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Lipid composition of functional domains of the lymphocyte plasma membrane

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Plasma membrane vesicles from calf T-lymphocytes were fractionated by affinity chromatography on Con A-Sepharose. One subfraction eluted freely from the affinity column (fraction 1), while a second one adhered specifically to the column (fraction 2). While both fractions were derived exclusively from the plasma membrane, fraction 2 carried the high-affinity receptor for the mitogen concanavalin A and was distinct from fraction 1 with respect to its polypeptide pattern and the content of some plasma membrane-associated enzymes, suggesting the existence of functional plasma membrane domains. These functionally distinct fractions showed different lipid composition. The adherent fraction was enriched in phosphatidylcholine, while the relative amount of phosphatidylethanolamine and phosphatidylserine was reduced. Furthermore, the relative amount of saturated fatty acids was enhanced in the phospholipids of the adherent plasma membrane fraction. This could be shown in total phospholipids, as well as in separated individual phospholipids. We could therefore demonstrate that lipid heterogeneity may exist in plasma membranes of cells without structural polarity. Similar results were obtained when T-lymphocytes were stimulated with the mitogen concanavalin A. The functional domain, consisting of the high-affinity concanavalin A receptor, several enzymes and distinct lipid compositional pattern, thus seems to constitute a relatively stable structural entity of the lymphocyte plasma membrane.

Introduction

Plasma membrane vesicles derived from calf thymocytes can be separated into two fractions by affinity chromatography on Con A-Sepharose [1]. Approx. 65% of the plasma membrane vesicles, which represent the bulk membrane, do not bind to Con A-Sepharose (designated fraction 1). About 25% of the vesicles do bind and contain high-affinity receptors for Con A [2]. It could be shown that the latter membrane fraction (fraction 2) is functionally distinct from the rest of the membrane. It is enriched in certain plasma membrane-bound enzymes, such as $(Na^+ + K^+)$-ATPase and lysophosphatide acyltransferase. Both enzymes are activated in the early phase of lymphocyte stimulation by mitogens. It was concluded that a close association existed between the high-affinity concanavalin A receptor and these enzymes, constituting a functional domain implicated in the activation of lymphocytes [3]. The in vitro activity of both enzymes may be modulated by changes in the fatty acid composition of the membrane phospholipids [4]. Analysis of the lipid composition of fraction 2 should thus clarify, whether a particular

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

lipid environment in the receptor domain is present.

In addition, it was of general interest to analyse, whether specific plasma membrane domains may demonstrate distinct phospholipid and fatty acid patterns. Heterogeneity of plasma membrane proteins is a well-documented fact and has been shown in murine T-lymphocytes [5] as well as in plasma membrane vesicles separated on Con A-Sepharose from fibroblasts [6].

However, there are minimal data available concerning the fine structure of plasma membrane lipids. Lipid domains were proposed to exist in platelet [7] and erythrocyte [8] plasma membranes as regions with different susceptibility to the action of phospholipases. Heterogeneity in anionic lipids could be detected by freeze-fracture cytochemistry, but could not be correlated with function [9]. Functional as well as structural heterogeneity was observed in liver cells, where the domains correlate with the orientation in the organ [10]. Plasma membrane vesicles from fibroblasts fractionated by Con A-Sepharose were found to be homogenous with respect to phospholipid and fatty acid composition [6].

In the present paper, we demonstrate that lipid heterogeneity does exist in the plasma membranes of lymphocytes, which represent cells without an apparent structural polarity.

Materials and Methods

Lymphocytes and plasma membranes: Affinity chromatography of plasma membrane vesicles. T-lymphocytes were prepared from calf thymus. A portion of the cells was incubated for 1 h in Hepes-buffered RPMI-1640 (Seromed) ($5 \cdot 10^7$ cells/ml) and stimulated with 5 μg/ml concanavalin A (Pharmacia). Cells were disrupted by the nitrogen cavitation method; plasma membranes were purified by differential centrifugation as described previously [11]. Membrane fractions were obtained by affinity chromatography on Con A-Sepharose (Pharmacia) [12].

Lysolecithin acyltransferase (Acyl-CoA : 1-acylglycero-3-phosphocholine O-acyltransferase, EC 2.3.1.23). To prevent inactivation of the enzyme on the affinity column, fractionation and all subsequent steps were done in the presence of 1 mM dithiothreitol. The enzymatic activity was determined as described in Ref. 2 with [^{14}C]-lysophosphatidylcholine and arachidonoyl coenzyme A as substrates. Protein was measured by its native fluorescence [13].

Phospholipids. Lipids were extracted as previously described [14]. The phospholipid composition was determined by one-dimensional thin-layer chromatography (TLC) and subsequent densitometric quantitation with cupric sulfate [14].

Fatty acid determination. Phospholipids were separated from neutral lipids by TLC on silica-gel plates (Schleicher and Schüll) with the solvent system hexane/ diethyl ether/ acetic acid (80 : 20 : 2). The phospholipids remain at the application site. They were scraped off and transmethylated in the presence of silica gel. The individual phospholipids were separated using the solvent system chloroform/ methanol/ acetic acid/0.9% NaCl [14]. To visualize the separated phospholipids, the plates were sprayed with a 10% solution of the fluorescence dye 1,6-diphenylhexatriene (Aldrich Chemicals) in petroleum ether [15]. Lipid spots were scraped off and transmethylated in the presence of silica gel. Transmethylation was performed with sodium methylate as described in Ref. 16. The fatty acid methylester was analysed by capillary gas-liquid chromatography (GLC) (Fractovap 4160, Erba Strumentazione, FFAP column (Jaeggi, Trogen, Switzerland), or Fractovap 2150, Erba Strumentazione, Sil 88 column (Chrompack), carrier gas H_2). Peaks were identified by standard fatty acid methylesters. Some peaks were only partially characterized by reduction with H_2; by this method the number of carbon atoms but not the number of double bonds could be determined. The peak area was calculated by a computer program from Spectra Physics.

Cholesterol. Cholesterol was determined with a test combination kit from Boehringer, Mannheim, as described [11].

Results

Phospholipid composition

The phospholipid composition of purified plasma membranes of calf thymocytes is shown in Table I. The data are in close aggreement with those reported by Ferber et al. [17] and similar to

TABLE I

PHOSPHOLIPID COMPOSITION OF PLASMA MEMBRANES AND SUBFRACTIONS

Phospholipids were separated by one-dimensional TLC and quantitated on the plate after spraying with cupric sulfate. Values are expressed as mol%. Data are means of two independent cell preparations, each measured in duplicate. Δ = mol% fraction 2 − mol% fraction 1.

	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-inositol	Phosphatidyl-serine	Sphingo-myelin
Control					
Plasma membrane	53.1 ± 0.5	23.5 ± 0.6	5.1 ± 0.8	10.0 ± 0.1	8.3 ± 1.7
Fraction 1	52.9 ± 1.0	24.7 ± 0.4	6.4 ± 0.5	9.5 ± 0.6	6.7 ± 1.5
Fraction 2	59.3 ± 1.2	20.5 ± 0.1	6.9 ± 0.1	6.5 ± 1.6	6.8 ± 1.6
Δ	+7.2 ± 1.4	−3.5 ± 0.7	−0.1 ± 0.9	−3.5 ± 0.4	−0.2 ± 0.5
Concanavalin A					
Plasma membrane	54.1 ± 1.2	22.2 ± 2.1	8.7 ± 1.2	9.2 ± 0.7	5.8 ± 1.0
Fraction 1	51.2 ± 0.7	25.2 ± 1.9	7.8 ± 0.4	9.0 ± 0.4	5.5 ± 0.9
Fraction 2	61.9 ± 1.9	17.8 ± 2.7	8.2 ± 0.1	7.1 ± 0.4	5.1 ± 1.3
Δ	+7.6 ± 1.8	−6.5 ± 0.1	+0.1 ± 0.2	−1.9 ± 0.1	−0.5 ± 0.3

those reported by Koizumi et al. [18] and Van Blitterswijk et al. [19] for the plasma membranes of mouse thymocytes. When the plasma membranes were fractionated on Con A-Sepharose, the phospholipid composition of fraction 1, the non-adherent fraction, closely resembled the total plasma membrane. This was expected, as this fraction comprises about 75% of the total plasma membrane. Fraction 2, the concanavalin A-binding fraction, however, showed a marked difference in the phospholipid composition (Tables I and II): most prominent was an increase in the phosphatidylcholine content by 7.2 ± 1.4% (four independent preparations), representing a relative gain of nearly 15% in phosphatidylcholine. By way of calculation, the increase must be accompanied by a decrease in the other phospholipid species. This, however, was not random: the percentage of sphingomyelin and phosphatidylinositol was similar in both subfractions, while the percentage of phosphatidylethanolamine and phosphatidylserine was decreased in fraction 2. This indicates that the

TABLE II

FATTY ACID COMPOSITION OF TOTAL PHOSPHOLIPIDS FROM PLASMA MEMBRANES AND SUBFRACTIONS

Phospholipids were separated from neutral lipids by TLC and transmethylated with sodium methylate. Fatty acid methylesters were separated by capillary GLC. Abbreviations: 20 : n = 3 distinct fatty acids with 20 carbon atoms and more than one double bond; 22 : n = 4 distinct fatty acids with 22 carbon atoms and more than one double bond; 18 : 1_c and 18 : 1_t = double bond in *cis* and *trans* position, respectively.

	14:0	16:0	16:1	18:0	18:1_c	18:1_t	18:2	18:3	20:0	20:n	20:4	22:n
Control												
Plasma membrane	0.8	25.5	2.1	19.0	25.5	4.6	10.7	0.5	0.3	4.0	5.4	1.7
Fraction 1	0.9	24.8	1.3	19.7	25.2	4.5	10.9	0.3	0.4	4.0	6.1	1.9
Fraction 2	0.1	30.5	1.5	24.7	25.1	6.8	6.2	1.0	0.6	1.7	1.9	0.6
Concanavalin A												
Plasma membrane	–	23.0	1.7	18.1	26.8	6.4	9.9	0.3	0.4	4.7	7.5	1.5
Fraction 1	1.0	23.3	2.1	18.7	28.2	5.9	11.1	0.4	0.4	4.6	7.2	2.3
Fraction 2	0.9	23.2	1.7	24.7	26.4	6.1	8.2	0.7	1.2	3.7	3.4	–

phospholipid composition of the plasma membrane domain contained in fraction 2 is distinct from the rest of the plasma membrane.

When lymphocytes are incubated at 37°C with concanavalin A, the receptors redistribute in the plane of the membrane to form micropatches and eventually caps [20]. One might thus have expected that the formation of vesicles and the distribution of the receptor domains might differ from the control cells. However, the phospholipid pattern of fraction 1 and fraction 2 derived from concanavalin A-incubated cells was not significantly different from the pattern obtained from control cells.

The cholesterol-to-phospholipid ratio was measured in control cells. As could be shown before [2], there was a slight but not statistically significant decrease in fraction 2 (plasma membrane: 0.76 ± 0.09; fraction 1: 0.84 ± 0.06; fraction 2: 0.72 ± 0.12; data of two independent experiments).

Fatty acid composition of total phospholipids

Total phospholipids were separated from neutral lipids by TLC. Their fatty acid composition was determined by GLC and is depicted in Table II. It is in accordance with the composition reported by Ferber et al. [17]. For ease of comparison, the fatty acid composition is characterized by the percentage of the main saturated fatty acids palmitic acid (16:0) and stearic acid (18:0). As can be seen clearly from Table III, there is a marked increase in saturated fatty acids in the phospholipids extracted from fraction 2.

The fatty acid composition of the phospholipids of plasma membranes derived from cells incubated with concanavalin A was similar to that of the control cells.

One might have expected some differences because the enzyme responsible for the fatty acid modulation of phospholipids in the 2-position, lysophosphatide acyltransferase, is activated in the cells incubated with concanavalin A and is localized predominantly in fraction 2 (Table IV and Ref. 2). 1 h, however, is too short a time period to detect changes due to the selective incorporation of polyunsaturated fatty acids by this enzyme, particularly when the fatty acids of both positions of the total phospholipids are analyzed.

Phosphatidylcholine is a phospholipid species containing a rather large portion of saturated fatty acids. As this phospholipid is enriched in fraction 2, one might raise the question that the changes in the fatty acid composition were due to the increase in the relative amount of phosphatidylcholine. Therefore, it was necessary to analyze the fatty acid composition of individual phospholipid species.

TABLE III

COMPARISON OF THE FATTY ACID COMPOSITION OF TOTAL PHOSPHOLIPIDS FROM PLASMA MEMBRANES AND SUBFRACTIONS

Values are the percentage of the saturated fatty acids (palmitic acid 16:0 and stearic acid 18:0). Data are means of four or five independent cell preparations.

	Control	Concanavalin A
Plasma membrane	41.1 ± 1.5	41.0 ± 2.6
Fraction 1	43.5 ± 2.7	40.0 ± 2.0
Fraction 2	51.4 ± 4.8	47.1 ± 1.9
Difference (fraction 2 − fraction 1)	8.8 ± 2.0	7.2 ± 1.0

TABLE IV

SPECIFIC ACTIVITY OF THE LYSOPHOSPHATIDE ACYLTRANSFERASE

The enzymatic activity (nmol/mg protein per min) was measured with [^{14}C]lysophosphatidylcholine and arachidonoyl coenzyme A as substrates.

	Control	Concanavalin A
Plasma membrane	17.7	24.8
Fraction 1	5.3	9.7
Fraction 2	21.8	42.9

Fatty acid composition of individual phospholipids

The individual phospholipid species were separated by one-dimensional TLC and the fatty acid composition of the main components phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine was determined (Table V). In phosphatidylethanolamine, a high percentage of polyunsaturated fatty acids was found. It was especially high because the plasmologen fraction, which comprises about 30% of the phosphatidylethanolamine in lymphocytes, was not separated by the method used. With our method, only the acyl fatty acid chains were

TABLE V

FATTY ACID COMPOSITION OF INDIVIDUAL PHOSPHOLIPIDS FROM PLASMA MEMBRANES FROM CALF THYMOCYTES

Data are means of two independent preparations, each analyzed in duplicate.

	14:0	16:0	18:0	$18:1_c$	$18:1_t$	18:2	18:3	20:0	20:*n*	20:4	22:*n*	polyenoic / saturated
Phosphatidylcholine												
Control	1.4	40.4	8.7	28.4	5.8	11.5	0.6	–	2.1	1.7	–	0.32
Concanavalin A	1.2	38.7	10.0	26.6	4.9	13.2	0.5	–	2.3	2.1	0.2	0.37
Phosphatidylethanolamine												
Control	–	7.6	15.9	28.6	3.9	12.5	0.3	0.5	7.5	15.1	7.8	1.82
Concanavalin A	–	5.8	17.7	27.9	3.3	12.1	–	–	7.5	16.6	8.9	1.92
Phosphatidylinositol												
Control	–	4.4	38.7	22.4	5.2	4.8	–	–	9.9	14.4	–	0.68
Concanavalin A	–	3.6	37.5	22.1	3.7	5.6	–	–	9.3	17.4	–	0.79
Phosphatidylserine												
Control	–	2.0	52.4	27.8	–	5.5	0.4	1.0	4.8	5.1	0.5	0.30
Concanavalin A	–	2.7	50.3	32.4	–	6.2	0.2	0.7	3.3	3.9	–	0.26

transmethylated, not the alkenyl or ether side-chains. Therefore, acylchains from position 2, which are generally more unsaturated than the acylchain from position 1 in the phospholipids of lymphocytes [21], were over-represented.

As the amount of fractionated plasma membrane was limited, the fatty acid composition of only the two main phospholipid species, phosphatidylcholine and phosphatidylethanolamine, was analyzed in the subfractions 1 and 2 (Table VI). A comparison of the two plasma membrane fractions shows that in fraction 2 both phospholipid species were enriched in saturated fatty acids and diminished in polyunsaturated fatty acids.

It could thus be clearly demonstrated that the different fatty acid composition measured in the

TABLE VI

COMPARISON OF THE FATTY ACID COMPOSITION OF PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE FROM PLASMA MEMBRANES AND SUBFRACTIONS

	Phosphatidylcholine		Phosphatidylethanolamine	
	control	concanavalin A	control	concanavalin A
Percentage of saturated fatty acids [a]				
Plasma membrane	54.3	57.2	32.4	32.1
Fraction 1	56.3	52.6	30.9	28.7
Fraction 2	66.8	63.5	38.2	39.9
Δ [b]	10.1 ± 4.7	8.5 ± 1.6	9.3 ± 4.4	11.1 ± 0.6
Percentage of polyunsaturated fatty acids [a]				
Plasma membrane	17.3	15.7	45.6	44.3
Fraction 1	20.4	16.8	42.3	44.8
Fraction 2	12.6	11.4	34.7	28.9
Δ [b]	-5.8 ± 1.3	-5.3 ± 2.1	-10.0 ± 2.9	-21.9 ± 4.3

[a] Data are means of a typical experiment.

[b] Data (% fatty acid in fraction 2 − % fatty acid in fraction 1) are means of three independent cell preparations.

total phospholipids was not solely due to an interspecies rearrangement, but was caused by an enrichment of phospholipids with a distinct fatty acid composition in the adherent fraction.

Discussion

The plasma membrane vesicles derived from thymocytes by the nitrogen cavitation method have been extensively characterized [1]. They are relatively small vesicles with an average diameter of about 70 nm [22]. Assuming that lymphocytes have a smooth surface, the number of vesicles derived from a single cell can be estimated to be 10000 as a minimum. In the literature, the reported number of lectin receptors per cell ranges between $0.5 \cdot 10^6$ and $10 \cdot 10^6$ molecules [20]. Even calculating with the low value, each vesicle may thus contain about 50 receptors for concanavalin A. It could be shown earlier that fractionation on Con A-Sepharose is due to two types of concanavalin A receptors, those with low affinity for concanavalin A in the nonadherent fraction and those with high affinity in the adherent fraction [2]. Both membrane fractions consist of pure plasma membranes oriented right-side-out [12]. This could be confirmed by protein analysis: both fractions show the same amount of actin as the plasma membrane but are distinct in their protein pattern (unpublished observations). Regarding concanavalin A-stimulated cells, it cannot be excluded that part of fraction 2 consists of plasma membranes internalized by the lymphocyte. As, however, fraction 2 derived from non-stimulated resting cells is not significantly different, it would implicate that preformed domains are internalized.

These plasma membrane domains thus characterized by function and protein pattern could now be shown to be structural domains with regard to lipid composition. We have shown by direct quantitative analysis that the concanavalin A-adherent fraction is enriched in phosphatidylcholine with a concomitant decrease in phosphatidylethanolamine and phosphatidylserine. The constant amount of sphingomyelin and phosphatidylinositol indicates that at least these three phospholipid species are involved in the composition of the domain. The plasma membrane vesicles obtained by the nitrogen cavitation method still contain a reasonable amount of bulk membrane. Thus, interpretation of the results should remain speculative. One may postulate, however, that a close association of certain phospholipids with the proteins of the concanavalin A receptor-enzyme complex may exist.

ATPases have been isolated from various cell types and require so called boundary lipids to regain enzymatic activity (reviewed in Ref. 23). In reconstitution experiments, a certain preference for phosphatidylserine could be shown, but other phospholipids were of similar efficiency. A direct analysis of the boundary lipids is presently not possible. Therefore, the slight decrease in phosphatidylserine observed in fraction 2 should not be interpreted as a contradiction to the reconstitution experiments, as the experimental conditions are too different.

With respect to the lysophosphatide acyltransferase, one could imagine that the incorporation of polyunsaturated fatty acids into the surrounding lipids may be more effective in a saturated phospholipid environment than in an already relatively unsaturated environment. In contrast to our findings, plasma membranes from fibroblasts fractionated on Con A-Sepharose showed different profiles of enzyme activities but no distinct lipid domains [6]. These data underline the idea that the lipid domains correspond to functional domains of lymphocytes, rather than simply binding sites for concanavalin A.

No statistically significant differences could be found between the plasma membrane fractions derived from concanavalin A-incubated cells and control cells. This indicates a stability of the proposed protein-lipid complex: the structural changes induced by concanavalin A on the plasma membrane of the thymocyte do not lead to a segregation of the receptor complex and the surrounding lipids. Slight differences could be observed between the adherent fractions MF 2, which, however, were not statistically significant. Although both adherent fractions are more saturated than the respective non-adherent fractions, fraction 2 from concanavalin A-incubated cells contained less saturated fatty acids and more polyunsaturated fatty acids than fraction 2 from control cells (Tables II and III). This effect may be ascribed to the enhanced activity of the lysophosphatide

acyltransferase, which is highly enriched in fraction 2 and shows a much higher activity in fraction 2 from concanavalin A-activated cells. The changes in the fatty acid composition of plasma membrane phospholipids are more pronounced after prolonged incubation of T-lymphocytes with concanavalin A (unpublished observations).

We could thus show that structural lipid domains exist in lymphocytes, a type of cell that does not show structural polarity. These structural domains are related to functional domains and may be important for the regulation of attached membrane proteins. Further research is necessary to confirm the hypothesis that lipid domains in general are of importance for the multiple functions of plasma membranes.

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