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CLUSTERING OF FATTY ACIDS IN PHOSPHOLIPID BILAYERS

H. HAUSER and W. GUYER

Eidgenössische Technische Hochschule, Laboratorium für Biochemie, ETH-Zentrum, CH-8092 Zürich (Switzerland)

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Summary

From electrophoresis experiments it is concluded that acidic phospholipids incorporated in liquid crystalline phosphatidylcholine bilayers at neutral pH are randomly distributed. The same is true for spin-labelled fatty acids. In contrast, long chain fatty acids are not fully ionized at neutral pH and appear to be clustered, i.e. they segregate out into patches. Only at pH>11 is the fatty acid-COOH group fully ionized and charge repulsion leads to a random distribution of the fatty acid within the plane of the bilayer.

There is unequivocal evidence for the lateral diffusion of membrane components being very fast [1,2]. Consequently the components of bilayers and biological membranes can be expected to interact so as to form clusters or patches of specialized regions. This phenomenon of clustering has been observed with a variety of mixed lipid systems [3—12]. Here we discuss the lateral distribution (mixing) of fatty acids in liquid crystalline egg phosphatidylcholine bilayers. This is important from the point of view that fatty acdis as intermediates of phospholipid metabolism are present though to a small extent in natural membranes and have been reported to affect the permeability and other membrane functions [13]. Furthermore deuterated and spin-labelled fatty acids have been widely used to probe the molecular organisation and motion of phospholipid bilayers and membranes.

Dispersions of pure lipids were made as described before [14]. Electrophoretic mobilities were measured in a commercially available particle electrophoresis apparatus (Rank Brothers, Cambridge, U.K.), and the surface chemical techniques were described previously [14—16].

Fig. 1 shows that incorporation of acidic lipids in egg phosphatidylcholine bilayers produced an increase in the negative surface charge density as

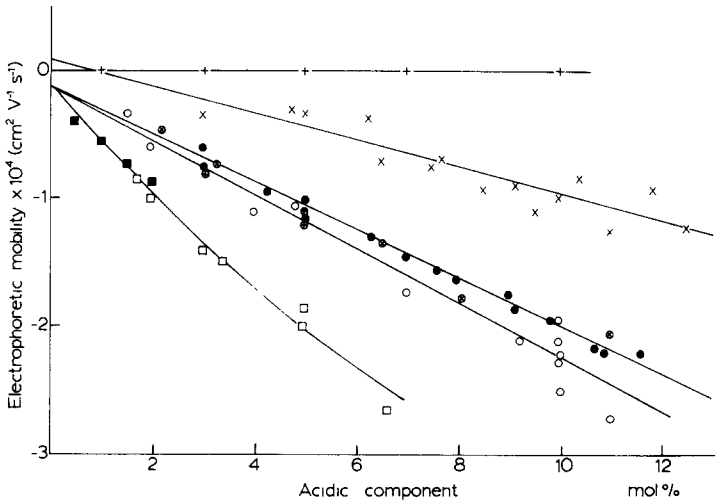


Fig. 1. Electrophoretic mobility (in $cm^2 \cdot V^{-1} \cdot s^{-1}$) of egg phosphatidylcholine liposomes containing acidic lipids. Chromatographically pure phosphatidylcholine and acidic lipids were mixed in $CHCl_3$/MeOH = 2:1 (by vol.) and dispersed in 0.025 M NaCl to which NaOH was added to adjust the pH or in 0.025 M NaCl, 5 mM sodium phosphate pH 8.0 as described before [14], +—+, stearic acid pH 5.5; x—x, stearic acid pH 8.1; ⊗—⊗, stearic acid pH 11.6; ○—○, phosphatidylserine from bovine spinal cord; ●—●, phosphatidylinositol extracted from frozen peas; □—□, cardiolipin from beef heart; ■—■, phosphatidic acid derived from egg phosphatidylcholine by phospholipase D treatment. The solid lines represent least squares fits to the experimental points.

evident from the increase in the electrophoretic mobility (ζ-potential). As shown before the electrophoretic mobility of pure phosphatidylcholine liposomes was zero over a large pH-range [17, 18]. The increment in electrophoretic mobility given by the slope of the linear relationship at low surface charge density (Fig. 1) is summarized in Table I. It is seen that the increment is related to the number of net negative charges per phospholipid molecule. Phosphatidylserine which is known to have one charge under the conditions of our measurements [19] gave an increment of —0.23 whereas phosphatidic acid or cardiolipin with two charges gave an increment of —0.42 (Table I). While the increment produced by spin-labelled fatty acids was in good agreement with that observed with negatively charged phospholipids, fatty acids such as stearic acid or oleic acid behaved differently. At pH 5.5 they did not impose a negative mobility on phosphatidylcholine bilayers and at pH$\approx$8 the increment in electrophoretic mobility was —0.11, i.e. about half of that measured for negatively charged phospholipids. Only at a pH>11 did the increment in electrophoretic mobility become equivalent to that of phospholipids with one net negative charge (Fig. 1, Table I).

Fig. 2 illustrates the difference in binding of $^{45}Ca^{2+}$ to close-packed monolayers of arachidic acid (area/molecule$\approx$20 Å^2) and to monolayers of phosphatidylserine (area/molecule of $\approx$70 Å^2). While phosphatidylserine was fully ionized at pH$\approx$6 [14, 18, 19] and the amount of $^{45}Ca^{2+}$ bound per phosphatidylserine reached a plateau at pH$\approx$7, arachidic acid was hardly ionized and hence $^{45}Ca^{2+}$ binding insignificant in that pH-range. The dissociation of the fatty acid -COOH group (and hence $^{45}Ca^{2+}$ binding) did not become appreciable until the pH rose above 9.5. Only then did the quantity of $^{45}Ca^{2+}$ bound

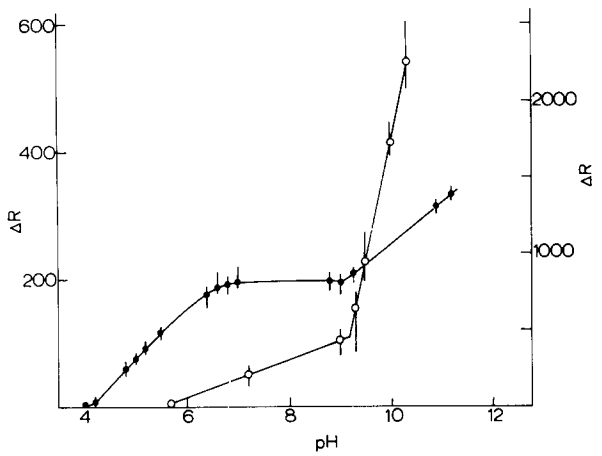


Fig. 2. $^{45}Ca^{2+}$ binding to monolayers of ox brain phosphatidylserine (monosodium salt) (●——●) and arachidic acid (○——○) as a function of pH. The bars represent the spread of 3—6 experiments. Monolayers were spread on 0.01 M NaCl solution containing 0.13 μ M $^{45}CaCl_2$ (specific activity = 1.56 kCi/mol) to a surface pressure π = 12 mN•m^{-1} as described in Ref. 19. Surface radioactivities R (in arbitrary units) were measured as described in Refs. 14—16.

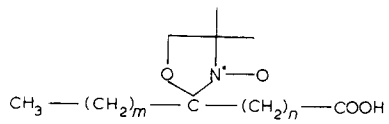
per fatty acid molecule approach the $^{45}Ca^{2+}$ / phosphatidylserine molar ratio measured at pH≈7.0. Consistent with the monolayer results of pure fatty acids is the finding that mixed monolayers of phosphatidylcholines and fatty acids failed to bind $^{45}Ca^{2+}$ at a pH≈7. This was true for monolayers of dipalmitoyl phosphatidylcholine containing palmitic, stearic or oleic acid and monolayers of dioleoyl phosphatidylcholine containing stearic or oleic acid up to molar ratios of about 1. Thus the pH-dependence of the interaction of $^{45}Ca^{2+}$ with close-packed monolayers of fatty acids parallels the pH-dependence of the electrophoretic mobility of mixed fatty acid-phosphatidylcholine particles (bilayers).

The experimental results presented above can be rationalized as follows. It has been shown [6, 7] that phosphatidylserine above its transition temperature is randomly distributed within liquid crystalline phosphatidylcholine bilayers. Hence its increment in electrophoretic mobility of —0.23 (Table I) is characteristic for a phospholipid with one net negative charge which is randomly distributed within the plane of the bilayer and evenly distributed over all layers constituting the multilamellar structure of phosphatidylcholine liposomes. Based upon measurements of the increment in electrophoretic mobility it can be concluded that the phospholipids and spin-labelled fatty acids of Table I are fully ionized at pH 8 and randomly distributed. Our electrophoresis experiments confirm the random distribution of spin-labelled fatty acids in phosphatidylcholine bilayers which has been established unequivocally from electron spin resonance work. The evidence is based on the fact that at molar ratios phospholipid/label>100 line broadening due to spin exchange is negligible [26].

On the other hand fatty acids incorporated in phosphatidylcholine bilayers are not fully ionized between pH 6—11 and behave as if present as a monolayer [20—25]. The parallel behaviour of long chain fatty acids in pure monolayers and when incorporated in bilayers leads us to believe that long chain fat-

TABLE I

Acidic lipids were mixed with egg phosphatidylcholine in $CHCl_3/CH_3OH = 2:1$ (by vol.) and dispersed in 0.025 M NaCl, 5 mM sodium phosphate, pH 8.0, as described before [14]. Alternatively 0.025 M NaCl was used and the pH adjusted by adding NaOH. The concentration of phosphatidylcholine was 0.2 mg/ml = $2.67 \cdot 10^{-4}$ M. Spin-labelled fatty acids are defined by the numbers m, n as follows:



Acidic lipid added to phosphatidylcholine	pH	Increment in electrophoretic mobility* (± S. D.)	Maximum number of net negative charges/molecule
Phosphatidylserine	8.1	−0.23 ± 0.02	1
Phosphatidylinositol	8.0	−0.20 ± 0.01	1
Phosphatidic acid	8.0	−0.42 ± 0.02	2
Cardiolipin	6.0	−0.42 ± 0.01	2
Stearic acid	5.5	0	
Stearic acid	8.1	−0.105 ± 0.02	
[$^2H_{35}$] Stearic acid (perdeuterated)	8.0	−0.10 ± 0.02	
Stearic acid	11.6	−0.22 ± 0.01	
Palmitic acid	8.0	−0.11 ± 0.01	
[2, 2 - $^2H_2$] Palmitic acid	8.0	−0.125 ± 0.01	1
Oleic acid	8.0	−0.12 ± 0.01	
Spin-labelled fatty acid (12, 3)	8.0	−0.23 ± 0.01	
(5, 10)	8.0	−0.23 ± 0.02	
(1, 14)	8.0	−0.23 ± 0.02	

*The increment in electrophoretic mobility (in $cm^2 \cdot V^{-1} \cdot s^{-1}$ per unit concentration of acidic lipid) is derived from the initial slope (as the concentration of acidic lipid goes to zero) of the linear relationship of electrophoretic mobility vs concentration of acidic lipid (see Fig. 1).

ty acids dissolved in bilayers are at least partially present as patches of monolayers, i.e. they segregate into patches (clusters). A predominantly random distribution in the partially undissociated form can be ruled out because it has been shown that the pK of single carboxyl groups in solution as well as in expanded monolayers at approx. 70 Å^2 /-COOH group [19] is between 4—5. Only at a pH>11 appear the fatty acid-COOH groups to be fully ionized, and charge repulsion causes then the clusters to disperse producing a random distribution. The difference in behaviour of spin-labelled and ordinary fatty acid is probably due to the bulky oxazolidine group in the hydrocarbon chain not allowing for close packing of the molecule (cf. work by Cadenhead et al. [27]). The finding that oleic acid, which is liquid at the temperature of our experiment behave similarly to stearic acid emphasizes the role the fatty acid-COOH groups play in clustering. The close alignment of fatty acid-COOH groups allowing for intermolecular hydrogen bonds to be formed may be responsible for the stabilisation of fatty acid clusters and may compensate for the entropy decrease accompanying the lateral phase separation.

Deuterated fatty acids together with deuteron magnetic resonance have been used to probe the structure and dynamics of phospholipid bilayers and membranes. Their use as probes is based on the assumption that they are randomly distributed within the bilayer causing minimal perturbation of the environment that they are meant to probe. From Table I it is clear that deute-

rated fatty acids behave like ordinary fatty acids, i.e. that they are clustered and not randomly distributed. In this case, the physicochemical properties of the probe will mainly or partly be determined by probe-probe interactions depending on the size of the cluster.

We can conclude that at physiological pH and above the T_c negatively charged phospholipids and spin-labelled fatty acids are randomly distributed in the plane of phosphatidylcholine bilayers. This is contrasted by the behaviour of long chain fatty acids which are clustered under these conditions. The effects of fatty acids upon membrane structure and function need to be reexamined in the light of clustering. Furthermore, the usefulness of deuterated fatty acids as molecular probes of the bilayer structure has to be tested carefully.

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