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CHARACTERIZATION OF FUNCTIONAL DOMAINS OF THE LYMPHOCYTE PLASMA MEMBRANE

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Highly purified plasma membranes of calf thymocytes were fractionated by means of affinity chromatography on concanavalin A-Sepharose into two subfractions; one (fraction 1) eluted freely from the affinity column, the second (fraction 2) adhered specifically to concanavalin A-Sepharose. Previous analysis showed that both subfractions were right-side-out (Resch, K., Schneider, S. and Szamel, M. (1981) Anal. Biochem. 117, 282–292). The ratio of cholesterol to phospholipid was nearly identical in plasma membrane and both subfractions. When isolated plasma membranes were labelled with tritiated $NaBH_4$, both subfractions exhibited identical specific radioactivities. After enzymatic radioiodination of thymocytes, the relative distribution of labelled proteins and externally exposed phospholipids was very similar in isolated plasma membranes and in both membrane subfractions, indicating the plasma membrane nature of the subfractions separated by affinity chromatography on concanavalin A-Sepharose. This finding was further substantiated by the nearly identical specific activities of some membrane-bound enzymes, Mg^{2+}-ATPase, alkaline phosphatase and γ-glutamyl transpeptidase. The specific activities of $(Na^+ + K^+)$-ATPase and of lysolecithin acyltransferase were several-fold enriched in fraction 2 compared to fraction 1, especially after rechromatography of fraction 1 on concanavalin A-Sepharose. Unseparated membrane vesicles contained two types of binding site for concanavalin A. In contrast, isolated subfractions showed a linear Scatchard plot; fraction 2 exhibited fewer binding sites for concanavalin A: the association constant was, however, 3.5-times higher than that measured in fraction 1. When plasma membranes isolated from concanavalin A-stimulated lymphocytes were separated by affinity chromatography, the yield of the two subfractions was similar to that of membranes from unstimulated lymphocytes. Upon stimulation with concanavalin A, Mg^{2+}-ATPase, γ-glutamyl transpeptidase and alkaline phosphatase were suppressed in their activities in both membrane subfractions. In contrast, the specific activities of $(Na^+ + K^+)$-ATPase and lysolecithin acyltransferase were enhanced preferentially in the adherent fraction (fraction 2). The data suggest the existence of domains in the plasma membrane of lymphocytes which are formed by a spatial and functional coupling of receptors with high affinity for concanavalin A, and certain membrane-bound enzymes, implicated in the initiation of lymphocyte activation.

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

Introduction

To permit isolation in a biochemically active form, plasma membranes of nucleated cells such as lymphocytes have to be disrupted. Upon cell disruption, the plasma membrane fragments into vesicles, the size of which depends on the method applied [1]. The possibility that these membrane vesicles are heterogeneous has stimulated attempts to fractionate them. As lectin-binding carbohydrates are exposed exclusively to the outer surface [2], affinity chromatography on insolubilized lectins has been attempted to separate membrane vesicles which had preserved their original orientation (i.e., right-side-out) from those, the orientation of which was inverted during the cell disruption procedure (i.e., inside-out) [3–5]. We have reported earlier that right-side-out-oriented membrane vesicles from lymphocytes can also be separated according to their different binding affinities for concanavalin A [6,7]. In these experiments, 'microsomal membranes' – which in lymphocytes consist of 60–80% plasma membranes – were used, isolated after nitrogen cavitation of calf thymus lymphocytes [8]. The interpretation of these experiments was limited by the fact that only semipurified plasma membranes could be analysed, as attempts to fractionate plasma membrane purified by density-gradient centrifugation with sucrose, Ficoll or CsCl originally resulted in inconsistent results due to aggregation of plasma membranes after removal of the gradient material. The difficulties in subjecting highly purified lymphocyte plasma membranes to affinity chromatography were recently overcome. The separated subfractions of the plasma membrane proved to be exclusively right-side-out-oriented [9]. Here we confirm and extend our previous observations with semipurified plasma membranes that by affinity chromatography on concanavalin A-Sepharose, domains of the lymphocyte plasma membrane are purified which bear high-affinity receptors for concanavalin A in close spatial association with two enzymes, $(Na^+ + K^+)$-ATPase and lysolecithin acyltransferase. In addition, upon stimulation of intact lymphocytes with concanavalin A, the activity of these enzymes is activated selectively, suggesting that the receptor-associated domains are implicated in the initiation of cell activation.

Materials and Methods

Lymphocytes

Calf thymus was obtained shortly after killing from the local slaughterhouse and kept in phosphate-buffered saline at room temperature. Each thymus was freed of adherent tissue, cut into small pieces by scissors and macerated gently by hand in phosphate-buffered saline in loosely fitting glass homogenizers (Potter-Elvejhem type) to yield intact lymphocytes. The cell suspensions were filtered twice through small glass funnels containing nylon wool (Leuko-Pak-Fenwall Lab., Travenol) to remove tissue remnants.

The thymus lymphocytes were sedimented (7 min at $350 \times g_{max}$) and resuspended in Hepes-buffered Dulbecco's modified Eagle's medium or in cell-disruption buffer (0.14 M KCl/0.02 M Hepes/0.25 mM $MgCl_2$ (pH 7.0)). All steps were carried out at room temperature, which resulted in optimal viability of the lymphocytes, which was always greater than 80–90%. In stimulation experiments, $5 \cdot 10^7$ lymphocytes/ml Hepes-buffered Dulbecco's modified Eagle's medium were incubated for 1 h with 10 μg/ml concanavalin A at 37°C in a waterbath. Then the cells were sedimented and resuspended in a cell-disruption buffer.

Isolation of plasma membranes

Lymphocytes (10^8 cells/ml in cell disruption buffer) were chilled in ice – if not stated otherwise, as in one experiment – and all subsequent steps were carried out strictly in the cold. The disruption of the cells and the isolation of plasma membranes has recently been described in detail [9]. Briefly, the lymphocytes were equilibrated for 20 min with 30 atm N_2 in an Artisan pressure homogenizer with gentle stirring, and then released dropwise. Immediately after the release, EDTA was added, to give a final concentration of 1 mM. Nuclei, large granules (mitochondria, lysosomes, aggregated plasma membranes) and a microsomal fraction containing vesicles derived from the plasma membrane and the endoplasmic reticulum were sedimented by differential centrifugation. After shocking the microsomal membranes hypotonically (0.02 M Hepes, pH 7.0) to remove trapped cytoplasmic protein, the membranes were suspended in plasma-membrane buffer (0.14 M KCl/

0.02 M Hepes (pH 7.0)) and layered on top of 35% (w/w) sucrose in plasma-membrane buffer. After centrifugation for 2 h at 250000 × g_{max} in a Beckman 60 Ti fixed angle rotor, the plasma membrane formed a band at the interface between sucrose and buffer which was collected by a Pasteur pipette. From the plasma membranes collected, the sucrose was removed by dialysis overnight against a gradient of a total of 2000 ml 20% sucrose (w/w) and plasma-membrane buffer, and finally for 4 h against 250 ml of plasma-membrane buffer. This slow removal of sucrose was essential to prevent the membranes from irreversible aggregation [9]. The plasma membranes were either processed immediately, or kept frozen at −80°C batchwise.

Affinity chromatography of plasma membranes on concanavalin A-Sepharose

Affinity chromatography was performed strictly at 4°C. The detailed procedure of the affinity separation of plasma membrane subfractions has been published recently [9]. Briefly, 50 ml of wet gel of concanavalin A-Sepharose (Pharmacia), washed thoroughly before use, were rinsed with 3-times the gel volume with plasma-membrane buffer, in a glass separation chamber as described in Ref. 9. To the wet gel, about 2 mg of plasma-membrane protein were added in 10 ml plasma-membrane buffer. Concanavalin A-Sepharose and membranes were mixed by stirring the gel for 2 min at 200 rpm with a motor driven stirrer. The membranes were then allowed to bind for 20 min, within which time the gel had sedimented. The plasma membrane fraction not binding to concanavalin A-Sepharose was eluted with plasma-membrane buffer at a flow rate of 2–3 ml/min, and designated fraction 1. After complete elution of fraction 1, the gel was washed with plasma-membrane buffer substituted with 0.1 M methyl α-mannoside. To dissociate the bound membranes, concanavalin A-Sepharose was stirred for 2 min at 200 rpm. The fraction was then eluted with plasma-membrane buffer containing 0.1 M methyl α-mannoside and was designated fraction 2. Elution of membrane protein was monitored with a spectrophotometer at 280 nm (UA5 ISCO instruments) and the fractions collected batchwise. Fraction 1 and fraction 2 were concentrated by ultracentrifugation for 120 min at 250000 × g_{max} (Rotor 60 Ti, Beckman instruments) and resuspended in plasma-membrane buffer.

Analytical procedures

Protein. Protein was measured by its native fluorescence as described earlier using an MPF 44 fluorescence spectrophotometer (Perkin-Elmer, Hitachi) [10].

Cholesterol. Cholesterol was measured enzymatically as described by Röschlau et al. [11] using a commercial test combination (Boehringer).

Phospholipid. Total phospholipid was measured as described by Zilversmit and Davis [12] with a commercial test kit (Boehringer). Separation of individual phospholipids: phospholipids were extracted by a modified method of Ways and Hanahan [13] as described earlier [14]. The phospholipids were separated by thin-layer chromatography on TLC plastic sheets Silica-gel 60, layer thickness 0.2 mm (Merck) with chloroform/methanol/acetic acid/0.9% NaCl (50 : 25 : 8 : 4, v/v) as originally described by Skipski [15]. The individual lipid fractions were visualized by exposure to iodine vapour. The areas containing a lipid fraction were cut out and counted in a liquid scintillation counter (Searle, Nuclear Chicago).

Radioactive labelling

$5 \cdot 10^7$ lymphocytes in 1 ml phosphate-buffered saline, substituted with 20 mM glucose, were incubated for 20 min at room temperature with 20 units lactoperoxidase (Boehringer), 80 units glucose oxidase (Boehringer) and 1 mCi $Na^{125}I$ (Amersham Buchler, IMS 30), a method similar to that described by Hubbard and Cohn [16]. The cells were then washed four times with excess phosphate-buffered saline supplemented with 10% foetal calf serum. Radioiodinated lymphocytes were then diluted with unlabelled lymphocytes.

Plasma membranes (1 mg protein in 5 ml plasma membrane buffer, supplemented with 20 mM glucose) were incubated for 30 min at room temperature with 100 units lactoperoxidase, 400 units glucose oxidase and 1 mCi $Na^{125}I$. The membranes were then washed twice by ultracentrifugation (60 min at 175000 × g_{max}) and dialyzed overnight in plasma-membrane buffer.

Membrane glycoproteins were labelled by

sodium borohydride reduction of galactose oxidase-treated membrane [17]. 10 ml plasma membranes containing 2 mg membrane protein were first reacted for 5 min with unlabelled $NaBH_4$ (2 mM) at room temperature. The plasma membranes were then centrifuged at $250000 \times g_{max}$ for 60 min and resuspended in 5 ml plasma-membrane buffer. The membranes were treated with neuraminidase (Sigma Type VI, 30 μg) for 20 min at room temperature, and subsequently with galactose oxidase (Boehringer, 25 units) for 15 min at 37°C. The aldehydes were reduced with tritiated $NaBH_4$ at a concentration of about 1 mCi/ml for 5 min at room temperature. NaB^3H_4 (Amersham Buchler, 8 Ci/mmol) was dissolved immediately before use in 0.01 M NaOH. The plasma membranes were washed twice in plasma membrane buffer (centrifugation 45 min at $250000 \times g_{max}$) and resuspended in 3 ml plasma-membrane buffer.

Binding of concanavalin A

Concanavalin A (Pharmacia) was labelled with ^{125}I (Amersham Buchler IMS 30) with slight modifications of the method of McConahy and Dixon [18] as described [19].

Binding studies were performed in a Beckman airfuge, in a total volume of 180 μl plasma membrane buffer. Membranes were added at concentrations of 40–50 μg/ml, concanavalin A (a mixture of labelled and unlabelled) at increasing concentrations up to 30 μg/ml. Nonspecific adsorption was determined in the presence of 0.1 M methyl α-mannoside. The reaction mixture was incubated for 30 min at 37°C, and then the membranes were spun down for 60 min at $150000 \times g_{max}$. An aliquot of the supernatant and the remainder in the centrifugation tube was counted in a gamma spectrometer (Searle, Nuclear, Chicago). The specifically bound concanavalin A was calculated as the amount of concanavalin A bound which inhibited by methyl α-mannoside. Unspecific adsorption (i.e., in the presence of 0.1 M methyl α-mannoside) was low, usually below 10% of total binding.

Enzyme assays

ATPases (EC 3.6.1.3) were measured in a total volume of 0.1 ml in the following test system (end concentrations): 0.15 M NaCl, 0.015 KCl, 0.02 M Hepes (pH 7.6), 2 mM $MgCl_2$, 2 mM ATP, and 10–40 μg of membrane protein. After 30 min of incubation at 37°C, the samples were diluted to 1.6 ml with bidistilled water and liberated phosphate was determined as described by Anner and Moosmeyer [20]. The enzyme insensitive to 10^{-3} M ouabain was designated as Mg^{2+}-ATPase, the sensitive enzyme as $(Na^+ + K^+)$-ATPase. Alkaline *p*-nitrophenylphosphatase (EC 3.1.3.1) was measured in a total volume of 1 ml in the presence of 5.5 mM *p*-nitrophenyl phosphate, 1 mM $MgCl_2$ (or 10 mM EDTA in controls), 1 M diethanolamine (pH 9.5), and 3–20 μg membrane protein. After incubation for 10 min at 37°C, the reaction was stopped by addition of 1 ml 1 M NaOH and extinction was measured at 405 nm. K^+ *p*-nitrophenylphosphatase (EC 3.1.3.41) was measured in a total volume of 1.0 ml, with *p*-nitrophenyl phosphate (Na^+-free) as substrate in a buffer comprising 0.1 M Tris HCl (pH 7.4)/5 mM $MgCl_2$/20 mM K^+. γ-Glutamyl transpeptidase (EC 2.3.2.2) was measured according to Szasz [21] using 1-γ-glutamyl-3-carboxy-4 nitroanilide as substrate with a commercial test combination (Boehringer). Lysolecithin acyltransferases (EC 2.3.1.23) were measured in a total volume of 1 ml containing 50 μM 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine (specific activity 0.5 mCi/mmol, made by mixing unlabelled (Sigma) and labelled (Amersham Buchler) substrate), 30 μM oleoyl-coenzyme A (Sigma) or arachidonoyl-coenzyme A, 0.14 M KCl, 20 mM Hepes (pH 7.4) and 8–40 μg membrane protein. After incubation for 10 min at 37°C, the reaction was stopped by addition of 5 ml methanol. The phospholipids were extracted as described and separated by thin-layer chromatography as described above. Enzyme activities were calculated from the conversion of the labelled precursor into phosphatidylcholine. Arachidonoyl-coenzyme A was synthesized according to Reitz et al. [22], using arachidonoyl chloride (Nu Chek Prep.) as substrate. Immediately after termination of the reaction, 1 mol/2 mol acyl-coenzyme A (2,6-di-*t*-butyl-4-methylphenol) were added as antoxidant and the acyl-coenzyme A's were dissolved in 0.14 M KCl/20 mM Hepes (pH 7.4) and were frozen batchwise at −80°C.

Results

Composition of plasma membranes and plasma membrane subfractions

The plasma membranes isolated from calf thymocytes by the method described above proved to be highly purified. This was shown by a high cholesterol-to-phospholipid ratio and a more than 30-fold enrichment of the specific activities of plasma-membrane marker enzymes such as γ-glutamyl transpeptidase or $(Na^+ + K^+)$-ATPase [9]. All enzymes measured in plasma membranes and their subfractions exhibited the highest specific activities in this cellular compartment compared to other cellular organelles. In addition, the purified plasma membranes were devoid of β-glucuronidase, acid phosphatase, or succinate dehydrogenase, which rules out contamination by lysosomes or mitochondria [8].

When sucrose-free purified plasma membranes were subjected to affinity chromatography on concanavalin A-Sepharose, two membrane fractions were recovered. One fraction, fraction 1 eluted freely from the affinity column, the second, fraction 2, was retained and could be recovered after mechanical dissociation in the presence of 0.1 M methyl α-mannoside, which competitively inhibits binding of concanavalin A to membrane receptors [23]. Fig. 1 shows a typical fractionation, as monitored by absorbance at 280 nm. As can be seen, substitution with methyl α-mannoside alone did not detach the membrane vesicles already bound, although it completely prevented the binding of membranes [7]. The membranes could easily be dissociated by mechanical forces, e.g., by stirring the gel gently (see Materials and Methods). From the membrane vesicles recovered after affinity chromatography, about 80% eluted in fraction 1 and 20% in fraction 2. In more than 20 separations, the amount of fraction 2 varied between 18 and 25%, thus showing the high reproducibility of the separation method. The total recovery of the membranes subjected to affinity chromatography under conditions used here was usually greater than 90%.

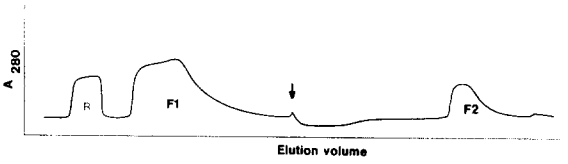


Fig. 1. Fractionation of plasma membranes on concanavalin A-Sepharose. 1 mg of plasma membrane protein was fractionated as described in Materials and Methods. The figure depicts absorbance at 280 nm recorded by an ISCO UA 5 spectrophotometer. At the elution volume indicated by the arrow, the buffer was substituted with 0.1 M methyl α-mannoside. *R*: absorbance of reference protein (200 μg).

In plasma membranes, and in the subfractions derived thereof, the content of cholesterol and phospholipid was very similar, resulting in a nearly identical high cholesterol-to-phospholipid ratio, as characteristic for plasma membranes [8] (Table I). This strongly suggested that both subfractions, fraction 1 and fraction 2, were derived from the plasma membrane. This was further substantiated by the identical labelling of plasma membranes and the two subfractions after radioiodination of intact lymphocytes (Table II). In this experiment, the cells were iodinated enzymatically by the lactoperoxidase method, then plasma membranes were isolated and subsequently fractionated into fraction 1 and fraction 2. Also, when membrane glycoproteins of isolated plasma membranes were labelled by reduction with tritiated $NaBH_4$ after

TABLE I

CHOLESTEROL AND PHOSPHOLIPID CONTENT OF PLASMA MEMBRANE SUBFRACTIONS

Data are means of values from 3–5 different plasma membrane preparations.

	Content (μmol/mg protein)		Molar ratio cholesterol/phospholipid
	Cholesterol	Phospholipid	
Plasma membrane	0.641	1.090	0.588
Fraction 1	0.638	1.085	0.588
Fraction 2	0.570	1.077	0.530

TABLE II

DISTRIBUTION OF SURFACE LABEL IN PLASMA MEMBRANE SUBFRACTIONS

Intact lymphocytes were surface-labelled by lactoperoxidase-catalyzed radioiodination as described in Materials and Methods. Then plasma membranes were isolated and fractionated into fraction 1 and fraction 2. Isolated plasma membranes were treated with neuraminidase and galactose oxidase, the oxidized carbohydrates were then reduced with NaB^3H_3 as described in Materials and Methods. The labelled plasma membranes were then fractionated into fraction 1 and fraction 2.

	Label (cpm/mg protein)	
	^{125}I	^3H
Plasma membrane	237578	283665
Fraction 1	240051	294827
Fraction 2	244564	308970

enzymatic oxidation by galactose oxidase, plasma membranes, as well as the subfractions, exhibited identical specific radioactivity (Table II). Essentially identical results were obtained when, instead of isolated membranes, intact lymphocytes were labelled in this way (data not shown).

Enzymatic radioiodination labels not only surface proteins, but also (externally exposed) lipids to an appreciable extent [24]. When lipids were extracted from isolated plasma membranes, fraction 1 and fraction 2, the relative distribution of individual phospholipids as well as their specific ^{125}I-labelling was very similar in all fractions (Goppelt, M., Szamel, M. and Resch, K., unpublished data).

Taken together, these data demonstrate that the fraction 1 and 2, separated by affinity chromatography on insolubilized concanavalin A, are both subfractions of the plasma membrane.

Distribution of enzyme activities in plasma membrane subfractions

The plasma membrane nature of fraction 1 and fraction 2 was also substantiated by the distribution of several plasma-membrane-bound enzymes [8,9]. Thus, γ-glutamyl transpeptidase, Mg^{2+}-ATPase, and alkaline *p*-nitrophenylphosphatase exhibited nearly identical specific activities in both membrane subfractions, as well as in unseparated plasma membranes (Table 4). Other membrane-specific enzymes, however, showed a clear-cut heterogeneous distribution. Thus, $(Na^+ + K^+)$-ATPase was enriched several-fold in fraction 2, as compared to fraction 1. Accordingly, the specific activity of K^+-dependent *p*-nitrophenylphosphatase – which measures part of the reaction sequence of $(Na^+ + K^+)$ATPase – could be measured only in plasma membranes and fraction 2. Lysolecithin acyltransferase, measured with two different substrates, was also present in higher activities in fraction 2 compared to fraction 1 (Table III).

When fraction 1 was rechromatographed under

TABLE III

DISTRIBUTION OF ENZYME ACTIVITIES IN PLASMA MEMBRANE SUBFRACTIONS

	Activity (nmol/mg protein per min) (37°C)		
	Plasma membrane	Fraction 1	Fraction 2
γ-Glutamyl transpeptidase	28 ±8 [a]	30 ±7	28 ±6
Alkaline *p*-nitrophenylphosphatase	127 [b]	90	135
Mg^{2+}-ATPase	60 ±11	76 ±11	63 ±16
$(Na^+ + K^+)$-ATPase	18.3±4.6	8.2±3.6	30.7±10.0
K^+-*p*-nitrophenylphosphatase	3.5	0	7.8
Oleoyl-coenzyme A: lysolecithin acyltransferase	5.0±0.7	3.5±1.1	8.3±2.7
Arachidonoyl-coenzyme A: lysolecithin acyltransferase	15.0±2.5	9.9±4.2	35.1±4.6

[a] Data are means of values from 5–12 different plasma membrane preparations ±S.D.
[b] Data are from a single plasma membrane preparation.

TABLE IV

DISTRIBUTION OF ATPases AND LYSOLECITHIN ACYLTRANSFERASE IN PLASMA MEMBRANE SUBFRACTIONS

Plasma membranes were separated into fraction 1 and fraction 2. Fraction 1 was then rechromatographed on concanavalin A-Sepharose into fraction 1′ and fraction 2′.

	Activity (nmol/mg protein per min)(37°C)				
	Plasma membrane	Fraction 1	Fraction 1′	Fraction 2	Fraction 2′
Mg^{2+}-ATPase	70.9	84.3	92.8	66.7	80.6
Mg^{2+}-ATPase + DOC [a]	76.9	93.1	94.3	74.5	89.3
$(Na^+ + K^+)$-ATPase	13.6	9.2	3.7	33.4	40.0
$(Na^+ + K^+)$-ATPase + DOC [a]	60.0	31.2	12.3	130.0	141.4
Oleoyl-coenzyme A: lysolecithin acyltransferase	3.9	3.5	1.7	7.3	7.3
Arachidonoyl-coenzyme A: lysolecithin acyltransferase	15.3	14.3	n.d.	40.0	n.d.
	13.6	n.d.	6.3	n.d.	29.9

[a] Desoxycholate, 0.05% end concentration per mg of protein.

conditions identical to those used for the fractionation of plasma membranes, part of the membrane vesicles (less then 10%) bound to concanavalin A-Sepharose. This fraction, designated fraction 2′, had enzyme activities similar to those of fraction 2. In contrast, in the rechromatographed fraction 1, designated fraction 1′, the specific activity of $(Na^+ + K^+)$-ATPase was further decreased, resulting in a more than 10-fold difference in the specific activities of the rechromatographed fractions (Table IV). Similarly, in rechromatographed 1′ fractions, the activity of lysolecithin acyltransferase was also decreased as compared to fraction 1, although the enrichment of fraction 2′ compared to fraction 1′ was smaller than with $(Na^+ + K^+)$-ATPase (Table IV). In addition, no enzyme activities of $(Na^+ + K^+)$-ATPase or lysolecithin acyltransferase were released during affinity chromatography or rechromatography from the membrane subfractions.

The differences in the activity of lysolecithin acyltransferase between different subfractions of the plasma membrane cannot be due to different accessibility of substrate, as lysophosphatidylcholine at concentrations of 50 μM, as used in the enzyme test, solubilizes membranes at least partially. In addition, increase of this substrate to 100 μM did not alter enzyme activities, although the membranes were totally solubilized (data not shown). To exclude differences in substrate accessibility in the case of $(Na^+ + K^+)$-ATPase, this enzyme was also measured in the presence of 0.05% desoxycholate per mg protein. As can be seen in Table IV, this detergent increased the specific activity; however, the relative distribution between the membrane subfractions remained unaltered.

Influence of disruption temperature on plasma membrane subfractions

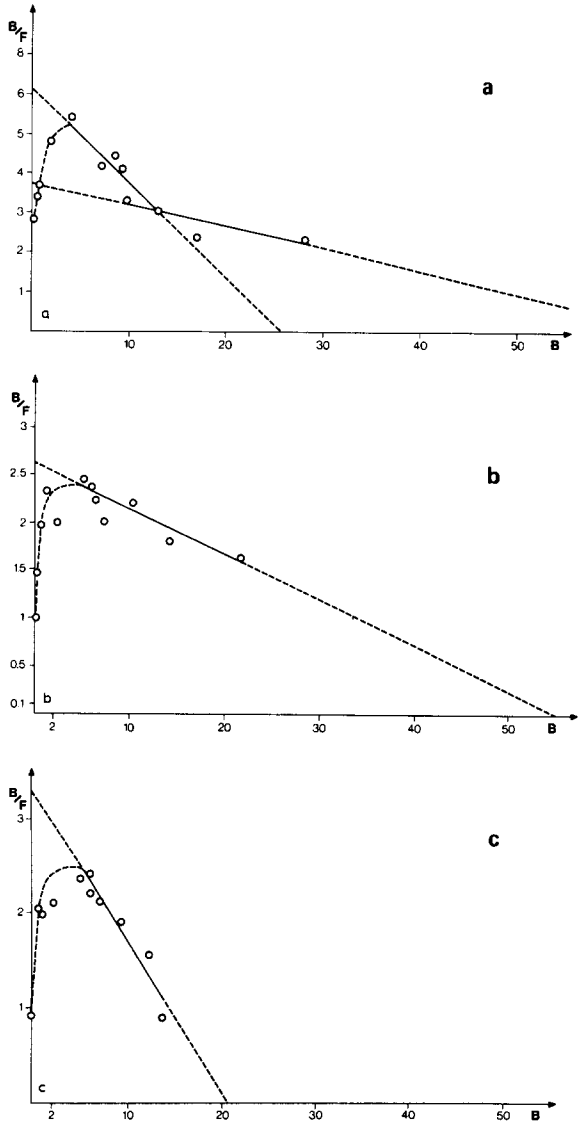
Routinely, lymphocytes were disrupted at 4°C. To exclude the possibility that temperature shifts are involved in the apparent membrane heterogeneities, lymphocytes were cultured after isolation from calf thymus for 2 h at 37°C, and then disrupted at the same temperature, taking care that the temperature was kept constant throughout. After disruption, the homogenate was chilled immediately and processed as usual. As depicted in Table V, identical membrane subfractions could be separated by chromatography on concanavalin A-Sepharose, whether cell disruption occurred at 4°C, or 37°C. The respective membrane fractions showed identical enzyme activities of Mg^{2+}-ATPase, as well as $(Na^+ + K^+)$-ATPase, irrespective of the temperature at which the lymphocytes were disrupted.

TABLE V

DISTRIBUTION OF MEMBRANE PROTEIN AND ATPases IN PLASMA MEMBRANE SUBFRACTIONS AFTER CELL DISRUPTION AT DIFFERENT TEMPERATURES

Thymus lymphocytes were disrupted by nitrogen cavitation strictly either at 4°C, or at 37°C, as indicated. Plasma membranes and membrane subfractions were then isolated as in Materials and Methods. Temperatures are those during the disruption of lymphocytes. Protein is expressed in mg, enzyme activities in nmol/mg protein per min (37°C).

	Plasma membrane		Fraction 1		Fraction 2	
	4°C	37°C	4°C	37°C	4°C	37°C
Protein	2.0	2.0	1.3	1.3	0.3	0.3
Mg^{2+}-ATPase	47.8	45.9	64.9	65.5	49.5	51.7
$(Na^+ + K^+)$-ATPase	19.1	22.9	8.7	7.3	24.8	22.9



Binding of concanavalin A to plasma membranes, and plasma membrane subfractions

Fig. 2 shows the specific binding of concanavalin A to plasma membranes and to the membrane subfractions 1, and 2. As it proved to be difficult to remove methyl α-mannoside from fraction 2 quantitatively, this fraction was eluted without the use of this sugar with constant stirring of the gel. By this method, an identical fraction 2 could be recovered [7]. The data in Fig. 2 are plotted according to Scatchard, calculations being made assuming a molecular weight for concanavalin A at pH 7.0 of 108 000. Binding of concanavalin A to all membrane vesicles showed positive co-operativity at low concentrations (up to 2–3 μg/ml concanavalin A). At concentrations above this, the binding to unseparated plasma membranes was complex. The Scatchard plots could best be fitted by assuming two distinct binding sites. One, with high affinity for concanavalin A (the apparent association constant being $2.5 \cdot 10^7\ M^{-1}$), was present in amounts of $1.46 \cdot 10^{15}$ sites per mg of protein. Plasma membranes also contained 3.79 ·

Fig. 2. Binding of concanavalin A to plasma membrane subfractions. Binding of ^{125}I-labelled concanavalin A to 40 μg/ml membrane protein was measured as described in Materials and Methods. The data are plotted according to Scatchard. In their linear parts as indicated by solid lines, the lines were fitted by linear least square regression the correlation coefficient being 0.924 for fraction 1, 0.955 for fraction 2, 0.843 and 0.968 for the corresponding parts of the Scatchard plot of the unseparated plasma membrane [41]. B = bound concanavalin A (μg/100 μg membrane protein); F = free concanavalin A (μg/ml). (a) Plasma membrane; (b) fraction 1; (c) fraction 2.

10^{15} low-affinity binding sites per mg of protein, the apparent association constant of which was $5.9 \cdot 10^6$ M^{-1}. In contrast, the separated plasma membrane subfractions showed a linear Scatchard plot above threshold concanavalin A concentrations. Fraction 1 bound $3.07 \cdot 10^{15}$ concanavalin A molecules/mg protein with an apparent association constant of $5.1 \cdot 10^6$ M^{-1}, fraction 2 $1.17 \cdot 10^{15}$ molecules/mg protein with an apparent association constant of $1.7 \cdot 10^7$ M^{-1}.

Characterization of plasma membrane subfractions from Concanavalin A-stimulated lymphocytes

Plasma membranes which had been isolated from thymus lymphocytes stimulated for 1 h with concanavalin A could also be separated by affinity chromatography on concanavalin A-Sepharose. Table VI shows a representative experiment out of a series of five experiments with similar results. The yield of the two subfractions 1 and 2 was similar to that of unstimulated lymphocytes (data not shown). Upon stimulation with concanavalin A, the specific activities of membrane-bound enzymes were altered in a specific pattern. ($Na^+ + K^+$)-ATPase and lysolecithin acyltransferase were activated predominantly in fraction 2. In contrast, Mg^{2+}-ATPase, alkaline *p*-nitrophenylphosphatase and γ-glutamyl transpeptidase were suppressed, and to a similar degree in plasma membranes and both membrane subfractions. Thus, the enzymes which were randomly distributed in the plasma membrane were affected similarly in different domains of the plasma membrane, and inhibited, whereas those enzymes predominantly located in the fraction containing the high-affinity receptor for concanavalin A, i.e., in fraction 2, were activated.

Discussion

Highly purified lymphocyte plasma membranes were fractionated by means of affinity chromatography on concanavalin A-Sepharose into two subfractions with different properties. Both subfractions isolated, fraction 1 which eluted freely and fraction 2 which was specifically retained by the affinity adsorbent, were derived from the plasma membrane. This was evidenced by a number of criteria. (i) The plasma membrane preparations used were highly purified [8,9]. (ii) Both subfractions had an identical cholesterol/phospholipid ratio (see Table I). (iii) Enzymatic surface radioiodination or labelling of surface carbohydrates by $NaBH_4$ reduction was identical (Table II, III). (iv)

TABLE VI

SPECIFIC ACTIVITIES OF MEMBRANE-BOUND ENZYMES IN PLASMA MEMBRANE SUBFRACTIONS OF CONCANAVALIN A-STIMULATED THYMOCYTES

10^{10} lymphocytes in 200 ml Hepes-buffered (pH 7.3) Dulbecco's modified Eagle's medium were stimulated for 60 min at 37°C with 10 μg/ml concanavalin A.

	Activities (nmol/mg protein per min)(37°C)					
	Plasma membrane		Fraction 1		Fraction 2	
	Control	Concanavalin A	Control	Concanavalin A	Control	Concanavalin A
($Na^+ + K^+$)-ATPase	15.2	32.6	4.9	5.9	23.3	42.6
($Na^+ + K^+$)-ATPas + DOC [a]	67.0	111.2	16.5	20.3	90.5	165.8
Oleoyl-Coenzyme A:lysolecithin acyltransferase	3.9	8.0	3.2	3.6	7.3	15.5
Arachidonoyl-Coenzyme A:lysolecithin acyltransferase	15.9	39.7	11.9	14.3	31.9	102.0
Mg^{2+}-ATPase	118.1	101.5	139.1	133.0	121.1	98.4
Mg^{2+}-ATPase + DOC [a]	126.4	106.6	148.8	139.7	129.6	103.4
Alkaline *p*-nitrophenylphosphatase	191.9	103.8	153.5	76.5	217.8	160.2
γ-Glutamyl transpeptidase	16.8	10.9	15.8	11.2	15.3	11.4

[a] Desoxycholate, end concentration 0.05% per mg of protein.

In both fractions a number of plasma membrane marker enzymes showed identical specific activities, including γ-glutamyl transpeptidase, alkaline phosphatase, or Mg^{2+}-ATPase (see Table III).

Upon cell disruption, fragments of the lymphocyte plasma membrane largely form vesicles. Upon cell disruption by nitrogen cavitation, the method used in the present experiments, fragments of the lymphocyte plasma membrane largely form small vesicles with a mean diameter of 70 nm [1]. Nitrogen cavitation has been reported to generally produce vesicles of right-side-out orientation [25]. This could be confirmed in lymphocytes, as matrix-bound sialidase liberated identical amounts of sialic acid from fraction 1 vesicles as from plasma membranes [9] excluding the possibility that this fraction not binding to concanavalin A-Sepharose does not expose carbohydrate to the outer surface.

Although both membrane subfractions share some properties – indicating their exclusive plasma membrane nature as discussed above – there also exist differences such as the specific activity of $(Na^+ + K^+)$-ATPase, and lysolecithin acyltransferase (see Table IV). These differences were even expanded after rechromatography, allowing in the case of $(Na^+ + K^+)$-ATPase the contention that this enzyme is exclusively present in fraction 2 (see Table IV). Apparent differences in enzyme activity of membranes could be due to differences in substrate accessibility. Enzyme 'latency' of sealed right-side-out vesicles has thus been discussed as the source of heterogeneous distribution in plasma membrane fractions [26]. This possibility appeared unlikely, as (i) in both subfractions, 1 and 2, the activity of Mg^{2+}-ATPase was identical, indicating that the plasma membrane vesicles obtained after nitrogen cavitation were at least 'leaky' for ATP (see Table IV). $(Na^+ + K^+)$-ATPase was also measured in the presence of 0.05% desoxycholate per mg of protein, a concentration of this detergent which maximally stimulates the activity of this enzyme (data not shown). Despite this increase, the relative distribution of enzyme activity in fraction 1 and fraction 2 was completely unchanged, indicating that it represented a true difference in the content of $(Na^+ + K^+)$-ATPase in plasma membrane subfractions. Similarly, as the determination of lysolecithin acyltransferase requires a substrate with detergent properties, i.e., lysophosphatidylcholine, and, thus, enzyme latency can be excluded, this enzyme protein is also highly enriched in at least those parts of the plasma membrane enriched in fraction 2.

Although thymus lymphocytes represent a population of pure T-lymphocytes, subfractionation of membrane vesicles obtained from cell suspensions could concern subpopulation of lymphocytes with different membrane properties. Using semipurified plasma membranes of calf thymus lymphocytes, we have shown earlier, [7] that by affinity chromatography on concanavalin A-Sepharose as used here, different areas of the plasma membrane of an individual cell are separated.

Thus, our data clearly demonstrate that lymphocyte plasma membranes are not entirely homogeneous, but contain domains consisting of (at least) two enzymes, $(Na^+ + K^+)$-ATPase and lysolecithin acyltransferase. These specialized membrane areas can be purified, or at least enriched, by specific separation procedures.

In our experiments, the cells were routinely disrupted at 4°C. Lowering the temperature below 30°C leads to disintegration of the cytoskeleton, predominantly the microtubules [27]. In addition, at temperatures below the transition temperature, which in biological membranes lies between 16–18°C, phase separation of lipids may occur [28]. When the lymphocytes were disrupted strictly at 37°C (after being kept for at least 1 h at the same temperature, which allows reformation of microtubules [27]), membrane subfractions could be isolated with properties identical to those from cell disruption at 4°C. This shows that microtubule integrity is not responsible for the observed membrane heterogeneity, suggesting that the domains of the lymphocyte plasma membrane are formed by interactions of their components themselves.

Fractionation of purified plasma membranes into fraction 1 and fraction 2 required the binding of surface receptors to matrix-bound concanavalin A [7]. As the orientation of the plasma membrane vesicles could be excluded as the separation principle (see above), fractionation could be due to the number of concanavalin A binding sites and/or to the affinity of the binding site-interaction. In fact, the fraction retained on concanavalin A-Sepharose, fraction 2, contained significantly less

binding sites. This fraction, however, exhibited binding sites with a higher (average) affinity for concanavalin A, favouring binding affinity as the separation principle (see Fig. 2). The existence of two types of binding site distinct in their affinity for concanavalin A corresponded well to the binding heterogeneity observed in unseparated plasma membranes. In the Scatchard plot, the binding data of unfractionated plasma membranes showed a bend. The binding sites with an apparently lower affinity for concanavalin A correlated well to those present on fraction 1, whereas the one with higher affinity closely resembled those present on fraction 2. Thus, in contrast to mere kinetic data, which cannot rule out other interpretations such as negative co-operativity [29], the separation of plasma membrane fractions according to their binding affinity demonstrates unambiguously that binding sites with high and low affinities exist in the plasma membrane of lymphocytes.

Concanavalin A and other mitogenic lectins are able to induce cell activation and proliferation in lymphocytes [30]. All mitogenic lectins bind to a great number of glycoproteins and even glycolipids [31]. So far, this has hampered the elucidation of the molecular nature of the mitogen receptor, as defined by the molecule(s) upon interaction of which with the mitogen, the lymphocyte is activated. More indirect evidence strongly pointed to a glycoprotein (or glycoproteins) distinct by a higher binding avidity for the mitogen [32]. Moreover, it appears likely that all mitogenic lectins, although exhibiting different oligosaccharide specificities, bind to the same receptor molecule(s) [31]. Taking into account that the mitogen receptor(s) has a higher avidity for a mitogenic lectin, fraction 2 would bear this receptor as part of specific plasma membrane domains.

The assumption that in the plasma membrane of lymphocytes there exist receptor-associated domains bears implications for the initiation of cell activations by mitogenic lectins such as concanavalin A. Such a role can indeed be inferred from experiments with concanavalin A-stimulated lymphocytes [33]. Upon stimulation of intact lymphocytes with concanavalin A, essentially identical subfractions were separated in isolated plasma membranes by affinity chromatography as in plasma membranes from resting cells. As under such conditions, the plasma membrane receptors are reorganized to form 'caps' [31], the receptors must have moved in the plane of the membrane together with their associated enzymes, indicating again strong forces in constructing this functional domain. The enzymes located in this domain, $(Na^+ + K^+)$-ATPase and lysolecithin acyltransferase, were exclusively activated, whereas the enzymes which were randomly distributed in the plasma membrane were inhibited and to a similar extent in the entire membrane (see Table VI). When concanavalin A in increasing concentrations was added to isolated microsomal membranes, i.e., semipurified plasma membranes, the changes in enzyme activities were either absent or different compared to the respective changes observed when intact cells were activated with this mitogen and, subsequently, the membranes were isolated and their catalytic activities analyzed [33]. This strongly suggests that the changes of enzyme activities found in plasma membranes of stimulated cells are not a modification of enzymes in situ, but due to an effect on the whole cell, mediated by the mitogen receptor.

We have shown recently that $(Na^+ + K^+)$-ATPase and lysolecithin acyltransferase are also functionally coupled. Blocking of the activation of $(Na^+ + K^+)$-ATPase by ouabain also prevented activation of lysolecithin acyltransferase [34], supporting a role of receptor associated domains in the initiation of lymphocyte activation.

The existence of specialized plasma membrane areas has long been known in cells with an evident polarity such as liver, intestinal or kidney epithelial cells. More recent evidence also suggested the presence of domains in cells lacking an obvious orientation, such as erythrocytes [35] or single-cell suspensions of liver-derived tumours [36]. In lymphocytes which exist as non-oriented single cells, plasma membrane heterogeneities have been shown morphologically as uropods, microvilli or by ultrastructural staining techniques including mitogen receptors or immunoglobulin [37–40]. Our data show that such domains can be purified, or at least enriched, by specific separation procedures, which opens the possibility for detailed biochemical analysis.

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