvant UCPs have been postulated to explain the uncoupling of ATP synthesis and oxydative phosphorylation [7].

The presence of cardiolipin in the asolectin lipid [6] may be important for the development of proton conductance, as well as for the proton transport by PA. The head group of cardiolipin could act on the membrane surface-collecting protons and donating them, via a water shuttle, to the hydrophobic environment and to transmembrane porters such as fatty acids [8].

Surprisingly, cardiolipin reduced SCC (Fig. 1B), G_H (Fig. 2B), and P_{H^+} (Fig. 3) of pure DPhPC membranes. G_{un} was also reduced by cardiolipin (Fig. 2B), suggesting a modification into the matrix organization similar to that induced by cholesterol. The treatment of membranes of DPhPC and cardiolipin with PA did not increase the proton selectivity. It thus appears that cardiolipin may not be a crucial factor per se, but rather has a contributing role when mixed with other naturally present lipids.

Cholesterol incorporation into asolectin membranes eliminates the PA-induced increase in proton selectivity: in asolectin plus cholesterol membranes, SCC (Fig. 1B), G_H (Fig. 2A), and P_{H^+} (Fig. 3) before and after treatment with PA were not significantly different ($1.7 \pm 0.7 \times 10^{-6}$ and $1.8 \pm 0.9 \times 10^{-6}$ cm s^{-1}, respectively.). It is known that cholesterol has a preference for saturated chain lipids, which has been implied in the formation of "rafts" [9]. Since PA is saturated, cholesterol/PA interactions in membranes with cholesterol modified by PA are possible; hence, the number of free PA molecules available to transport protons would be reduced, as well as the mobility of PA and, consequently, its flip-flop rate.

PA also failed to increase the proton selectivity of pure DPhPC and DPhPC plus cholesterol membranes. In such membranes, PA was not effective to increase PD (Fig. 1A), SCC (Fig. 1B), and G_H (Fig. 2B). Futhermore, neither PA nor cholesterol caused a significant change of P_{H^+} of DPhPC membranes (Fig. 3). The P_{H^+} of DPhPC membranes before and after treatment with PA were $3.3 \pm 2.5 \times 10^{-6}$ and $4.4 \pm 2.1 \times 10^{-6}$ cm s^{-1}, whereas the P_{H^+} of DPhPC plus cholesterol membranes were $6.4 \pm 3.5 \times 10^{-6}$ and $9.2 \pm 4.0 \times 10^{-6}$ cm s^{-1}, respectively. It seems that the environment of the DPhPC hydrophobic matrix, independently of cholesterol content, is not favorable to H^+ transport by PA. Possibly in DPhPC membranes, as well as in asolectin plus cholesterol membranes, the efficiency of PA in increasing the proton permeability could be favored by carrier proteins that would facilitate PA anion translocation.

Fig. 2A and B demonstrates that the most remarkable effect of cholesterol on asolectin and DPhPC membranes was the reduction in unspecific conductance, G_{un}, which explains the higher level of PD in membranes with cholesterol (Fig. 1A). Those effects are consistent with findings that cholesterol greatly reduces the permeability of lipid membranes to many different substances [10–13], which is generally attributed to its ability to induce order in lipid chains. Furthermore, the putative action of cholesterol in generating an ordered environment is quite general and does not depend on the particular lipid head group or the nature

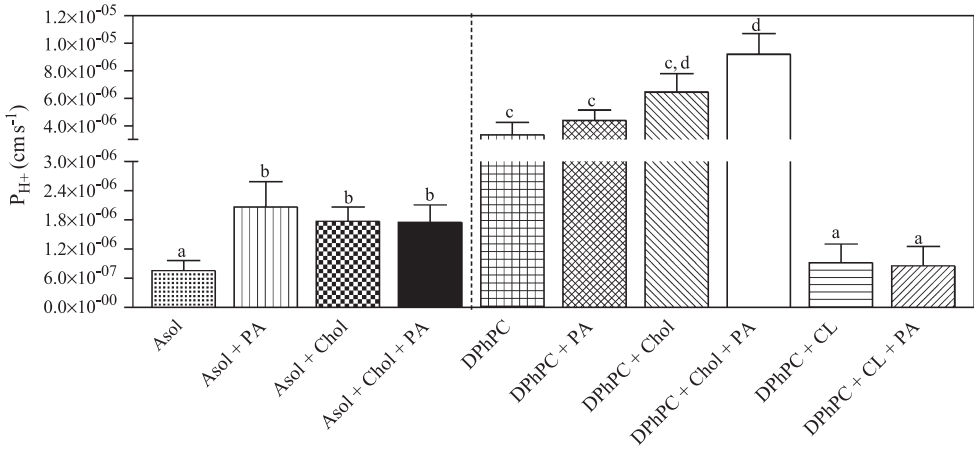

Fig. 3. Effect of palmitic acid and cholesterol on proton permeability (P_{H^+}). P_{H^+} was calculated (Eq. (7)) under the two experimental conditions described in Fig. 1. (A) P_{H^+} of asolectin and asolectin plus cholesterol membranes before (Asol, Asol + Chol) and after (Asol + PA, Asol + Chol + PA) PA addition, respectively. (B) P_{H^+} of DPhPC, DPhPC plus cholesterol, and DPhPC plus cardiolipin membranes before (DPhPC, DPhPC + Chol, DPhPC + CL) and after (DPhPC + PA, DPhPC + Chol + PA, DPhPC + CL + PA) PA addition, respectively. Results shown in (A) and (B) are mean ± S.E.M. Means with different letters are statistically different.

of acyl chains. Cholesterol provides an effective physical barrier to passive transport of molecules across membranes, which is important to secure active transport processes.

Together, our results indicate that the transport of H^+ induced by PA is sensitive to the composition of the lipid matrix, suggesting that the requisites for PA being able to function as a proton shuttle are the presence of cardiolipins and the lack of cholesterol, a condition that resembles that in mitochondria, where the inner membrane contains cardiolipin but not cholesterol and where fatty acids are able to uncouple oxidative phosphorylation by increasing the proton conductance [7]. A reasonable scenario would be that cells make use of altering the membrane cholesterol fraction in order to control their membrane permeability to H^+, especially in conditions that require a more efficient membrane barrier property. On the other hand, PA would be an adjunct factor in controlling proton permeability of membranes with low cholesterol content.

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