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Lipid bilayer dynamics in plasma and coated vesicle membranes from bovine adrenal cortex. Evidence of two types of coated vesicle involved in the LDL receptor traffic

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Pure coated vesicles have been prepared from the bovine adrenal cortex and two homogeneous populations have been separated, one of large diameter (100 nm) and one of small diameter (70 nm). The chemical composition in lipids and proteins of coated vesicles has been compared with that of partially purified plasma membranes and evidences a higher protein/lipid ratio and a higher concentration in phosphatidylethanolamine and unsaturated fatty acids. Evaluation of the lateral diffusion of pyrene in the lipid bilayer of coated vesicles as compared to uncoated vesicles evidences a slowing-down effect of clathrin. Measurements of lipids' rotational diffusion by time-resolved fluorescence indicate a decrease in the order parameter of the lipids in the coated vesicles due to clathrin. A hypothesis is proposed for a possible role of the clathrin coat in the concerted motion of lipids and proteins toward coated pits and in the mechanism of formation of coated vesicles. Separation of the large from the small coated vesicles made it possible to reveal different protein components in the two types of vesicle by electrophoresis and autoradiograms of the [γ-^{32}P]adenosine triphosphate- (ATP-) treated vesicles. Visualisation of the low-density lipoprotein receptor by ligand blotting and enzyme-linked immunosorbent assay (ELISA) techniques indicates an increased low-density lipoprotein receptor binding capacity in small coated vesicles as compared to large ones and plasma membranes.

Introduction

Steroidogenesis constitutes the main function of mammalian adrenocortical cells with cholesterol as the first substrate of the corticosteroid biosynthetic pathway This whole process is therefore dependent on two membrane processes a long-range hormonal stimulation by the adrenocorticotropic pituitary hormone (ACTH) and a short range process, the internalisation of cholesterol carried by the plasmatic low-density lipoprotein [1]

Since it has been shown that all the steroidogenic enzymes in adrenocortical cells are membrane proteins [2] dependent on mitochondrial and endoplasmic reticulum membrane structure and dynamics [3], it was of interest to analyse further the plasma membrane processes related to

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Abbreviations SDS, sodium dodecyl sulfate, Suramin, hexasodium-bis(*m*-aminobenzoyl-*N*-amino-*p*-methylbenzoyl-1-naphthylamino-3,6,8-trisulfonate) carbamide, LDL, low-density lipoproteins, Mes, 4-morpholineethanesulfonic acid, EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, DMSO, dimethylsulfoxide, Pna, (9,11,13,15)-octadecatetraenoic acid, ELISA, enzyme-linked immunosorbent assay

the entry into the cell of LDL carrying the cholesterol the formation of the coated pits where the LDL receptors cluster and their transformation into coated vesicles under the influence of the specific coat protein clathrin

In the current study we have isolated these vesicles from the adrenocortical cells, have analysed their chemical composition in comparison with the whole plasma membrane and have given more insight into the role of the clathrin network in the lipid bilayer structure and dynamics For the first time we have separated two different types of coated vesicle which could correspond to those described cytochemically as originating from the plasma membranes and the Golgi apparatus [4] The presence of the LDL receptor has been evidenced in these different membrane materials representing different steps of the internalisation process of LDL and its receptor

Experimental procedures

Materials

Suramin (Germanin) is a Bayer 205 product Triton X-100, Amido black 10B, bovine serum albumin and a high molecular weight SDS kit were from Sigma SDS was obtained from BDH Pyrene was from Aldrich Parinaric acids (*cis* and *trans*) were purchased from Molecular Probes (Oregon) DC kiesel gel platten 60 were from Merck β-D-Octylglucopyranoside came from Riedel Rouge Ponceau S was obtained from Sebia 3,3′-Diaminobenzidine tetrachloride was from Interchim Nitrocellulose paper, 0 45 μm pore size, was obtained from Bio-Rad All other chemicals were analytical grade Merck products

LDL and rabbit LDL antibodies were kindly provided by M Ayrault-Jarrier Goat anti-rabbit antibody horseradish peroxidase conjugate was from Pasteur Production

Methods

Biological material preparations were carried out at 4°C The cortical part of bovine adrenal glands was carefully dissected

Plasma membrane preparations The extraction step giving the crude membrane fraction was carried out as described by Schlegel and Schwyzer [5] This crude fraction obtained in 1 mM $NaHCO_3$/ 0 25 M sucrose (pH 7 4) was layered on the top of a sucrose density discontinuous gradient (15%, 19%, 22 5%, 26%, 35%, 45% 50%, w/w) and centrifuged at 90000 × *g* for 1 5 h The membranes were collected at the 22 5–26% sucrose interface and extensively dialyzed against 1 mM $NaHCO_3$ (pH 7 4) or buffer A 100 mM Mes (pH 6 5)/1 mM EGTA/0 5 mM $MgCl_2$/200 mM NaCl/3 mM NaN_3

Purification of coated vesicles Coated vesicles were isolated according to Nandi et al [6] with some minor modifications Briefly, a typical preparation was made with 10–12 adrenal glands corresponding to 50–60 g wet weight of cortex tissue Homogenisation was done in 150 ml of buffer A with a Waring Blendor (1 min, 3 × 15 s on, 15 s off) The suspension was centrifuged at 15000 × *g* for 45 min The pellet was discarded and the supernatant centrifuged at 105000 × *g* for 1 h The supernatant was discarded and the pellet homogenised in 150 ml of buffer A with a Dounce (pestle B) The homogenate was centrifuged at 10000 × *g* for 10 min The pellet was discarded The 105000 × *g* and 10000 × *g* steps were repeated twice Aliquots of 2 5 ml of the last 10000 × *g* supernatant were layered on the top of a 31 ml discontinuous gradient made with buffer A in ^2H_2O and sucrose at (w/w) 0% (5 ml), 5% (6 ml), 12% (7 ml), 16% (7 ml) and 24% (6 ml) Gradients were centrifuged at 90000 × *g* for 1 5 h Two fractions were collected, at the 12–16% interface (1 15–1 18 density range) and at the 16–24% interface (1 18–1 21 density range) The fractions were extensively dialysed against buffer A In some cases the 12–16% fraction was layered on a second ^2H_2O sucrose gradient identical to the first

Electron microscopy A drop of the sample was placed on a 200-mesh Formwar carbon coated grid and allowed to air-dry Samples were negatively stained with 2% uranyl acetate Micrographs were taken on a JEOL 100 CX electron microscope

Protein content The protein content was determined according to Peterson [7]

Lipid analysis of plasma membranes and coated vesicles The extraction of phospholipids was done using chloroform/methanol according to Folch et al [8] as modified by Rouser and Fleischer [9]

The phosphorus content of total lipid extract was determined using the method of Ames and Dubin [10] Phospholipids were separated by thin-layer chromatography according to Hess and Talheimer [11] using a silica-gel 60 plate The areas of the different phospholipids revealed by iodine staining were scraped off and the phosphorus content was determined using the method of Chen et al [12] modified by Rouser et al [13] Cholesterol was determined on the chloroform/methanol extract by an enzymatic assay according to Ott et al [14] The analysis of the different classes of fatty acids was made on the same lipid extract after separation by gas column chromatography according to van Wijngaarden [15]

SDS-polyacrylamide gel electrophoresis Slab gel electrophoreses (14 × 8 5 × 0 15 cm) on either 6 or 7 5% polyacrylamide were carried out according to Laemmli [16] Plasma membrane or coated vesicle samples were solubilised in the Laemmli buffer and boiled for 3 min in the presence of a reducing agent (2-β-mercaptoethanol) 60–150 μg of protein were applied at the bottom of each slot Gels were calibrated with a high molecular weight SDS kit Electrophoreses were run for 4–5 h at 15°C and 35–50 mA/slab gel Proteins were stained with Coomassie brilliant blue R 250 (0 2%) and destained with a methanol/acetic acid solution (10 10, v/v)

Ligand blotting For further transfer of proteins from slab gels to nitrocellulose paper, SDS-gel electrophoresis samples were solubilised in Laemmli buffer containing 40 mM octylglucopyranoside and incubated for 1 h at 37°C without reducing agent Amounts of proteins layered on top of the gel varied from 20 to 80 μg Electrophoreses were run under conditions identical to those described above Electrophoretic transfer of proteins from slab gels to nitrocellulose paper was carried out at 20 V for 16 h or at 35 V for 7 h, according to Towbin et al [17] and Burnette [18] To control protein transfer, nitrocellulose paper was transiently stained with a Rouge Ponceau solution The nitrocellulose paper was incubated in buffer B (50 mM Tris HCl (pH 8 0)/150 mM NaCl/2 mM Ca^{2+} and bovine serum albumin 5%) and then with the LDL at various concentrations in the same buffer for 1 h (as indicated in the figures) The visualisation of LDL binding was done using the ELISA technique After extensive washing nitrocellulose paper was further incubated with LDL-rabbit antibodies in buffer B After washing, a second incubation was made with goat antirabbit antibodies conjugated with peroxidase and washed again extensively All the washings were performed in buffer B containing 0 2% Triton X-100 The substrate used was 3,3'-diaminobenzidine tetrachloride, resulting in staining of the specific component

Fluorescence intensity measurements Fluorescence intensity measurements were made on a Jobin Yvon JY3C fluorimeter Pyrene monomer and excimer emissions were measured at 390 and 470 nm, respectively, with excitation at 346 nm The emission of *cis*- and *trans*-parinaric acids was measured at 410 nm with an excitation wavelength of 320 nm

Lifetime measurements Fluorescence lifetimes of pyrene (monomer and excimer) and parinaric acids were determined using a single-photon counting fluorimeter equipped with an RCA photomultiplier The electronic devices were an Ortec System 9200 coupled with an Ortec 6240 multichannel analyzer interfaced with a PDP 10 computer The flash was obtained from a 9352 ns light pulser (Edinburgh Instruments) The optical system (Applied Photophysics, PS7) was equipped with two $f/4$ monochromators for excitation and emission wavelengths The temperature was controlled by circulating water from a thermostatically controlled bath through a compartment surrounding the cell This equipment and the method of data analysis employed have been described elsewhere [19] The lifetime of pyrene excimer was measured using concentrated pyrene solutions in which the dimer is the predominant form

Steady-state anisotropy of parinaric acid Fluorescence polarization measurements were performed with a SLM E450 fluorimeter Excitation and emission wavelengths were 320 and 410 nm respectively Fluorescence anisotropy (r) was obtained by measuring intensity of the parallel and perpendicular components of the fluorescence emission, and using the equation

$$r = (I_{VV}\ G - I_{VH})/(I_{VV}\ G + 2I_{VH})$$

where G is an instrumental correction factor The absorbance of the suspension was always less than 0 15 and a blank was used for light-scattering corrections

Anisotropy decay measurements Time-resolved emission anisotropy was obtained with the Applied Photophysics system described above and elsewhere [19,20] The emission decay curves $I_{VV}(t)$ and $I_{VH}(t)$ of the sample, from which a blank was subtracted when needed, were collected successively for time periods in the range of 5–10 min, depending on sample intensity Methods of calculation were as previously described [21]

A series of measurements was also performed at Oregon State University (Corvallis, U S A) The equipment used was similar for electronics (ORTEC), the flash was a picosecond dye laser, coupled to an Apple II computer Data analysis was performed by standard, non-linear least-squares regression technique, or by the method of moments as described by Isenberg and Small [22] A PDP 11 computer was used

Incorporation of the fluorescence labels 1–10 μl of 1 mM pyrene in dimethyl sulfoxide (DMSO) were added to the vesicle suspension and incubated for 1 h at room temperature to give a molar ratio of probe/lipid in the range 0 01–0 08 The parinaric acids were dissolved in ethanol and added to vesicle suspensions under nitrogen or argon, all solutions were maintained in a nitrogen or argon atmosphere The probe/lipid molar ratio for the parinaric acids was of the order of 1 300 With both pyrene and parinaric acid, controls were performed to check that no fluorescence arose from binding of the probe to the clathrin coat Both probes are hydrophobic molecules which penetrate but do not cross the lipid bilayer Moreover, parinaric acids remain attached to the lipid polar heads by their charged carboxyl group

Pyrene diffusion measurements The formation of an excited complex (excimer) by a collision between an excited and an unexcited pyrene monomer is a diffusion-controlled process in the fluid state of the membrane [23] Experiments were performed above 25°C only and linearity between pyrene concentration and I'/I ratio was controlled for all experiments The intensity ratio, I'/I, (excimer intensity (I') at 470 nm and monomer intensity (I) at 390) is a measurement of the collision rate of the pyrene molecules, i e , of the lateral mobility of the probe within the membrane

The method for calculation of the lateral diffusion coefficient (D) has been described previously [24]

Results

Characterisation of adrenocortical coated vesicles and comparison with plasma membranes

Fig 1 shows an electron micrograph of a mixture of coated vesicles different in size, obtained at the 1 15–1 18 density range interface after the first ^2H_2O sucrose gradient The characteristic polygonal structure of the clathrin lattice surrounding the lipid vesicles is observed Coated vesicle diameter ranges from 70 to 120 nm The chemical composition of coated vesicles and partially purified plasma membrane preparations is given in Table I An increase in the protein weight percent is observed from plasma membranes to coated vesicles Among the lipids, an enrichment in phosphatidylethanolamine is accompanied by an increase in arachidonic acid (20 to 26% and 20 to 24%, respectively) from plasma membranes to

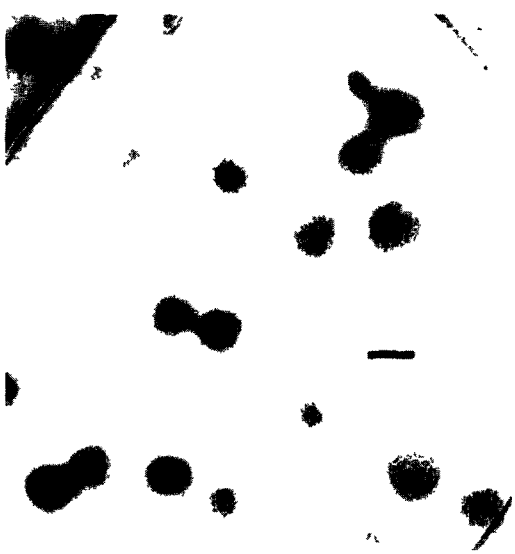

Fig 1 Electron micrograph of a mixture of coated vesicles obtained at the 1 15–1 18 density range interface after the first ^2H_2O gradient Negative staining with 2% uranyl acetate Bar represents 100 nm

TABLE I

CHEMICAL COMPOSITION OF PARTIALLY PURIFIED PLASMA MEMBRANES AND COATED VESICLES EXPRESSED IN WEIGHT%

Data are mean values of five different preparations each determination made in duplicate

	Standard deviation	Plasma membranes	Coated vesicles
Proteins	±1 46	73	79
Lipids	±0 6	27	21
Phospholipids			
sphingomyelin	±0 1	4 7	6 3
phosphatidylcholine	±0 8	50 6	42 6
phosphatidyl-ethanolamine	±0 3	20 2	26 2
phosphatidylinositol-phosphatidylserine	±0 2	16 6	20 2
Cardiolipin	±0 1	2 3	3 25
Cholesterol	±0 5	8	10 4
Arachidonic acid	±0 9	20	24

coated vesicles A higher percentage of cholesterol is observed in the coated vesicles as compared to the plasma membranes A low clathrin over total protein ratio (w/w) appears characteristic of the bovine adrenal coated vesicles, in agreement with previous data [25]

Polypeptide components of coated and uncoated vesicles

SDS-polyacrylamide gel electrophoresis of plasma membranes, coated vesicles and clathrin are shown in Fig 2 On the clathrin and coated vesicle lanes, polypeptide components already described for the clathrin coat of vesicles from different organs [26] are observed the major 180 kDa component and the 110, 36 and 33 kDa components The 55 and 53 kDa peptides, identified as α- and β-tubulins [27], are also present

For the first time, on these adrenal coated vesicle preparations, a 190 kDa peptide distinct from the 180 kDa component is revealed on the coated vesicle lane A 160 kDa component, which could be the LDL receptor under Laemmli reducing conditions, as described previously [28], is also observed on the coated vesicle lane

Uncoated vesicles have been prepared from the

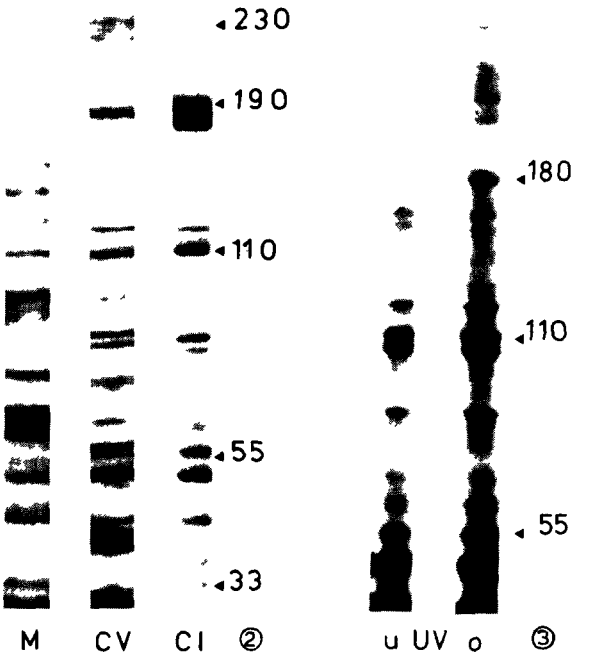

Fig 2 7 5% SDS-polyacrylamide gel electrophoresis of plasma membranes (M), mixture of coated vesicles (CV) and clathrin (Cl) Coated vesicles are dialyzed overnight against 100 mM Mes buffer (pH 6 5), pelleted at 105 000 × g for 1 h The pellet was dissolved in Laemmli buffer Clathrin was prepared by dialysis overnight of a mixture of coated vesicles against 2 M urea/10 mM Tris-HCl (pH 8 5) After centrifugation at 105 000 × g for 1 h the supernatant (clathrin) was dialysed against the same buffer and treated according to Laemmli In all cases, the Laemmli buffer contained 2-β-mercaptoethanol Gels were stained with Coomassie brilliant blue R 250 Arrow-heads are M_r ($\times 10^{-3}$)

Fig 3 6% SDS-polyacrylamide gel electrophoresis of uncoated vesicles (UV) prepared from the mixture of coated vesicles Lane u stripping has been obtained by dialysis against 2 M urea/10 mM Tris-HCl (pH 8 5) followed by sedimentation at 105 000 × g for 1 h Pellet containing uncoated vesicles was resuspended in 10 mM Tris-HCl (pH 8 5) and dialysed against the same buffer Lane o stripping has been obtained by dialysis against 500 mM Tris-HCl (pH 8 5) without urea followed by sedimentation at 105 000 × g for 1 h Pellet containing uncoated vesicles is resuspended in 10 mM Tris-HCl (pH 8 5) Arrowheads are M_r ($\times 10^{-3}$)

coated vesicles obtained after the first sucrose gradient A comparison has been made between uncoated vesicles stripped off either with 2 M urea/10 mM Tris-HCl (pH 8 5), or with 500 mM Tris-HCl buffer (pH 8 5) SDS-gel electrophoresis of both preparations is shown in Fig 3 It appears

that at pH 8 5, 2 M urea strips off more proteins from coated vesicles than 500 mM Tris-HCl does The 180 kDa clathrin component is the main polypeptide removed from the vesicles by urea Indeed, as can be seen on lane u, the 180 kDa component has completely disappeared, while the 190 kDa is still present

Lipid dynamics within coated and uncoated vesicles and the role of clathrin

Lateral diffusion of pyrene These studies have been performed with coated vesicles obtained after the first ^2H_2O-sucrose gradient and with uncoated vesicles stripped off in the presence of 2 M urea to avoid any side-effect of the clathrin remaining on the uncoated vesicles

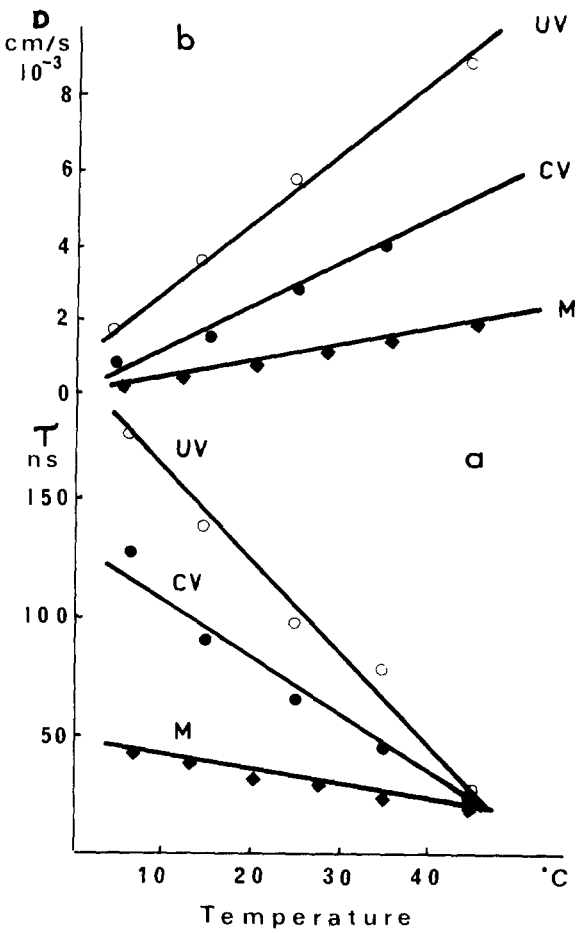

Fig 4 Pyrene lifetime (a) and lateral diffusion coefficient (b) in plasma membranes (M ◆), coated vesicles (CV, ●) and uncoated vesicles (UV ○) prepared with 2 M urea as described in Experimental procedures

The lateral diffusion coefficient calculated from the rate of pyrene excimer formation as a function of pyrene/lipid ratio [29] indicates a decrease in the rate of lipids lateral diffusion in the presence of clathrin around coated vesicles as compared with uncoated vesicles Furthermore, it is worthwhile noticing that the lifetime of the pyrene excimer is longer in the uncoated vesicles than in the coated vesicles (Fig 4), in agreement with the data obtained with bovine brain coated vesicles [24] The presence of the clathrin lattice in interaction with its hook protein, the 110 kDa component [30], embedded in the vesicles lipid bilayer induces a slowing down of the lipid lateral diffusion

Rotational diffusion of the parinaric acid isomers Time-resolved fluorescence measurements carried out with *cis*- and *trans*-parinaric acid isomers (*cis*-Pna–*trans*-Pna) as probes, allow evaluation of the rotational motion and constraints in the different phase domains of the lipid bilayer, since the *trans* isomer partitions preferentially in the gel phase and the *cis* isomer partitions mainly in the liquid crystal phase and around the proteins The measurements of the anisotropy decay of Pna isomers in coated and uncoated vesicles at different temperatures demonstrate that the clathrin coat in interaction with membrane proteins, decreases the long-term residual anisotropy (r_∞) and therefore the order parameter of the lipids mainly around the proteins as probed by the *cis*-Pna (Table II)

Separation of large and small coated vesicles Two different populations of vesicles have been described in the cell the large one generated from the plasma membrane coated pits, and the small one localised around the Golgi cisternae [4] For the first time, the large vesicles have been sep-

TABLE II

LIMIT ANISOTROPY VALUES (r_∞) OF ADRENAL COATED VESICLES (CV) AND UNCOATED VESICLES (UV) MEASURED WITH *cis*- AND *trans*-Pna, AT DIFFERENT TEMPERATURES

T (°C)	*cis*-Pns		*trans*-Pna	
	CV	UV	CV	UV
10	0 04	0 21	0 15	0 20
27	0 07	0 19	0 18	0 19
36	0 03	0 18	0 17	0 11

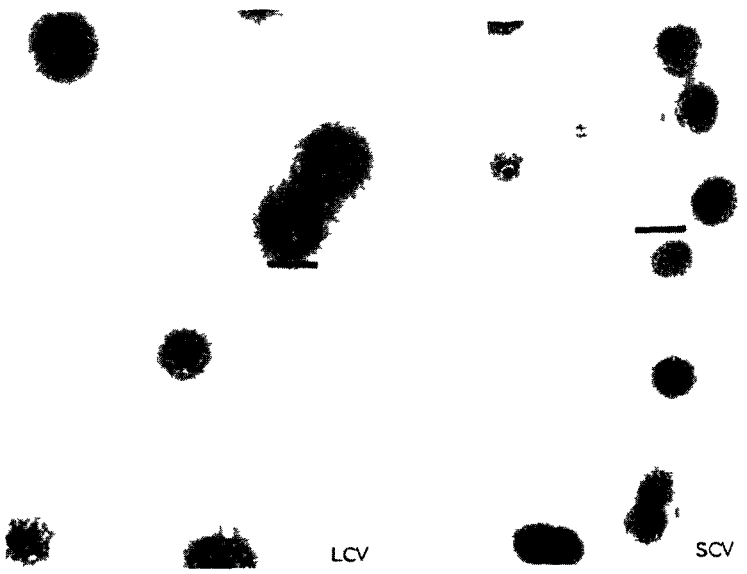

Fig 5 Electron micrographs of large (LCV) and small (SCV) coated vesicles negatively stained with 2% uranyl acetate The two types of vesicle were obtained after a second ^2H_2O-sucrose gradient large coated vesicles at the 1 17–1 21 interface and small coated vesicles at the 1 15–1 17 interface Quantitative estimation over a large number of preparations of the contamination by large coated vesicles (over 90 nm) of the small coated vesicles preparation indicates a value of 3 2% Bars represent 100 nm

arated from the small ones (Fig 5) using a second density gradient (see Experimental procedures) A symmetric pattern is observed for the size distribution of each population with a maximum at 90–110 nm in diameter for large coated vesicles and at 70–80 nm for small coated vesicles (Fig 6) A quantitative estimation over a large number of preparations of the contamination by large coated vesicles (over 90 nm) of the small coated vesicles preparation leads to a value of 3 2%, which supports a high homogeneity of the preparation Electrophoresis of these two types of vesicle with the same total amount of protein layered on the gel (Fig 7) evidences a difference in the staining intensity of the 180 kDa component, much stronger in the small coated vesicles than in the large ones The staining intensity of the 190 kDa component is identical in both types of vesicle A 88 kDa peptide appears to be characteristic of the large coated vesicles as well as two high-molecu-

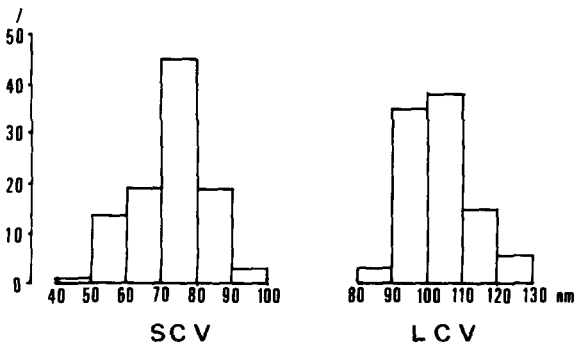

Fig 6 Size distribution pattern of the two types of vesicle large and small coated vesicles (LCV SCV)

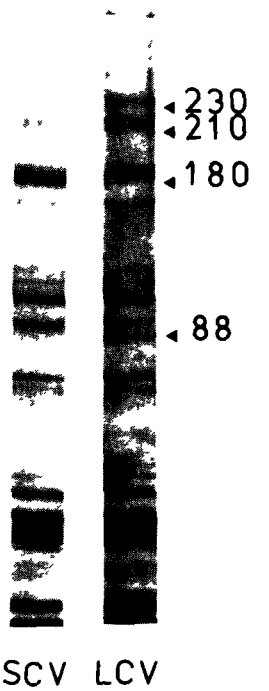

Fig 7 7 5% SDS-polyacrylamide gel electrophoresis of large (LCV) and small (SCV) coated vesicles obtained after the second ^2H_2O sucrose gradient Arrowheads are M_r ($\times 10^{-3}$)

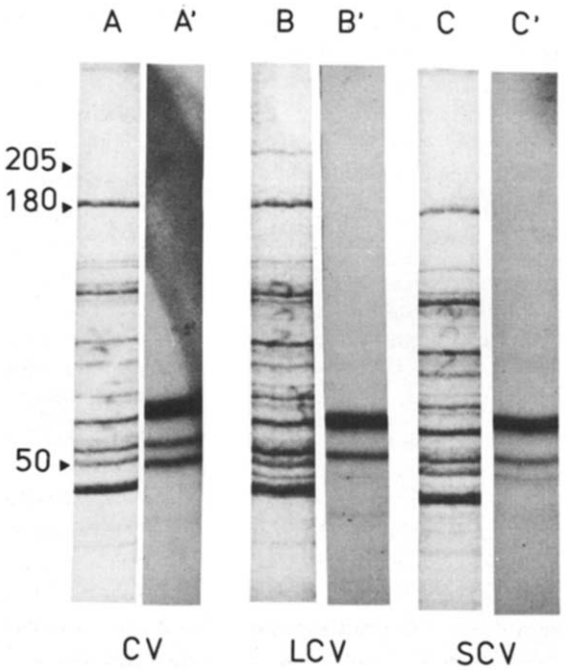

Fig 8 7 5% SDS-polyacrylamide gel electrophoresis of mixed coated vesicles (CV) large (LCV) and small (SCV) coated vesicles [γ-^{32}P]ATP treated either Coomassie blue stained (lanes A, B C respectively) or revealed by autoradiography (lanes A' B' C' respectively) Arrowheads are M_r ($\times 10^{-3}$)

lar-mass species, of 210 and 230 kDa, which could be peptides from the degraded LDL

Autoradiograms performed on the two different populations of vesicles, either mixed coated vesicles or those separated by the second ^2H_2O-sucrose gradient (large and small coated vesicles) treated with [γ-^{32}P]ATP to detect the previously described phosphokinase activity [31] are shown in Fig 8 in comparison with the original Coomassie blue-stained SDS gel electrophoresis of the same material Analysis of the different lanes indicates that a strongly phosphorylated peptide with an apparent mass of 58 kDa is present in both large (lane B') and small coated vesicles (lane C') This result confirms the presence in vesicles from adrenocortical cells of a phosphoprotein kinase activity, as has been described in brain coated vesicles [31,32] A 52 kDa component is also phosphorylated in both types of vesicle, but appears much weaker Only in small coated vesicles can a faintly phosphorylated peptide of 50 kDa be revealed All these components are revealed by a very short exposure of the film under conditions where no unspecific background is present None of these phosphorylation appears Ca^{2+}- or/and cAMP-dependent (data not shown) This is in agreement with the data of Puszkin et al [32] on the brain coated vesicle kinase

Identification of LDL receptor

ELISA-ligand blotting of adrenal large coated vesicles, small coated vesicles and plasma membrane preparations have been performed as al-

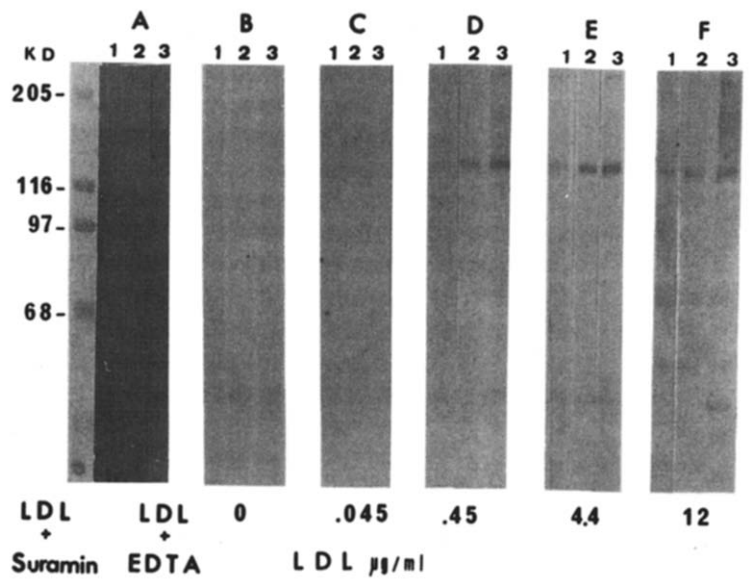

Fig 9 ELISA ligand blotting of plasma membranes (1) large coated vesicles (2) and small coated vesicles (3) in all lanes, A–F After electrophoretic transfer of proteins from slab gels to nitrocellulose paper, the paper was incubated with LDL at different concentrations for 1 h Lane A, left, molecular weight markers, and (1, 2 3) blots with 4 4 µg/ml LDL and either 7 mM Suramin or 10 mM EDTA The same patterns are obtained with both inhibitors LDL concentrations (µg/ml) in lanes as follows B none C 0 045 D, 0 45 E 4 4, F 12

ready described [33] in order to characterise the LDL receptor in these fractions As shown in Fig 9, under the Laemmli electrophoresis conditions but in the absence of 2-β-mercaptoethanol, the LDL receptor appears as a 130 kDa component The specificity of binding to the LDL receptor is demonstrated with large coated vesicles, small coated vesicles and the plasma membranes by (i) the absence of binding in the presence of EDTA, chelating agent of Ca^{2+} required for the binding, and (ii) the inhibition of the LDL binding by Suramin Visual inspection of LDL binding under saturating conditions evidences an increase in the LDL-receptor activity from the whole membrane preparations to large coated vesicles and then to small coated vesicles (lanes E, 1, 2, 3) Urea treatment of coated vesicles giving uncoated vesicles does not decrease the receptor binding capacity, indicating that clathrin does not play any role in the binding of LDL (data not shown)

Discussion

The present work is a step in the study of steroidogenesis regulation by ACTH in bovine adrenal cortex In steroidogenesis, internalisation of cholesterol via LDL, its carrier through receptor-mediated endocytosis, represents an important step initiated at the plasma-membrane level It was therefore essential to gain a detailed understanding at the molecular level of the plasma membrane structure and dynamics leading to the formation of the coated vesicles and endosomes, organelles involved in the LDL endocytosis and receptor cycle

The clustering of the receptor in the coated pits is a prerequisite for the endocytosis of LDL in adrenal cortex, as demonstrated by Lehrman et al [34] with molecular biology experiments on the LDL receptor genes, the coated pits acting as molecular filters [35]

In the plasma membrane the coated pits account for only 2% of the total area Therefore, a physical-chemical study of an homogeneous coated vesicle preparation derived from coated pits in comparison with a preparation of the whole plasma membrane could shed some light on lipid bilayer dynamics involving lipid and protein segregation Such a study could suggest a possible mechanism for the role of the coated pits as molecular filters in the plasma membrane

The data presented here indicate that the clustering of the LDL receptor in the coated vesicles formed by the pinching off of the coated pits is accompanied by a segregation of lipids namely phosphatidylethanolamine This phospholipid is present in a higher amount in coated vesicles than in plasma membrane, leading to a higher proportion of arachidonic acid in coated vesicles Other differences are observed between plasma membranes and coated vesicle lipids, namely phosphatidylserine and inositol and sphyngomyelin, which are also in a higher percentage in the coated vesicles, while phosphatidylcholine is present in a lower percent in coated vesicles than in the whole plasma membrane It is worth noting that such a difference controlled over five different preparations cannot be ascribed to the presence of LDL in different amounts in the lipid extract of coated vesicles and membrane Indeed, since the LDL cluster with their receptors into the coated pits and coated vesicles, they should represent a higher percentage among the lipids of coated vesicles than among those of the membrane According to Skipski [36], the human LDL phospholipid composition is characterized by a very low level of PI-PS and PE (2–10%) and a very high PC and sphingomyelin (75 and 20%, respectively) A higher amount of LDL in coated vesicles would produce a result opposite to the one actually obtained for the phospholipid composition of coated vesicles as compared to the membranes Therefore, it can be inferred that the global percentage of lipids from LDL extracted and measured is about the same in the membranes and in the coated vesicles Also, the higher proportion of cholesterol found in the coated vesicles cannot be due to the presence of LDL, either Therefore an actual segregation should occur among the lipids in the coated pits and if it is limited to one of the lipid layer in the membrane it would initiate an asymmetry inducing plasma membrane invagination [37] and leading to coated pit formation

According to Lehrman et al [34] the selective migration of the LDL receptor towards coated pits is a function of its cytoplasmic domain This part of the receptor would bind to one of the proteins covering the cytoplasmic part of the

coated pits and coated vesicles

The data obtained in the present work by comparing the lateral and rotational diffusion in the lipid bilayer of coated and uncoated vesicles indicate that clathrin slows down the lateral diffusion and decreases the order parameter for the rotational diffusion of the acyl chains, in agreement with previous results obtained with bovine brain coated vesicles [24] These data strongly support the hypothesis that the cytoplasmic domain of the LDL receptor interacts with the clathrin network bound to the plasma membrane by the 110 kDa hook protein, leading to clusters of the LDL receptors in the coated pits

Identification of the proteins in the coated vesicles as compared with those of the plasma membranes indicates in coated vesicles a segregation of a protein kinase and its substrates, of the 110 kDa component which serves as clathrin hook, of the α- and β-tubulins, in addition to the LDL receptor, while many of the membrane proteins are absent in these organelles

Numerous histological observations [4] have indicated the existence in the animal cells of two different populations of coated vesicles, the larger near the plasma membrane and the smaller around the Golgi cisternae The purification method used in the present work has allowed us to separate a population of large coated vesicles with a size distribution in which 75% are of 90–110 nm in diameter from a population of small coated vesicles with a sharp maximum of 48% at 70–80 nm in diameter

The differences between these two populations lie not only in their size but also in their protein composition, as demonstrated by Coomassie blue stained SDS-polyacrylamide gel electrophoresis and autoradiograms performed with [γ-^{32}P]ATP to study the protein kinase activity If the phosphorylated peptides are not those described commonly in the brain coated vesicles [31], the presence of a strongly phosphorylated peptide of 58 kDa in both types of coated vesicle confirms the presence in the adrenocortical cell coated vesicles of a kinase activity, Ca^{2+}- and/or cAMP-independent The presence of a 50 kDa phosphorylated peptide only in small coated vesicles further supports the difference between the two populations of coated vesicles Whether the difference is only in the substrates of the kinase or in the nature of the enzyme itself cannot be answered at present, but work is in progress to solve this problem

The visualisation and quantification of the LDL receptor binding activity in the plasma membrane, large and small coated vesicles indicate a higher activity of the receptor in small than in large coated vesicles and in the plasma membranes The presence of internalised LDL in large coated vesicles and their apparent absence in small coated vesicles suggest that large coated vesicles could actually be involved in the LDL receptor internalisation process and small coated vesicles in the transport of neosynthesised receptor and its recycling from the endosomes to the plasma membranes

These data open the way for a systematic study of the two isolated populations of coated vesicles from adrenocortical cells and their role in the membrane cellular traffic

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References

1 Boggaram V, Funkenstein, B, Waterman, M R and Simpson, E R (1984–85) Endocrine Res 10, 387–409
2 Simpson, E R and Waterman, M R (1983) Can J Biochem Cell Biol 61, 692–707
3 De Paillerets, C, Gallay, J and Alfsen, A (1984) Biochim Biophys Acta 772, 183–191
4 Steven, A C, Hainfeld, F J and Wall, J S (1983) J Cell Biol 97, 1714–1723
5 Schlegel, W and Schwyzer, R (1977) Eur J Biochem 72, 415–424
6 Nandi, P K, Irace, G, Van Jaarsveld, P P, Lippoldt, R E and Edelhoch, H (1982) Proc Natl Acad Sci USA 79 5881–5885
7 Peterson, G L (1977) Anal Biochem 83, 346–356
8 Folch, J, Lees, M and Sloane-Stanley, G H (1957) J Biol Chem 226, 497–509
9 Rouser, G and Fleischer, S (1967) Methods Enzymol 10, 385–406

10 Ames, B N and Dubin, D T (1960) J Biol Chem 235, 769–775
11 Hess, H H and Talheimer (1965) J Neurochem 12, 193–204
12 Chen, P S Toribara, T Y and Warner, H (1956) Anal Chem 28, 1756–1758
13 Rouser, G , Fleischer, S and Yamamoto, A (1969) Lipids 5 494–496
14 Ott P , Bingeli Y Brodbeck, Y (1982) Biochim Biophys Acta 685, 211–213
15 Van Wijngaarden, D (1967) Anal Chem 39, 849–853
16 Laemmli, U K (1970) Nature 227, 680–685
17 Towbin, H , Staehelin, T and Gordon, J (1979) Proc Natl Acad Sci USA 76 4350–4354
18 Burnette W N (1981) Anal Biochem 112 195–203
19 Gallay, J , Vincent, M , De Paillerets C , Rogard, M and Alfsen, A (1981) J Biol Chem 256, 1235–1241
20 De Paillerets, C Gallay, J , Vincent, M , Rogard, M and Alfsen, A (1984) Biochim Biophys Acta 772, 183–191
21 Vincent, M , de Foresta, B , Gallay, J and Alfsen, A (1982) Biochemistry 21, 708–716
22 Isenberg, I and Small, E W (1982) J Chem Phys 77, 2799–2805
23 Galla, H J and Luisetti, J (1980) Biochim Biophys Acta 596, 108–117
24 Alfsen A , De Paillerets, C Prasad, K , Nandi, P K , Lippoldt, R E and Edelhoch, H (1984) Eur Biophys J 11, 129–136
25 Woods, J W , Woodward, M P and Roth, T F (1978) J Cell Sci 30, 87–97
26 Bretscher, M S (1982) Science 224, 681–696
27 Pfeffer, R S , Drubin, D G and Kelly, R G (1983) J Cell Biol 97, 40–47
28 Brown, S M , Anderson, R G W and Goldstein, J L (1983) Cell 32, 663–667
29 Galla, H J and Sackmann, E (1974) Biochim Biophys Acta 339, 103–115
30 Prasad, K , Alfsen, A , Lippoldt, R E , Nandi, P D and Edelhoch, H (1984) Arch Biochem Biophys 235, 403–410
31 Pauloin, A Loeb, J and Jollès, P (1984) Biochim Biophys Acta 799, 238–245
32 Schook, W and Puszkin, S (1985) Proc Natl Acad Sci USA 82, 8039–8043
33 Weintraub, H , Bomsel, M , De Paillerets, C , Néant I Ayrault-Jarrier, M and Alfsen, A (1985) FEBS Lett 184, 263–267
34 Lehrman, M A , Goldstein, J L , Brown M S , Russel, D W and Schneider, W J (1985) Cell 41, 735–743
35 Goldstein, J L , Anderson, R G W and Brown, M S (1979) Nature 279, 679–685
36 Skipski, V P (1972) in Blood Lipids and Lipoproteins Quantitation, Composition and Metbolism (Nelson, J L , ed), Wiley Interscience, New York
37 Sackmann, E , Kotulla, R and Heiszler, F J (1984) Can J Biochem Cell Biol 62, 778–788