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Separation of plasma membrane domains of calf thymocytes by affinity chromatography on ouabain-Sepharose

Marta Szamel [a,b], Margarete Goppelt-Strübe [a], Wolfgang Bessler [c], K.H. Wiesmüller [d] and Klaus Resch [a]

[a] *Division of Molecular Pharmacology, Department of Pharmacology and Toxicology, Medical School Hannover, Hannover (F.R.G.),* [b] *Department of Biochemistry, Semmelweis University Medical School, Budapest (Hungary),* [c] *Institute for Microbiology, University of Tübingen, Tübingen (F.R.G.) and* [d] *Institute for Organic Chemistry, University of Tübingen, Tübingen (F.R.G.)*

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Highly purified plasma membranes of calf thymocytes were fractionated by means of affinity chromatography on ouabain-Sepharose. By the method used two subfractions were obtained, one eluting freely from the affinity gel (MF_{1oua}) and a second specifically retained by matrix-bound ouabain (MF_{2oua}), with a total recovery of 95 per cent. Fractionation required the binding of matrix-bound ouabain to its plasma membrane receptor, i.e. $(Na^+ + K^+)$-ATPase. Increasing the temperature and binding time did not significantly alter the fractionation of plasma membranes into the two subfractions. Both plasma membrane subfractions separated by ouabain-Sepharose were of plasma membrane origin, as revealed by the identical specific activities of several membrane bound enzymes, γ-glutamyl transpeptidase, alkaline phosphatase and Mg^{2+}-ATPase in unseparated plasma membranes and in both subfractions, and by the identical amounts of the cytoskeletal protein actin in unseparated plasma membranes and subfractions. The plasma membrane subfractions MF_{1oua} and MF_{2oua} showed different structural and functional properties. In SDS-polyacrylamide gel electrophoresis polypeptides of 170, 150, 110, 94, 39, and 30 kDa were several-fold enriched in the adherent fraction, MF_{2oua}. The phospholipid fatty acid composition of the plasma membrane subfractions proved to be different, as well. MF_{2oua} contained significantly higher amounts of saturated fatty acids as compared to MF_{1oua}. The specific activities of $(Na^+ + K^+)$-ATPase, Ca^{2+}-ATPase and lysolecithin acyltransferase were highly enriched in the adherent fraction MF_{2oua}, as compared to MF_{1oua}. The data suggest that by the means of affinity chromatography on ouabain-Sepharose plasma membrane domains of the lymphocyte plasma membrane can be isolated, most probably implicated in the initiation of lymphocyte activation.

Introduction

$(Na^+ + K^+)$-ATPase is an ubiquitous integral constituent of cellular plasma membranes. The enzyme represents the monovalent cation pump and is the unique and specific receptor for cardiac glycosides such as ouabain [1]. Ouabain has been

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; SDS, sodium dodecylsulphate.

Correspondence: K. Resch, Medizinische Hochschule Hannover, Abteilung Molekularpharmakologie, Konstanty-Gutschow-Strasse 8, D-3000 Hannover 61, F.R.G.

shown to prevent the mitogenic activation of lymphocytes [2]. As concentrations of this drug effectively suppressed the induction of macromolecular synthesis in lymphocytes, by mitogens such as concanavalin A, which did not affect ion transport, this suggested that $(Na^+ + K^+)$-ATPase is involved in the growth regulation of cells such as lymphocytes independent of its ion pumping activity [3].

In previous studies we have shown that in lymphocytes, cells with no apparent polarity, the plasma membrane is not entirely homogeneous, but exhibits a mosaic structure. On the basis of affinity chromatography on Con A-Sepharose two subfractions of the plasma membrane could be separated [4]. The fraction binding specifically to Con A-Sepharose and carrying receptors with high affinity for concanavalin A (designated MF2), was distinct from the non-binding bulk fraction (designated MF1) [5]. Thus several enzymes were several-fold enriched in MF2, including $(Na^+ + K^+)$-ATPase [4,5]. In addition, the protein pattern as well as the phospholipid composition proved to be different in both plasma membrane subfractions, suggesting the existence of structural domains in the plasma membrane of lymphocytes. As concanavalin A is a potent mitogen for lymphocytes by binding to high-affinity receptors, we suggested that these domains are implicated in the regulation of growth and division of lymphocytes [4,5].

The high specificity of the interaction between $(Na^+ + K^+)$-ATPase and ouabain prompted the idea to fractionate highly purified lymphocyte plasma membranes also by binding to insolubilized ouabain. Here we report that by affinity chromatography on ouabain-Sepharose a membrane subfraction could be isolated, which appeared to be structurally as well as functionally identical to that obtained by affinity chromatography on Con A-Sepharose.

Materials and Methods

Isolation of plasma membranes by differential and sucrose gradient centrifugation

Plasma membranes of calf thymocytes were isolated as described earlier [4,6]. Thymus was freed of adherent tissue, cut into small pieces and macerated gently in a loosely fitting glass homogenizer to yield the lymphocytes. Cell suspensions were filtered twice through small funnels containing nylon wolls (Leuko-Pak, Fenwall Lab., Travenol) to remove tissue remnants. Lymphocytes were then sedimented and suspended in cell disruption buffer (0.14 mol/l KCl, 0.02 mol/l Hepes (pH 8.0), 0.25 mmol/l $MgCl_2$). All steps until the final cell suspension were carried out at room temperature, which resulted in optimal viability of the lymphocytes.

The lymphocytes were chilled in ice and all further steps were carried out strictly in the cold (unless stated otherwise for some experiments). Lymphocytes ($2 \cdot 10^8$ cells/ml in cell disruption buffer) were disrupted in an Artisan pressure homogenizer by nitrogen cavitation, 30 atm for 20 min, and then released dropwise. Immediately after release the cell homogenate was made 1 mmol/l with EDTA. Large granules (containing nuclei, mitochondria and lysosomes) were sedimented for 20 min at $20000 \times g$, the microsomal fraction containing vesicles derived from the plasma membrane and the endoplasmic reticulum was sedimented by $250000 \times g_{max}$ for 1 h. After shocking the microsomal membranes hypotonically (0.02 mol/l Hepes (pH 8.0)) to remove trapped cytoplasmic protein, the membranes were suspended in plasma membrane buffer (0.02 mol/l Hepes (pH 8.0), 0.14 mol/l KCl) and layered on top of 35% (w/w) sucrose in plasma membrane buffer. After centrifugation for 2 h at $250000 \times g_{max}$ the plasma membrane formed a band at the interface between sucrose and buffer. Plasma membranes were collected, the sucrose was removed by dialysis overnight against a agradient of a total of 2000 ml 20% sucrose (w/w) in 0.02 mol/l Hepes, 0.14 mol/l NaCl (pH 7.4), and finally for 4 h against 250 ml of the same buffer. This slow removal of sucrose was essential to prevent membranes from irreversible aggregation. The preparation obtained by this method contained plasma membrane vesicles with a mean diameter of 50–100 nm. The plasma membranes were either processed immediately or kept frozen at $-80\,^\circ C$ batchwise.

Preparation of ouabain-Sepharose

5 g CH-Sepharose 4B were swollen in 75 ml 0.5 mol/l NaCl and washed with 1 l of 0.5 mol/l

NaCl and 0.5 l destilled water. 760 mg ouabain (Boehringer, Mannheim) were dissolved in 20 ml of 65% dioxane/35% destilled water. CH-Sepharose and ouabain were mixed and 19 ml water was added (pH 5.3). 820 mg carbodiimide (Merck) dissolved in 1 ml destilled water was added dropwise, pH was adjusted to 4.7, and the reaction mixture shaken for 48 h at room temperature. Thereafter, it was washed consecutively with 0.1 mol/l H_2CO_3 buffer (pH 10), distilled water and 0.1 M phosphate buffer (pH 5.5), containing 1 mol/l NaCl and 0.01% NaN_3. The latter buffer was also used for storage at 4° C. Before use ouabain-Sepharose was washed with 0.02 mol/l Hepes, 0.14 mol/l NaCl, 2 mmol/l $MgCl_2$ (pH 7.4).

Isolation of plasma membrane fractions from cell homogenates by affinity chromatography on ouabain-Sepharose

Lymphocytes were homogenized by the nitrogen cavitation method as described above with the exception that a disruption buffer containing 0.02 mol/l Hepes (pH 7.4), 0.14 mol/l NaCl and 0.5 mmol/l $MgCl_2$ was used. The cell homogenate was centrifuged by $500 \times g$ for 10 min to get rid of the nuclei and the nucleus-free homogenate (500 g supernate) processed to affinity chromatography. 10 ml ouabain-Sepharose (25×27 mm column) was rinsed with at least three times the gel volume with 0.02 mol/l Hepes (pH 7.4), 0.14 mol/l NaCl, 0.5 mmol/l Mg^{2+}-ATP and then the buffer was left at a level just keeping the gel wet. To 10 ml ouabain-Sepharose 5 mg nucleus-free cell homogenate (500 g supernate) was added. Ouabain-Sepharose and cellular proteins were mixed thoroughly by stirring for 2 min with a motor driven stirrer (200 rpm). After mixing, ouabain-Sepharose and membranes were kept for 60 min at 4° C. The fraction, not binding to ouabain-Sepharose, was eluted with 0.02 mol/l Hepes, 0.14 mol/l NaCl (pH 7.4) (fraction 1), the proteins bound to immobilized ouabain were eluted with 0.02 mol/l Hepes, 0.14 mol/l KCl (pH 7.0) (fraction 2).

Affinity chromatography of plasma membrane vesicles on ouabain-Sepharose

To 10 ml ouabain-Sepharose pretreated as described above, 1 mg plasma membranes was added in 2–3 ml 0.02 mol/l Hepes (pH 7.4), 0.14 mol/l NaCl. Ouabain-Sepharose and membranes were mixed thoroughly by stirring with a motor-driven stirrer (200 rpm). After mixing, ouabain-Sepharose and membranes were kept for 60 min at 4° C (except for the experiments indicated).

The fraction of plasma membranes not binding to ouabain-Sepharose was eluted with 0.02 mol/l Hepes (pH 7.4), 0.14 mol/l NaCl, 2 mmol/l $MgCl_2$. Elution velocity was set to a level, which did not cause perturbation of the gel, usually 2–3 ml/min. The membrane fraction eluting freely from the affinity gel, was designated MF_{1oua}. After complete elution of MF_{1oua}, the mebrane material bound to ouabain-Sepharose was eluted with 0.14 mol/l KCl in 0.02 mol/l Hepes (pH 7.0). The bound fraction was designated MF_{2oua}. In some experiments (as indicated) a third fraction was collected with constant stirring of the gel and designated MF_{3oua}. Elution of membrane protein was followed with a spectrophotometer at 280 nm (Uvicord II, LKB), and the fractions collected batchwise. With the exception of the experiments where the influence of temperature was studied the entire separation procedure was carried out strictly at 4° C. Membrane subfractions were concentrated by ultracentrifugation for 2 h at 250000 $\times g_{max}$ and resuspended in plasma membrane buffer.

Protein determination

Protein was measured by its native fluorescence as described earlier [7] using a fluorescence spectrometer, SP 500 (Aminco) or by the method described by Lowry et al. [8].

Radioactive labelling

Plasma membrane glycoproteins were labelled by sodium borohydride reduction of galactose oxidase-treated membranes [9]. 10 ml plasma membranes containing 2 mg membrane protein were first reacted for 5 min with unlabelled $NaBH_4$ (2 mmol/l) 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 oxydase (Boehringer, 25 units) for 15 min at 37° C. The aldehydes were reduced with

tritiated $NaBH_4$ at a concentration of 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 mol/l NaOH. The plasma membranes were washed twice in plasma membrane buffer (centrifugation 60 min at $250\,000 \times g_{max}$) and resuspended in 0.02 mol/l Hepes (pH 7.4), 0.14 mol/l NaCl, as indicated.

Enzyme assays

ATPases (EC 3.6.1.3) were measured as described earlier [10], the enzyme sensitive to 1 mmol/l ouabain was designated $(Na^+ + K^+)$-ATPase, the insensitive enzyme Mg^{2+}-ATPase. Ca^{2+}-ATPase was measured as described by Lichtman et al. [11]. Liberated phosphate was determined by the method of Sanui [12]. Alkaline *p*-nitrophenyl phosphatase (EC 3.1.3.1), and γ-glutamyl transpeptidase (EC 2.3.2.2) were measured as described earlier [4,5], with 5 mmol/l *p*-nitrophenylphoshate and 1-γ-glutamyl-3-carboxy-4-nitroanilide as substrates, respectively. Lysolecithin acyltransferase (EC 2.3.1.23) was measured as described earlier [4,5], with 50 μmol/l lysolecithin (1-[1-^{14}C]palmitoyl-*sn*-glycerol-3-phosphocholine, specific activity 0.5 mCi/mmol, made by mixing unlabelled (Sigma) and labelled (Amersham Buchler) substrate) and 30 μmol/l arachidonoyl-coenzyme A as substrates. The phospholipids were extracted as described and separated by thin-layer chromatography [13]. Enzyme activities were calculated from the conversion of the labelled precursor into phosphatidylcholine. Arachidonoyl-coenzyme A was synthesized according to Reitz et al. [14].

Fatty acid determination

Lipids were extracted as described previously [15]. Phospholipids were separated from neutral lipids by thin-layer chromatography on silica gel plates (Schleicher and Schüll) with the solvent system hexane/diethyl ether/acetic acid (80 : 20 : 2, v/v). The phospholipids remained at the starting line. They were scraped off and transmethylated in the presence of silica gel. Transmethylation was performed with sodium methylate as described [16]. The fatty acid methyl esters were analysed by capillary GLC (Fractovap a 4160, ERBA Strumentatione, FFAP column, carrier gas H_2). Peaks were identified by standard fatty acid methyl esters. Some peaks were only partially characterized by the 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.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out according to Laemmli [17]. Stacking gel was 5% and running gel 12.5% in acrylamide (Bio-Rad). Electrophoresis was performed at a constant current of 40 mA for 3 h. 150 μg of membrane protein was solubilized in sample buffer (0.625 mol/l Tris (pH 6.8), 2% SDS, 5% β-mercaptoethanol, and 10% glycerol) at 100 °C for 3 min. Gels were stained with Coomassie brilliant blue-R-250 (Sigma), 0.1% in methanol/acetic acid/water (3 : 6 : 75, v/v) for 30 min at 60 °C and destained by 7% acetic acid overnight.

Results

Separation of plasma membranes from the nucleus-free cell homogenate by affinity chromatography on ouabain-Sepharose

When a nucleus-free cell homogenate ($500 \times g$ supernate) of calf thymocytes was processed by affinity chromatography on ouabain-Sepharose in the presence of 0.02 mol/l Hepes, 0.14 mol/l NaCl, 0.5 mmol/l MgATP (pH 7.4), 90% of the applied protein eluted freely from the affinity gel, whereas 5% were specifically bound by ouabain-Sepharose and could be eluted by 0.02 mol/l Hepes, 0.14 mol/l KCl (pH 7.0). Table I summarizes the characteristics of both fractions obtained by affinity chromatography on matrix-bound ouabain.

The specific activities of several membrane bound enzymes, e.g. of $(Na^+ + K^+)$-ATPase, γ-glutamyl transpeptidase, and lysolecithin acyltransferase, were several-fold enriched in the adherent fraction, showing that by the help of ouabain-Sepharose plasma membranes, containing the specific ouabain receptor $(Na^+ + K^+)$-ATPase were isolated. Pretreatment of the affinity gel with 0.14 mol/l KCl or with 1 mmol/l ouabain prevented the separation, e.g. the total protein was recovered as a single fraction (data not shown).

TABLE I

CHARACTERIZATION OF PLASMA MEMBRANE FRACTIONS ISOLATED BY AFFINITY CHROMATOGRAPHY ON OUABAIN-SEPHAROSE FROM CALF THYMOCYTES

Calf thymocytes were homogenized as described in Materials and Methods. Cell homogenate was centrifuged at 500 × *g* for 10 min and the nucleus free homogenate (500 × *g* supernate) applied to the affinity column. Fraction 1 and 2 were eluted as described in Materials and Methods. Results are means of triplicates. γ-GT, γ-glutamyl transpeptidase; LAT, lysolecithin acyltransferase; n.d., not detectable.

	Protein (mg)	Specific activities (nmol·(mg protein)$^{-1}$·min^{-1}; 37 °C)			
		Mg^{2+}-ATPase	$(Na^+ + K^+)$-ATPase	γ-GT	LAT
Homogenate	7.75	8.2	2.3	0.59	2.8
500 × *g* supernate	5.00	11.4	3.2	0.82	3.9
Fraction 1	4.50	4.2	n.d. [3]	n.d.	2.4
Fraction 2	0.28	132.4	53.8	13.50	28.9

Separation of palsma membrane subfractions by ouabain-Sepharose

When plasma membrane vesicles of calf thymocytes were allowed to bind to ouabain-Sepharose in the presence of 140 mmol/l NaCl, 0.5 mmol/l MgATP in 20 mmol/l Hepes (pH 7.4), for 60 min, 75% of the plasma membrane material eluted freely from the affinity gel (MF_{1oua}), whereas 20% were specifically bound by ouabain-Sepharose and could be eluted by 140 mmol/l KCl in 20 mmol/l Hepes (pH 7.0) (MF_{2oua}) (Fig. 1). Binding of ouabain to its receptor, e.g. $(Na^+ + K^+)$-ATPase, was an absolute requirement for fractionation, as membranes equilibrated with ouabain-Sepharose in the presence of 1 mmol/l free ouabain or 140 mmol/l KCl could be eluted in a single fractin (data not shown).

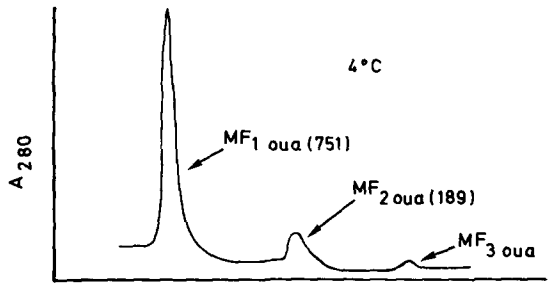

Fig. 1. Separation of plasma membrane subfractions by ouabain-Sepharose. 10 ml ouabain-Sepharose 4B was washed and activated as described in Materials and Methods. 1 mg plasma membrane was allowed to bind in the presence of 20 mmol/l Hepes, 140 mmol/l NaCl, 0.5 mmol/l MgATP (pH 7.4) for 60 min. MF_{1oua} was eluted by 140 mmol/l NaCl, 2 mmol/l $MgCl_2$, 20 mmol/l Hepes (pH 7.4) and MF_{2oua} by 20 mmol/l Hepes, 140 mmol/l KCl (pH 7.0). The temperature was kept constant during the entire separation procedure. The figure represents the drawing of the absorbance recorded with a spectrophotometer (UA5 ISCO Instruments) at 280 nm. Numbers in parenthesis represent the amount of protein in micrograms. Total recovery is based on colorimetric protein determination.

To easily follow the distribution of plasma membranes into subfractions the membranes were labelled with tritiated $NaBH_4$. Under the binding conditions described above, 95% of the membrane protein were recovered (Table II). In addition to the non-adherent fraction, MF_{1oua}, and the adherent one, MF_{2oua}, a third minor fraction could be eluted by mechanical forces, e.g. by stirring of the affinity gel. As only a very small amount of protein was found as MF_{3oua}, this indicates that the procedure used for the elution of MF_{2oua}, nearly quantitatively released the membrane vesicles bound to ouabain-Sepharose. As can be seen in Table II, approximately 1% of the membrane material applied was found remaining in the affinity gel.

Conditions influencing the separation of membrane vesicles on ouabain-Sepharose

In a set of experiments, various conditions were analysed, which could effect separation of membrane fractions by ouabain-Sepharose.

To achieve optimal binding conditions, the concentration of NaCl and that of MgATP were varied between 80 to 140 mmol/l or 0.5 to 5 mmol/l, respectively. As can be seen in Table III, neither variations of the NaCl concentrations in this range, nor the elevation of the concentration

TABLE II

FRACTIONATION OF NaB^3H_4 LABELLED PLASMA MEMBRANES ON OUABAIN-SEPHAROSE

1 mg plasma membrane (containing $2.63 \cdot 10^5$ cpm) was fractionated on ouabain-Sepharose as described in Materials and Methods. The numbers in parenthesis are percentages of recovered radioactivity.

Counts per min ($\times 10^{-3}$)				
MF_{1oua}	MF_{2oua}	MF_3	ouabain-Sepharose [a]	Recovery (%)
199 (75.5)	49 (18.6)	4.9 (1.9)	2.6 (1.0)	97

[a] Amount of label remaining in the gel.

of MgATP influenced significantly the amount of membrane vesicles bound. In order to find out the concentration of KCl required to desorb the bound membrane vesicles a continuous KCl gradient ranging from 0 to 0.14 mmol/l KCl in 0.02 M Hepes (pH 7.0), was used. Ionic strength was kept constant using choline chloride. As shown in Fig. 2, 0.04 mol/l KCl was effective in releasing adhered plasma membrane vesicles from the affinity gel.

In the absence of KCl, addition of 1 mmol/l ouabain to the elution buffer was also effective to recover this fraction from the affinity column (data not shown).

Binding time

To determine the effect of time on the binding of plasma membranes to ouabain-Sepharose, membranes were incubated for different lengths of time with matrix bound ouabain and separated into the subfractions (MF_{1oua} and MF_{2oua}) after different time intervals. The amount of plasma membranes binding to ouabain-Sepharose and therefore being recovered in MF_{2oua}, was maximal after 60 min equilibration and did not increase after longer incubation times (Table III). A similar binding time was required when affinity chromatography was carried out at 4 °C or 25 °C (data see below).

Temperature

In these experiments the temperature was kept constant during the whole separation procedure, and binding of plasma membranes allowed for 60 min. At 4, 25, and 37 °C, the same amount of membrane protein was bound by ouabain-Seph-

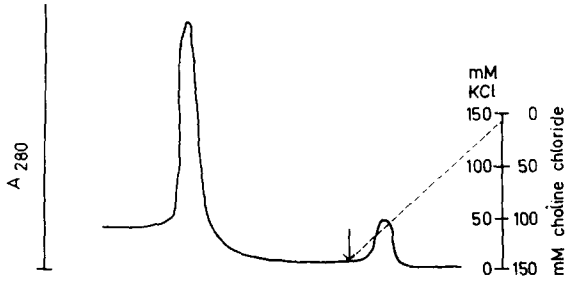

Fig. 2. Effect of potassium on the elution of plasma membrane vesicle bound to ouabain-Sepharose. 1 mg plasma membrane protein was separated by affinity chromatography on ouabain-Sepharose as described in Materials and Methods (binding time: 60 min, 4 °C). MF_{1oua} was eluted by 20 mmol/l Hepes, 140 mmol/l NaCl (pH 7.4). MF_{2oua} was eluted by a continuous KCl gradient ranging from 0 to 140 mmol/l. Ionic strength was kept constant by choline chloride. Results represent a typical experiment from three similar ones.

TABLE III

EFFECT OF SODIUM AND Mg^{2+} ATP ON THE BINDING OF CALF THYMOCYTE PLASMA MEMBRANES TO OUABAIN-SEPHAROSE

Concentration (mmol/l)		Time (min)	Membrane protein bound to ouabain-Sepharose i.e. recovered in MF_{2oua} (μg)
NaCl	Mg^{2+}ATP		
80	0.5	60	192
100	0.5	60	186
120	0.5	60	190
140	0.5	60	195
140	1.0	60	187
140	2.0	60	183
140	5.0	60	194
140	0.5	20	125
140	0.5	30	165
140	0.5	120	182

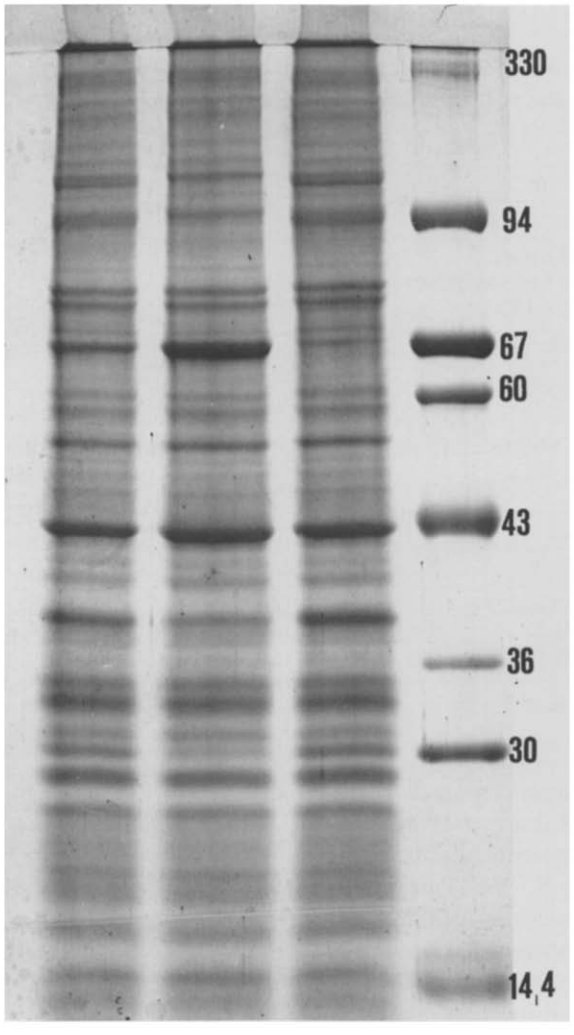

Fig. 3. Polypeptide composition of the plasma membrane subfractions separated by affinity chromatography on ouabain-Sepharose. Highly purified plasma membranes from calf thymocytes were fractionated into MF_{1oua} and MF_{2oua} by affinity chromatography on ouabain-Sepharose, as described in Materials and Methods. Plasma membranes and subfractions were concentrated by ultracentrifugation and taken up in sample buffer, containing 0.1% Bromophenol blue for SDS-polyacrylamide gel electrophoresis. 150 μg protein per gel was run at 40 mA constant current for 3 h. Gel slabs were stained by Coomassie brilliant blue and destained by acetic acid as described in Materials and Methods. Marker proteins used: thyroglobulin subunit (330 kDa), phosphorylase *b* (94 kDa), albumin (67 kDa), catalase (60 kDa), ovalbumin (43 kDa), lactate dehydrogenase (36 kDa), carbonic anhdrase (30 kDa), α-lactalbumin (14.4 kDa). Slots from left to right: plasma membrane, MF_{1oua}, MF_{2oua}.

arose and recovered in MF_{2oua}. There were small variations in the amount of MF_{1oua} recovered, which was highest at 4° C and slightly decreased with increasing temperature (data not shown). Accordingly, with the increased recovery of MF_{1oua} total recovery of membrane protein was highest at 4° C, being consistently approx. 95%.

On behalf of these data all further separation experiments were carried out at 4° C, binding allowed for 60 min in a buffer containing 20 mmol/l Hepes (pH 7.4), 140 mmol/l NaCl, 0.5 mmol/l MgATP, and the adherent fraction MF_{2oua} eluted with 140 mmol/l KCl in 20 mmol/l Hepes (pH 7.0).

Characterization of the plasma membrane subfractions obtained by affinity chromatography on ouabain-Sepharose

The subfractions of lymphocyte plasma membranes obtained after affinity chromatography exhibited different structural and functional properties. Table IV depicts the separation experiments, in which enzyme activities were measured. The specific activities of γ-glutamyl transpeptidase, alkaline phosphatase and MG^{2+}-ATPase were nearly identical in the unseparated plasma membranes and in the subfractions MF_{1oua} and MF_{2oua}. In contrast, some enzymes, i.e. Ca^{2+}-ATPase, acyl-CoA : lysolecithin acyltransferase and $(Na^+ + K^+)$-ATPase itself, were highly enriched in the adherent fraction MF_{2oua} as compared to the non-adherent one.

Characterization of the polypeptide pattern of calf thymocyte plasma membranes in their subfractions

As can be seen in Fig. 3, the overall polypeptide pattern of the plasma membrne and of the subfractions MF_{1oua} and MF_{2oua} were broadly similar. Among the major cytoskeletal proteins, actin (molecular mass 46 kDa), which was identified by comigration with a purified marker, was present in both subfractions in identical amounts. However, some plasma membrane proteins were markedly enriched in MF_{2oua} compared to MF_{1oua}. The most strinking differences were observed in the amounts of 170, 150, 110, 94, 39, and 30 kDa polypeptides. 5 of 6 polypeptides proved to be glycoproteins, the 39 kDa polypeptide was shown to be nonglycosylated as it failed to be labelled by $NaBH_4$ reduction and was not stained by glycoprotein staining

TABLE IV

PROPERTIES OF THE PLASMA MEMBRANE SUBFRACTIONS

Results are means ± S.D. of three separate preparations. γ-GT, γ-glutamyl transpeptidase; AP, alkaline phosphatase; LAT, acyl-CoA : lysolecithin acyltransferase; n.d., not detectable.

	Marker enzyme activities (nmol·(mg protein)$^{-1}$·min^{-1}; 37 °C)					
	Mg^{2+}-ATPase	$(Na^+ + K^+)$-ATPase	γ-GT	LAT	Ca^{2+}-ATPase	AP
Plasma membrane	132.0 ± 6.6	40.3 ± 2.1	12.6 ± 0.6	19.9 ± 1.1	13.3 ± 0.6	215 ± 10
MF_{1oua}	167.2 ± 8.3	18.0 ± 0.9	12.8 ± 1.0	14.0 ± 0.7	n.d.	238 ± 11
MF_{2oua}	139.6 ± 6.9	67.5 ± 3.4	12.6 ± 0.8	53.1 ± 2.5	51.2 ± 2.5	229 ± 11

TABLE V

PHOSPHOLIPID FATTY ACID COMPOSITION OF THE PLASMA MEMBRANE SUBFRACTIONS OBTAINED BY AFFINITY CHROMATOGRAPHY ON OUABAIN-SEPHAROSE

	Composition						
	16:0	18:0	18:1	18:2	20:n [a]	20:4	22:n [a]
Plasma membrane	11.9	23.2	21.7	11.6	9.8	14.5	7.3
MF_{1oua}	10.7	25.8	23.7	11.2	9.6	12.2	6.9
MF_{2oua}	18.4	28.4	29.2	11.1	6.3	5.3	2.6

[a] 20:n, 22:n, three distinct peaks, each with more than one double bound.

(Szamel, M., Kaever, V. and Resch, K., in the press). MF_{1oua} contained several polypeptides of apparent molecular masses of 65, 60 and 57 kDa that were present at significantly higher amounts as compared to MF_{2oua}.

Characterization of the phospholipid fatty acid composition of plasma membrane subfractions

As can be seen in Table V, the phospholipid fatty acid composition of the plasma membrane subfractions MF_{1oua} and MF_{2oua} proved to be different, as well. Whereas the amounts of the saturated fatty acids, palmitic and stearic acid, were enhanced in the phospholipids of MF_{2oua}, a concomitant decrease in the amounts of unsaturated fatty acids was observed in the adherent fraction MF_{2oua} as compared to the non-adherent fraction MF_{1oua}. In contrast, no significant differences were detectable in the phospholipid composition of the subfractions (data not shown).

Discussion

Affinity chromatography with insolubilized ligands has been established as a useful tool for easily separating different cellular proteins [18]. Making use of the very specific interaction between ouabain and its cell surface receptor, $(Na^+ + K^+)$-ATPase, we here succeeded in isolating fractions of cellular organelles, i.e. the plasma membrane from a nucleus-free homogenate of calf thymocytes. This was achieved in a one-step procedure, by chromatography on ouabain-Sepharose. Approximately 90% of the cellular protein added to the affinity gel were not retained, only about 5% were bound specifically in the absence of K^+ ions, and were eluted in the presence of KCl above 40 mmol/l. That the fraction adhering to ouabain-Sepharose was derived from the plasma membrane was evidenced by the fact that a number of plasma membrane marker enzymes were enriched 10–15-fold.

A number of methods have been developed to isolate plasma membranes in a purified form, including differential and gradient ultracentrifugation [4,5,6,23]. Compared to these, affinity chromatography on ouabain-Sepharose is much simpler, and especially much less time consuming. In addition the overall recovery of plasma membrane material was even higher than those obtained by

established methods. Although the membranes isolated by affinity chromatography appear to be derived exclusively from the plasma membrane, they do not represent the entire plasma membrane, but rather specific domains thereof. This was initially suggested by the finding that some plasma membrane bound enzymes, including $(Na^+ + K^+)$-ATPase, exhibited higher specific activities when compared to conventionally purified plasma membrane, whereas others were similar (see Table I and Table IV).

Indeed, using affinity chromatography on ouabain-Sepharose, plasma membranes, which had been highly purified by differential and sucrose gradient ultracentrifugation [4,5], could be further fractionated. By the method used, two plasma membrane subfractions were obtained, one eluting freely from the affinity gel, MF_{1oua}, the second one being retained specifically by ouabain-Sepharose, MF_{2oua}. The total recovery in both fractions was 95%, MF_{1oua} containing about 75%, and MF_{2oua} containing about 20% of the membrane protein applied to the gel (Fig. 1).

Binding of ouabain to its membrane receptor, $(Na^+ + K^+)$-ATPase, proved to be required for the separation of the plasma membrane subfractions. We have shown earlier that chromatography on the matrix Sepharose-4B alone yielded a single, non-adherent fraction [19]. More specifically, binding of plasma membrane material to ouabain-Sepharose could be prevented completely either by excess free ouabain (1 mmol/l), or by high concentrations of potassium. This indicated that the fractionation was due to affinity chromatography.

There exist several possibilities that might be responsible for the observed separation of plasma membrane subfractions on ouabain-Sepharose. (i) The fractions were derived from different membranes, i.e. plasma membranes versus internal membranes. However, plasma membranes as used in these experiments, were highly purified as shown by a high cholesterol to phospholipid ratio, as well as a more than 30-fold enrichment of plasma membrane marker enzymes such as alkaline phosphatase, γ-glutamyl transpeptidase, lysolecithin acyltransferase, or $(Na^+ + K^+)$-ATPase [4,5]. Accordingly, the plasma membranes were devoid of enzyme activities of γ-glucuronidase, acid phosphatase or succinate dehydrogenase ruling out contamination with intracellular membrane components like mitochondria or lysosomes [4,5]. Moreover, some plasma membrane associated proteins, such as actin (see Fig. 3), or plasma membrane bound enzymes, such as γ-glutamyl transpeptidase, Mg^{2+}-ATPase, or alkaline phosphatase, were represented in identical amounts in plasma membranes, as well as in MF_{1oua} or MF_{2oua}. This strongly suggests that both subfractions are derived from an identical cellular compartment, i.e. the plasma membrane. (ii) Fractionation was due to different orientation the plasma membrane vesicles. This theoretical possibility is ruled out by the finding that specific membrane proteins segregated differently into the membrane subfractions (see Fig. 3 and Table IV). In addition, we have shown previously that plasma membrane vesicles obtained after disrupting the cells by nitrogen cavitation as used here preserved their original orientation, i.e. were exclusively right-side out [4]. (iii) Plasma membranes from different cells having different affinities towards ouabain were separated. Such a possibility appears unlikely as thymus lymphocytes consist nearly exclusively of T-lymphocytes, thus representing a homogeneous cell lineage [20]. Moreover, intact thymus lymphocytes all bound to ouabain-Sepharose (data not shown), indicating that the plasma membrane as a whole of all cells would be retained on ouabain-Sepharose. (v) The membrane fractions originated from different areas of the plasma membranes of an individual lymphocyte.

Taken together, the results discussed above strongly suggest, that both subfractions, MF_{1oua} and MF_{2oua}, indeed resemble different domains of the the plasma membrane. This is supported by the distinct properties of both membrane subfractions. Thus the specific activities of certain plasma membrane bound enzymes, $(Na^+ + K^+)$-ATPase, Ca^{2+}-ATPase, and lysolecithin acyltransferase, were highly enriched in MF_{2oua}, and depleted in MF_{1oua} (see Table IV). The subfractions not only showed differences in their functional properties, but also in their polypeptide and phospholipid fatty acid composition. MF_{2oua} contained a set of proteins with apparent molecular masses of 170, 150, 94, 39, and 30 kDa, that were present at significantly lower amounts in the non-binding

fraction MF_{1oua}. The latter fraction, on the other hand, contained higher amounts of several polypeptides in the 50–65 kDa range. Moreover, the phospholipid fatty acid composition was also different. While the amount of saturated fatty acids was significantly higher in MF_{2oua} as compared to MF_{1oua}, concomitantly the content of unsaturated fatty acids was decreased in the fraction (see Table V). Thus, by means of affinity chromatography on ouabain-Sepharose a subfraction of highly purified plasma membranes was isolated, the properties of which suggest the existence of membrane domains consisting of a set of membrane (glyco)proteins, among others of some functionally important enzymes, embedded in a phospholipid milieu, distinct from that of the bulk membrane.

Previously, we have obtained two different plasma membrane subfractions by affinity chromatography on Con A-Sepharose, termed MF1 for the non-binding and MF2 for the binding fraction [4,5]. The fact, that $(Na^+ + K^+$-ATPase, together with Ca^{2+}-ATPase and lysolecithin acyltransferase segregated highly preferentially in MF2, prompted us to approach plasma membrane subfractionation also by means of binding to its high-affinity ligand ouabain. The biochemical composition as well as the functional properties of the plasma membrane subfractions obtained by affinity chromatography on Con A-Sepharose were nearly identical to those isolated by ouabain-Sepharose. Hence, the identical functional ultrastructure of MF2 and MF_{2oua} strengthens the idea of the domain structure of the T-lymphocyte plasma membrane.

By fractionation of plasma membrane vesicles on Con A-Sepharose the adherent fraction (MF2) bore the high-affinity mitogen receptor(s) embedded in its natural environment, thus representing receptor associated domains [4,5,19,21]. Upon concanavalin A stimulation only the enzymes associated with the high-affinity mitogen receptor were activated suggesting the implication of plasma membrane domains in the regulation of lymphocyte activation.

Further analysis of functional plasma membrane domains may help to understand the role of the plasma membrane in regulating cell activity.

As many cellular processes involve $(Na^+ + K^+)$-ATPase, our results thus might be of general interest, as affinity chromatography on ouabain-Sepharose could serve as a useful tool in isolating membrane vesicles, highly enriched in $(Na^+ + K^+)$-ATPase activity also in other cell types.

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