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Polyunsaturated fatty acids are enriched in the plasma membranes of mitogen-stimulated T-lymphocytes

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Plasma membranes were purified from T-lymphocytes from rabbit thymus stimulated with concanavalin A. Lipids were extracted and the fatty acid composition of the individual phospholipid species was determined by gas-liquid chromatography. Compared to the plasma membranes derived from control cells, the plasma membranes from mitogen-stimulated cells were enriched in polyunsaturated fatty acids. This increase in unsaturation was found in phosphatidylcholine, phosphatidylinositol and phosphatidylserine, while the fatty acid composition of phosphatidylethanolamine was not significantly altered. The phospholipid composition remained almost unchanged during the period of stimulation. The molar ratio cholesterol to phospholipid was decreased. These changes in the lipid composition of plasma membranes from mitogen-stimulated T-lymphocytes are discussed with regard to functional implications.

Introduction

Resting T-lymphocytes may be stimulated to proliferation and differentiation by mitogenic lectins such as concanavalin A. Immediately after binding of the mitogen to its receptor, multiple changes in the metabolism of the plasma membrane are observed, which may be attributed to the process of signal transmission across the membrane. Among these are alterations in the phospholipid metabolism (reviewed in Ref. 1): an increased turnover of phosphoinositides leads to the generation of two second messengers, inositol trisphosphate and diacylglycerol. Besides this, an increased turnover of long-chain fatty acids of phospholipids in the plasma membrane could be demonstrated [2,3], which may lead to an altered fatty acid composition of phospholipids in the plasma membrane and a concomitant change of physicochemical properties of the membrane. The fatty acid composition of the phospholipids has been shown to modulate, among others, the activities of enzymes which take part in the regulation of the ionic milieu of the cell [4,5]. Thus changes in the long-chain fatty acid composition may be involved in a long-lasting support of lymphocyte activation.

It could be shown that the reacylating enzyme,

Abbreviations: PL, total phospholipid; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethansulphonic acid.

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the lysophosphatide acyltransferase (acylCoA: 1-acylglycero-3-phosphatidyl-*O*-acyltransferase), was activated in T-lymphocytes only minutes after mitogen binding [6] and remained activated for several hours. The enzyme preferentially acylated unsaturated long-chain fatty acids to lysophospholipids [6]. This could also be demonstrated by studies on the incorporation of exogenous radioactive fatty acids into the lipids of T-lymphocytes: polyunsaturated fatty acids such as arachidonic acid or linoleic acid were incorporated more quickly than saturated fatty acids such as palmitic acid [2]. Activation of the lysophosphatide acyltransferase should thus lead to a change of the fatty acid composition of the plasma membrane phospholipids. This, however, cannot be shown with labelled fatty acids, because their distribution will possibly not reflect the complete plasma membrane but only those pools of fatty acids that are readily accessible to exogenous fatty acids. In the present study we therefore investigated in detail the fatty acid composition of the phospholipids from highly purified plasma membranes of resting and mitogen-stimulated T-lymphocytes.

Materials and Methods

Stimulation of T-lymphocytes

T-lymphocytes were prepared from the thymus of rabbits (New Zealand white, about 3 months old) as described previously [7]. The cells were suspended in Hepes-buffered RPMI 1640 (Gibco) in the presence or absence of concanavalin A (Pharmacia) for 4 h. Incubation conditions were chosen with respect to an optimal stimulation of the lysophosphatide acyltransferase, measured by an enhanced incorporation of exogenously added radioactive fatty acid. I: $5 \cdot 10^7$ cells/ml medium, 5 μg/ml concanavalin A; II: $2 \cdot 10^7$ cells/ml medium supplemented with 5 μg/ml bovine serum albumin, essentially fatty acid free, 2 μg/ml concanavalin A. Using these cell densities and concentrations of concanavalin A the cells were activated to proliferation as measured by the incorporation of radioactive uridine into RNA. In some experiments, the albumin containing medium was supplemented with 2.5 μg/ml (10 nmol/ml) palmitic acid and 1.25 μg/ml (4 nmol/ml) arachidonic acid [8]. Plasma membranes were prepared from the cells by nitrogen cavitation and subsequent differential centrifugation as described earlier [7]. These plasma membranes could be shown to be highly purified and almost devoid of intracellular membrane vesicles.

Depending on the rabbit, about $5 \cdot 10^9$ to $1.5 \cdot 10^{10}$ cells were obtained from one thymus. The yield of plasma membranes was about 1 mg protein per 10^{10} cells. Thus 2–4 incubations could be performed in order to get the amount of plasma membranes (about 500 μg protein) required for the analytical investigations.

Phospholipid analysis

Lipids were extracted as described previously [9]. The phospholipid content of the lipid extracts was determined by mineralization followed by the estimation of liberated phosphate by means of malachite green as described earlier [9] with poly(vinyl alcohol) (average M_r 14000, Aldrich Chemicals, F.R.G.) as dye solvent.

Neutral lipids were separated from phospholipids by thin-layer chromatography on silica sheets (Schleicher & Schuell, F.R.G.) with the solvent system petroleum ether/diethyl ether/acetic acid (50/50/1, v/v); phospholipids were separated with the solvent system chloroform/methanol/acetic acid/0.9% NaCl (15/25/8/2.5, v/v). The lipids were charred with $CuSO_4/H_3PO_4$ and subsequently quantitated by scanning the thin-layer plates with a laser densitometer [9].

Cholesterol determination

Cholesterol was determined in the lipid extracts with a test combination kit from Boehringer-Mannheim (F.R.G.) with the modifications described by Ott et al. [10].

Fatty acid determination

Lipids were separated on glass plates using the solvent systems described above. To visualize the individual phospholipids, the plates were sprayed with a 10% solution of the fluorescent dye, 1,6-diphenylhexatriene, in petroleum ether [11]. Lipid spots were scraped off and transmethylated in the presence of silica gel. Transmethylation was performed with sodium methylate [12]. By this method, only acyl-linked fatty acids are hydrolysed and methylated. The fatty acid methyl ester

were analysed by capillary gas-liquid chromatography (Fractovap 4160, ERBA Strumentazione, FFAP column (Jaeggi, Trogen, Switzerland), carrier gas H_2). Peaks were identified by standard fatty acid methyl esters. 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.

Statistic analysis

Each preparation was analyzed at least in duplicate. The mean values of individual preparations were used for Student's *t*-test analysis.

Results

Fatty acid composition of phospholipids from plasma membranes of T-lymphocytes

T-lymphocytes from rabbit thymus were incubated in medium RPMI 1640 for 4 h. Lipids were extracted from plasma membranes derived from these cells. The fatty acid composition of the total phospholipids or the individual phospholipids was determined by gas-liquid chromatography. As an example, the profile of the fatty acid methyl esters from phosphatidylethanolamine is shown in Fig. 1. The peaks indicated as 22:*n* could not be identified directly. By comparison with data from the literature [13], it is likely that

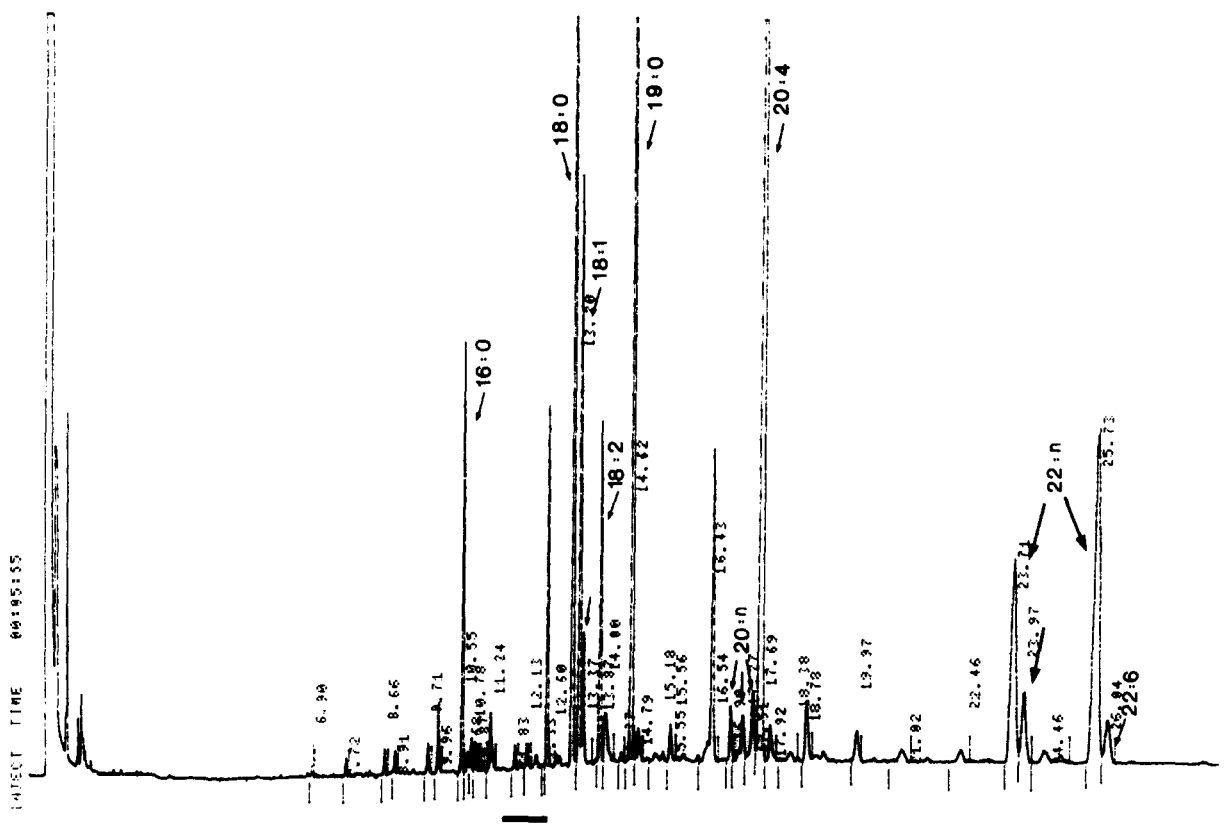

Fig. 1. Typical GLC profile of fatty acid methylesters derived from the fatty esters of phosphatidylethanolamine. For GLC conditions see Materials and Methods. The analysis of the data is shown in Table I.

they correspond to 22:4 ($n-6$), 22:5 ($n-6$) and 22:5 ($n-3$), respectively. A typical example of an analysis of the fatty acid composition of resting thymocytes is given in Table I.

Changes in the fatty acid composition upon stimulation of the T-lymphocytes with concanavalin A

T-lymphocytes were stimulated for 4 h with mitogenic doses of concanavalin A. The fatty acid composition of the phospholipids extracted from plasma membranes from stimulated and control cells was compared. For the sake of clarity, only three parameters are depicted: the percentage of saturated fatty acids (palmitic acid and stearic acid), the percentage of arachidonic acid, and the percentage of other polyunsaturated fatty acids (Table II).

Changes in the fatty acid composition could be detected in two of the individual phospholipids analyzed: the content of saturated fatty acids was decreased in phosphatidylcholine and phosphatidylinositol and the content of arachidonic acid and the polyunsatured fatty acids was increased concomitantly in the plasma membranes derived from stimulated cells. A smaller increase was also found in phosphatidylserine, while the fatty acid composition of phosphatidylethanolamine remained unchanged.

In order to mimic in vivo conditions a little closer, T-lymphocytes were incubated with palmitic acid and arachidonic acid bound to serum albumin. As a control, cells were incubated with serum albumin alone. The content of palmitic acid remained unchanged in the phospholipids of the plasma membrane, when the cells were incubated

TABLE II

COMPARISON OF THE FATTY ACID COMPOSITION OF PHOSPHOLIPIDS FROM PLASMA MEMBRANES DERIVED FROM MITOGEN-STIMULATED AND UNSTIMULATED T-LYMPHOCYTES

T-lymphocytes were incubated in the presence and absence of mitogenic doses of concanavalin A in Hepes-buffered RPMI as described in Materials and Methods. Highly purified plasma membranes were prepared by differential centrifugation. After lipid extraction the fatty acid composition of the individual phospholipids was determined by gas liquid chromatography. Data shown are means ± S.E. of five preparations. ** $P < 0.01$, * $P < 0.05$. Δ, Con A − control.

	Control	Con A	Δ
Percent saturated fatty acids			
PC	60.3 ± 1.0	54.9 ± 0.6	−5.4 ± 1.4 *
PI	48.2 ± 0.5	44.9 ± 1.3	−3.3 ± 1.1
PS	52.8 ± 0.8	52.1 ± 1.1	−0.7 ± 1.5
PE	19.3 ± 0.6	19.6 ± 1.3	+0.3 ± 0.8
Percent arachidonic acid			
PC	5.6 ± 0.5	8.2 ± 0.7	+2.6 ± 0.5 **
PI	37.2 ± 0.5	39.2 ± 0.8	+2.0 ± 0.7 **
PS	13.1 ± 0.6	14.9 ± 0.6	+1.7 ± 1.1
PE	38.0 ± 0.7	37.4 ± 0.6	−0.6 ± 0.4
Percent other polyunsaturated fatty acids			
PC	13.0 ± 0.4	16.3 ± 0.7	+3.3 ± 0.3 **
PI	4.7 ± 0.4	6.7 ± 0.6	+2.0 ± 0.8
PS	18.4 ± 1.2	19.9 ± 1.0	+1.5 ± 1.8
PE	30.0 ± 0.9	32.1 ± 1.5	+2.1 ± 0.9

TABLE I

FATTY ACID COMPOSITION OF PHOSPHOLIPIDS FROM PLASMA MEMBRANES DERIVED FROM T-LYMPHOCYTES FROM RABBIT

T-lymphocytes were incubated in medium with defatted serum albumin for 4 h. Lipids were extracted from their purified plasma membranes and separated by thin-layer chromatography. The fatty acid composition was determined by gas-liquid chromatography as described in Materials and Methods. Data shown are percentages of fatty acid from a typical example out of a series of five similar ones. $18:1_c$ and $18:1_t$ are isomers with the double bond in *cis* and *trans* position, respectively. $20:n$ is the sum of three peaks with 20 carbon atoms and an unknown number of double bonds. $22:n$ are peaks with 22 carbon atoms and an unknown number of double bonds, tentatively identified as 22:4, 22:5 (n-6) and 22:5 (n-3), respectively.

	16:0	16:1	18:0	$18:1_c$	$18:1_t$	18:2	18:3	20:n	20:4	22:n	22:n	22:n	22:6
PL	25.8	1.4	19.3	11.1	1.9	7.9	0.1	2.7	20.1	3.0	1.1	4.9	0.7
PC	53.1	2.4	8.2	12.8	2.5	9.7	0.3	2.0	6.3	0.4	–	0.5	–
PI	3.4	1.2	45.2	5.9	1.3	1.2	–	3.7	37.3	0.8	–	–	–
PS	1.3	1.0	52.8	10.1	0.8	4.1	–	5.2	14.2	4.1	1.6	4.0	0.6
PE	5.4	1.9	15.7	7.6	1.6	5.5	0.2	3.2	38.3	6.4	2.6	10.2	1.2

with fatty acids. There was a slight, but not significant increase in arachidonic acid in phosphatidylcholine and phosphatidylinositol (Table III).

Upon stimulation, the content of saturated fatty acids was decreased in phosphatidylcholine and phosphatidylinositol as described above. A concomitant increase of unsaturated fatty acids was observed in phosphatidylcholine. In phosphatidylinositol, the relative amount of the most abundant unsaturated fatty acid, arachidonic acid, was further enhanced, while the other polyunsaturated fatty acids remained unchanged. The opposite was true for phosphatidylserine, where arachidonic acid was slightly decreased and the other polyunsaturated fatty acids were enhanced. These changes were qualitatively the same when the cells were incubated in the presence or absence of exogenous fatty acids

Changes in the lipid composition of plasma membranes derived from mitogen-stimulated lymphocytes

The phospholipid composition and the ratio cholesterol to phospholipid were determined in plasma membranes derived from cells incubated in medium supplemented with serum albumin. The phospholipid composition of plasma membranes from mitogen-stimulated lymphocytes was only slightly changed: the relative content of

TABLE IV

LIPID COMPOSITION OF PLASMA MEMBRANES FROM MITOGEN-STIMULATED AND NON-STIMULATED T-LYMPHOCYTES

Lipids were extracted from the purified plasma membranes and the content of cholesterol and phospholipid determined as described in Materials and Methods. The content of the individual phospholipids is expressed as mole percent. The amount of lysophospholipids was in the range of 1 to 2% and not included in the calculation. Data shown are means ± S.E. of three preparations. The difference of the molar ratio cholesterol to phospholipid was significant with $P < 0.001$ ($n = 5$).

	Control	Con A
Sphingomyelin	12.4 ±1.1	10.8 ±1.0
Phosphatidylcholine	44.5 ±0.7	47.5 ±0.7
Phosphatidylinositol	8.7 ±0.7	8.2 ±0.4
Phosphatidylserine	11.7 ±1.0	10.6 ±1.5
Phosphatidylethanolamine	22.7 ±1.0	22.9 ±0.7
Cholesterol to phospholipid molar ratio	0.59±0.06	0.44±0.04

TABLE III

CHANGES IN THE FATTY ACID COMPOSITION OF PLASMA MEMBRANES DERIVED FROM MITOGEN-STIMULATED T-LYMPHOCYTES INCUBATED WITH EXOGENOUS FATTY ACIDS

T-lymphocytes were incubated with or without exogenous arachidonic acid and palmitic acid (see Materials and Methods and Table II). Δ, Con A − control.

	Medium with albumin			Medium supplemented with fatty acids		
	control	Con A	Δ	control	Con A	Δ
Percent saturated fatty acid						
PC	61.6±1.4	54.3±1.4	−7.4±1.1 **	60.8±3.3	55.6±3.6	−4.2±2.1
PI	46.4±0.5	43.5±0.3	−2.9±0.8 *	48.4±1.6	44.9±0.5	−4.2±1.5 *
PS	50.0±0.6	49.6±0.8	−0.4±0.5	51.0±1.4	49.6±1.4	−1.4±0.3
PE	21.2±0.9	22.1±0.8	+0.9±1.1	23.6±1.1	22.5±2.3	−1.1±1.1
Percent arachidonic acid						
PC	4.3±0.3	6.6±0.2	+2.3±0.1 **	5.4±1.1	7.5±1.2	+2.1±0.3 *
PI	27.5±1.5	37.3±0.6	+9.7±1.5 *	29.2±3.2	34.2±2.9	+5.0±2.8
PS	15.4±1.4	13.2±0.9	−2.2±1.0	15.9±1.9	14.8±1.0	−1.0±0.6
PE	33.5±1.2	32.3±1.0	−1.2±1.0	33.2±1.0	34.2±1.7	+1.0±0.8
Percent other polyunsaturated fatty acids						
PC	12.6±1.0	14.8±1.1	+2.2±1.0	11.7±1.1	14.7±1.0	+3.0±0.3 **
PI	8.7±1.0	7.3±1.0	−1.4±1.7	7.6±0.9	7.8±2.3	+0.2±1.5
PS	17.3±1.0	21.0±1.5	+3.8±0.8 **	18.2±0.7	22.4±1.0	+4.2±0.7 *
PE	33.9±1.1	30.9±0.8	−2.9±0.9 *	30.6±1.5	31.4±1.8	+0.8±0.9

phosphatidylcholine was increased and the relative content of sphingomyelin decreased (Table IV). The ratio cholesterol to phospholipid was significantly decreased in plasma membranes from stimulated cells.

Discussion

Activation of T-lymphocytes to proliferation and differentiation is a long-lasting process and it has been postulated that changes in the lipid metabolism of the plasma membranes of these cells are involved in the molecular mechanism of a continuing supported of cellular stimulation. Several lines of evidence point to this interpretation: (1) the reacylating enzyme lysophosphatide acyltransferase is activated already minutes after binding of the mitogen to the cells and remains activated for hours [6]; (2) unsaturated fatty acids are incorporated increasingly into stimulated cells, measured as incorporation of labelled fatty acids [2]; (3) the enzyme, lysophosphatide acyltransferase, could be shown to be in close structural relation with the mitogen receptor [14]; (4) inhibition of the enzyme correlated with an inhibition of cell proliferation [15]. It was thus tempting to analyze the lipid composition and especially the fatty acid composition of plasma membranes from stimulated T-lymphocytes, in order to investigate, whether the overall composition reflected the changes supposed by incorporation studies.

Earlier studies with T-lymphocytes derived from calf thymus had shown that 1 h of stimulation was too short to detect significant changes in the fatty acid composition of the phospholipids [16]. Therefore, the cells were incubated for 2 and 4 h, with 4 hours revealing significant effects.

The composition of the fatty acids of the total phospholipids differed markedly from the one published previously by Roozemond and Urli [17]. The content of saturated fatty acids was much higher in their plasma membranes than in the ones analyzed in the present study. This discrepancy may be due to the different strains of rabbits used or to different analytical techniques. Only limited data are available on the fatty acid composition of individual phospholipids from plasma membranes. The data published for lipids derived from plasma membranes from mouse thymocytes [18,19] or calf thymocytes [16] are similar to those obtained for thymocytes from rabbit, with the main exception that the relative content of 20:4 (arachidonic acid) is much higher, while the content of linoleic acid (18 : 2) is reduced in the phospholipids of plasma membranes from rabbit lymphocytes. Linoleic acid (18 : 2) may be converted to arachidonic acid (20 : 4) [5], and different activities of the converting enzymes may lead to variable amounts of these two polyunsaturated fatty acids.

An effect of stimulation could be detected in certain phospholipid species: the relative content of saturated fatty acids was decreased and the content of polyunsaturated fatty acids increased in phosphatidylcholine and phosphatidylinositol, the effect being most evident in phosphatidylinositol. In phosphatidylserine the alterations were less prominent, an increase in the polyunsaturated fatty acid content was nearly compensated by a decrease in arachidonic acid. These results were consistent with the data obtained when T-lymphocytes from calf were incubated for only 1 h [16]. The changes occurred independent of culture conditions, i.e., exogenous fatty acid supply was not necessary as a source of arachidonic acid. The intracellular source of the polyunsaturated fatty acids has not yet been determined, but intracellular transport and exchange reactions are described in many cell types, including lymphocytes. The absolute amount of the alterations was 2–10%. It has to be considered, however, that most of the exchange reactions occur with the fatty acids of position 2, while the first position remains mostly unaltered. As both positions were analysed simultaneously, the percentage of phospholipids involved in the alterations may be nearly doubled.

When the cells were incubated with exogenous fatty acids (palmitic acid and arachidonic acid), no change in the fatty acid composition of plasma membranes from control cells or stimulated cells could be observed compared to cells incubated with albumin containing medium. It is known from studies with radiolabelled fatty acids that fatty acids are readily taken up and acylated to neutral lipids and phospholipids under the experimental conditions used [3]. Thus it has to be concluded that during the 4 h of incubation the homeostasis of the lipid composition of the plasma

membrane is maintained or rather regulated.

Both findings, the changes in the fatty acid composition of the membrane phospholipids upon mitogenic stimulation and the constancy in the membrane upon addition of exogenous fatty acids, fit well to the fact that many membrane bound enzymes are modulated in their activities by the fatty acid composition of the surrounding lipids. It thus seems to be of functional importance for the cells to keep the fatty acid composition of membrane lipids under strict control.

The phospholipid composition remained nearly unchanged with a small increase in phosphatidylcholine and a concomitant decrease in sphingomyelin in the plasma membranes of cells treated with concanavalin A. Similar results were found in calf thymocytes [16]. A reduction of the content of sphingomyelin in connexion with a reduced ratio cholesterol to phospholipid was shown to give rise to an increase in 'membrane fluidity' [20,21]. An increase in membrane fluidity, measured as a decrease in fluorescence polarization with the probe, 1,6-diphenylhexatriene, was not observed in the plasma membranes from stimulated rabbit T-lymphocytes (unpublished observation). This might be interpreted as an indication that the changes in the lipid composition described above do not occur randomly, but might be most pronounced in certain domains of the plasma membrane of T-lymphocytes. Such domains were described in the plasma membranes of calf thymocytes, consisting of a high-affinity binding site for concanavalin A, distinct proteins and a lipid composition, which differed from the bulk membrane [14,16].

We could thus show that, besides general changes such as the reduced cholesterol-to-phospholipid ratio, specific enzymatically regulated alterations occur in the plasma membranes of activated T-lymphocytes that may be involved in the maintenance of the activated state.

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