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Phosphatidylcholine and cholesterol inhibit phosphatidate-mediated calcium traversal of liposomal bilayers

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Rates of phosphatidic acid- (PA-) mediated Ca^{2+}-traversal are maximal in 'passive bilayers' void of lipid CO and OH groups: dietherphosphatidylcholine (diether-PC) or OH-blocked cholesterol liposomes. Phosphatidylcholine (PC) as bilayer matrix causes 99% inhibition, while 45 mol% cholesterol in passive bilayers inhibits by about 70%. Possibly, the absence of CO and OH groups causes a dehydration of the 'hydrogen belts', i.e., the membrane strata occupied by hydrogen bond acceptors (CO of phospholipids) and donors (OH of cholesterol, sphingosine) and thereby facilitates the formation of dehydrated $Ca(PA)_2$, the ionophoric vehicle; or (our preferred explanation) PC engages in a (non-ionophoric) Ca(PA · PC) complex and thus reduces the concentration of the ionophore, while cholesterol competes with Ca^{2+} for the CO groups of phosphatidic acid by hydrogen-bonding. The Ca^{2+}-traversal rates realized in bilayers with modified hydrogen belts lend support to the speculation that a $Ca(PA)_2$ ferry may be of physiological importance, e.g., in membranes (such as myelin) containing much ether phospholipid (plasmalogen); and that Ca^{2+}-membrane association and traversal may be controlled by the composition of the hydrogen belts.

Membrane lipids fall into different classes not only according to their hydrophobic (e.g., fatty acids, sterols) and hydrophilic (e.g., phosphorylcholine, galactose) parts, but also according to the composition of their 'hydrogen belts' [1,2], i.e., the regions containing the lipid CO and OH groups. Little is known about reason and purpose of hydrogen belt variations, but their importance is demonstrated by reports on lipid-lipid (cholesterol to lysophosphatidylcholine) [3] and lipid-protein hydrogen bonding (lysophosphatidylcholine to glucose-6-phosphatase; diacylglycerol to protein kinase C) [4,5]. Here we report that phosphatidic acid-mediated traversal of membranes by Ca^{2+} depends dramatically on the composition of the hydrogen belts.

Phosphatidic acid alone among phospholipids can transport Ca^{2+} ion through a lipid bilayer [6–9], the vehicle being dehydrated $Ca(PA)_2$ [6]. The cation is held in a coordination complex with participation of the lipid CO oxygens: if these are eliminated, as in diether-PA, the rate of ionophoresis drops by 95% [6]. But not only the calcium-carrier, phosphatidic acid, also the liposomal matrix phospholipid influences Ca^{2+} traversal. On testing diether-PC (i.e., phosphatidylcholine without CO groups) as a matrix, we observed that the Ca^{2+} traversal rate (and, by implication, $Ca(PA)_2$ formation) increased 100-fold over the rate obtained in a PC matrix. An entirely different bilayer system [10] consisting of OH-blocked cholesterol, free of CO, OH, or charged groups,

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Abbreviations: PA, phosphatidic acid; PC, phosphatidylcholine; PS, phosphatidylserine.

TABLE I

RATES OF TRANSFER OF Ca^{2+} THROUGH BILAYERS OF LIPOSOMES CONTAINING 10 MOL% PHOSPHATIDIC ACID

Egg phosphatidylcholine (PC) was isolated by the method of Singleton et al. [14]. Dietherphosphatidylcholine (diether-PC), i.e., 1-hexadecanyl-2-oleyl-*sn*-glycerophosphocholine, was synthesized [15]. Phosphatidic acid (PA) was prepared from egg PC [16]. 3-*O*-Methoxyethoxyethoxyethylcholesterol (OH-blocked cholesterol) was synthesized [17]. Cholesterol-*O*-methyl ether purchased from Sigma. All lipids were pure as judged by thin-layer chromatography. Arsenazo-III dye purchased from Sigma was purified [18]. Chloroform/methanol (1 : 1) solutions of 50 μmol lipid were evaporated under vacuum at 70°C for 1 h while flushed with N_2 every 10 min. The lipid film was dispersed in 2 ml of 72.5 mM NaCl/72.5 mM KCl/5 mM Hepes/Tris buffer (pH 7.45) containing 40 μmol Arsenazo-III. Small liposomes were prepared by sonication under Argon to clearing, which was achieved in 30 (for PC) to 240 min (for cholesterol liposomes). Liposomes with trapped dye were isolated on Sepharose 4B [18]. Phosphorus and cholesterol were estimated by the methods of Marinetti [19] and Zlatkis et al. [20]. To a cuvette containing 3.0 μmol liposomal lipid, with trapped dye, in buffer (total volume 2.0 ml) were added 30 μl of 0.203 M $CaCl_2$ (final $[Ca^{2+}] = 3$ mM) and the increase of optical absorbance at 650 nm was recorded continuously. The increase of absorbance by light scattering due to the coagulation of liposomes was determined under identical conditions except for the omission of the dye, and subtracted. The rate of Ca-Arsenazo III complex formation was quantitated from the initial slope of absorbance with a molar extinction coefficient [7] of $2.06 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$. Surface factor, *F*. For comparison, Ca^{2+}-traversal rates have to be reduced to the same phosphatidic acid concentration; this varies between the different liposomes. In our assay, only the phosphatidic acid on the outside takes part in complexing calcium, and the outside fraction of total phosphatidic acid depends on the curvature, or diameter, of the vesicle. Further correction is necessitated by the so-called condensation effect of cholesterol in a bilayer [21,22] which will tend to increase the concentration of phosphatidic acid (at constant 10 mol%). Finally, this increased concentration must be squared because complex formation is second order regarding phosphatidic acid. From data available elsewhere [3,10] the correction factors can be calculated: 0.35 for the diether-PC/cholesterol liposomes;0.23 for the all-cholesterol liposomes. These controls were performed: leakage of Ca^{2+} (in above liposomes without phosphatidic acid, or with 10 mol% PS instead of phosphatidic acid; leakage of dye, or Ca^{2+}-dye complex, from all liposomes with and without phosphatidic acid, by change of absorbance on addition of EDTA [9], and by removal of liposomes by Sepharose separation after 2 h incubation with Ca^{2+}. All these controls were negative. PA, phosphatidic acid; PC, phosphatidylcholine; blocked cholesterol, *O*-methyoxyethoxyethoxyethylcholesterol.

	Composition of liposomes: 10 mol% phosphatidic acid, 90 (45/45) mol% of lipids as listed	mmol Ca^{2+} transported $\cdot min^{-1} \cdot (mol\ PA)^{-1}$	*F*	mmol Ca^{2+} transported $\cdot min^{-1} \cdot (mole\ PA)^{-1} \times F$	% inhibition
A	Diether-PC	27.8 ± 2.3 (4)	1	27.8 ±2.3	–
B	*O*-Methylcholesterol/ blocked cholesterol	140.4 ±27.6 (5)	0.23	32.2 ±6.4	–
C	PC	0.27± 0.03 (5)	1	0.27±0.03	99
D	Diether-PC/cholesterol	25.5 ± 6.9 (4)	0.35	8.9 ±2.4	68
E	Blocked cholesterol/ cholesterol	25.6 ± 2.8 (4)	0.23	5.9 ±0.6	78

generated the same high activity. In both systems, free cholesterol depressed traversal rates by 70%.

The results are collected in Table I. 'Passive' bilayers, which contain no CO or OH groups in their hydrogen belts (other than the CO groups of the ionophore, PA), show the highest Ca-transfer rates (Table I, A and B). When CO groups are introduced to the bilayer matrix, by replacing diether-PC with normal PC, inhibition of 99% results (Table I, C). Calcium traversal is also influenced by the OH group of cholesterol in the membrane; replacement of the *O*-methylcholesterol of Table I, B by cholesterol results in 78% inhibition (Table I, E). Similar inhibition by cholesterol, 68%, is exhibited in the diether-PC/cholesterol bilayer (Table I, D). The inhibition is not due to an increase in the viscosity of the bilayer; though viscosity will increase from A to D, it does not from B to E (Table I), since the bilayer cores are identical.

The similarity of traversal rates obtained in two entirely different 'passive bilayer' systems, and the equal degree of inhibition by cholesterol in both systems, suggest that in these bilayers maximal Ca-traversal rates have been attained, i.e., that these matrices serve as mere solvents without in-

fluencing the formation of the $Ca(PA)_2$ complex.

The highest Ca-traversal rate in diether-PC is obtained with calcium at 3–5 mM concentration; higher concentrations of Ca^{2+} give partial inhibition (Fig. 1). In the PC-matrix, on the other hand, rates increase up to about 7 mM Ca^{2+} and then drop very slowly. At all Ca^{2+} concentrations up to 20 mM the rates are considerably higher in diether-PC than in PC. The apparent K_m for $Ca(PA)_2$ is 2 mM Ca^{2+} in diether-PC, 6 mM Ca^{2+} in PC. A number of possible explanations come to mind regarding these differences in Ca-dependence (e.g., phase separation of phosphatidic acid; involvement of the phosphocholine group), but none of them is convincing; Fig. 1 remains, for now, partly unexplained. It should be noticed that the Ca-concentrations which cause inhibition are considerably above physiological (mammalian extracellular) levels, about 3 mM [11]. Fig. 1 does show the large difference, two orders of magnitude, between calcium transport rates in the bilayer systems below a calcium concentration of 5 mM; a difference created by presence or absence of the CO groups of the matrix lipid.

We can assume that Ca^{2+}-traversal rates are a measure of the concentration of the ionophore, $Ca(PA)_2$, in the membrane. Formation of this complex requires dehydration of the headgroup of phosphatidic acid as well as of the Ca ion [6]. Removal of CO and OH groups from the membrane matrix may lead to a dehydration of the hydrogen belts and thus facilitate the dehydration steps leading to formation of the complex. We believe, however, that a more distinct explanation can be provided by postulating the existence of a Ca(PA · PC) complex. Such mixed Ca(phospholipid)$_2$ complexes have been proposed [12] and evidence for Ca(PA · PC) and several similar complexes has recently been supplied by Reusch [13]. In a passive bilayer, all phosphatidic acid is engaged in $Ca(PA)_2$ formation; in a bilayer containing PC, the species Ca(PA · PC) will also be present. Assuming random pairing of these phospholipids we find that in our system – with 10% phosphatidic acid and 90% PC – there would be only one-tenth of the total phosphatidic acid available to associate with another phosphatidic acid. Since Ca(PA · PC), because of the hydrophilic choline headgroup, is not a membrane-crossing ionophore, and since $Ca(PA)_2$ formation is second order regarding phosphatidic acid, we can expect a 100-fold decrease of the Ca-traversal rate: precisely the experimental finding (Table I, A and C).

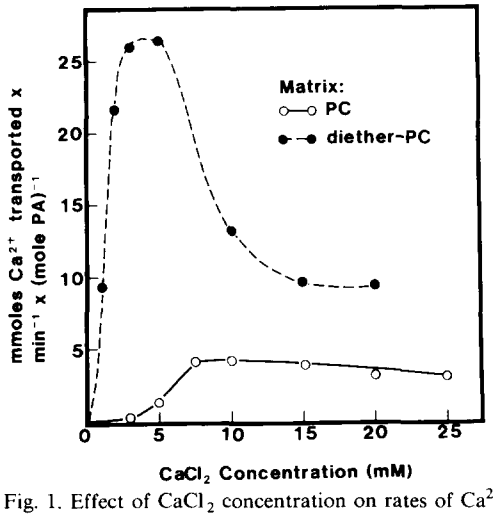

Fig. 1. Effect of $CaCl_2$ concentration on rates of Ca^{2+} traversal in liposomes containing 10 mol% PA and 90 mol% matrix lipid (diether-PC or PC). Conditions as in Table I.

The inhibitory effect of the cholesterol OH group (Table I, D and E) is most simply explained by its competing with Ca^{2+} for the CO group oxygens of phosphatidic acid, by virtue of hydrogen-bonding.

In summary, the results underline the importance of the 'hydrogen belt' composition of bilayers. The rates of Ca^{2+}-traversal in the "passive" bilayers (11 min for 0.5 mol Ca/mol external phosphatidic acid) are high enough to bolster the speculation that in biological membranes with special hydrogen belt structures (e.g., myelin) a $Ca(PA)_2$ ferry might be of physiological significance, and that the natural variation of membrane lipid hydrogen belts (e.g., compare PC, plasmalogen, sphingolipid) might have the function of controlling the passage and storage of calcium.

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