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Influence of the clathrin coat on the membrane lipidic organization of endocytic vesicles: a fluorescence study

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Abstract

Endocytic coated vesicles (CV) were purified from bovine brain, and uncoated vesicles (UV) were obtained from the latter by dialysis against 1 M Tris. Membrane dynamics were explored in both vesicle populations using two complementary fluorescence approaches: diphenylhexatriene fluorescence anisotropy to account for rotational lipid movements, and pyrene excimerization with a phosphatidylcholine pyrene derivative for translational motion. It was concluded that membrane fluidity was considerably higher in UV than in CV, and that adding bulk coat proteins (adaptors + clathrin) to uncoated vesicles re-established the low fluidity found in coated vesicles. However, adding coat protein constituents separately had no effect.

Keywords: Clathrin; Endocytosis; Coated vesicle; Uncoated vesicle; Membrane fluidity; Fluorescence anisotropy; Diphenylhexatriene; Pyrene excimer

1. Introduction

Coated vesicles [1–5] are distinct organelles involved in transport mechanisms in endocytosis, exocytosis and membrane recycling. Clathrin triskelions and adaptors first assemble on the cytoplasmic side of the cell membrane to form coated pits, where specific receptors and their ligands then concentrate. Coated pits further invaginate and pinch-off to form intracellular coated vesicles which convey the accumulated receptor-ligand complexes towards inner cell compartments. Finally, coated vesicles rapidly reorganize: while the ingested material is eventually delivered to appropriate targets, dissassembled clathrin triskelions return to the plasma membrane for a further endocytic cycle.

Coated vesicles in their native form are easily isolated, for instance from bovine brain homogenate. Furthermore, the coat proteins can be dissociated from the vesicles by treatment with protonated amines [6], and the resulting uncoated vesicles then purified by centrifugation. Coat proteins can themselves be separated into their constituents, clathrin and adaptors. The aim of the present work was to investigate vesicle membrane dynamics, i.e., to examine the influence of the protein coat on the vesicle membrane lipid organization. To that end we adopted two fluorescence approaches: (i) the formation of excimers by the phospholipidic pyrene derivative PyPC; (ii) the fluorescence anisotropy of the hydrophobic probe diphenylhexatriene (DPH). Both methods allow membrane fluidity levels to be assessed, the first accounting for translational and the second for rotational movements in the bilayer. This study was intended to shed light on the interaction mechanisms between the cell membrane and the coat proteins, which initiate the coated pit formation. These mechanisms involve protein-protein recognition [7,8], but protein–lipid interactions may occur too.

Abbreviations: CV, coated vesicles; DPH, diphenylhexatriene; Mes, 4-morpholinoethanesulfonic acid; PyPC, 3-palmitoyl-2-(1-pyrene-decanoyl)-L-α-phosphatidylcholine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TMA-DPH, 1-(4-(trimethylammonio)phenyl-1,6-phenyl-1,3,5-hexatriene; UV, uncoated vesicles.

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2. Materials and methods

2.1. Coated vesicle preparation and purification

Coated vesicles were prepared and purified according to the method of Nandi et al. [9], modified in our laboratory as described in [10]. The method includes the homogenization of freshly recovered bovine brains, differential centrifugation and sucrose cushion centrifugation. Purified vesicles were stored at 4°C under nitrogen atmosphere in 100 mM Mes (pH 6.5); 0.2 mM EGTA; 0.5 mM $MgCl_2$; 0.02% NaN_3; 1 mM PMSF; 0.1% β-mercaptoethanol, and could be used up to one month after the preparation, with low-speed centrifugation before starting the experiments.

2.2. Uncoated vesicle preparation and coat protein separation and purification

The method used in our laboratory [10] is based on extracting the coat proteins by dialysis against 1 M Tris (pH 7.0), with 1 mM EDTA and 0.1% β-mercaptoethanol. Uncoated vesicles are then centrifuged for 50 min at $100\,000 \times g$. The presence of clathrin and adaptors in the supernatant is monitored with SDS-PAGE [11]. A remarkable improvement in the extraction of coat proteins, is obtained by performing the dissociation–centrifugation sequence twice (see Results).

Clathrin and adaptors were separated on a S300 (Pharmacia) column. Adaptors were purified on a hydroxyapatite column after fixation with 10 mM phosphate in 0.5 M Tris. Tris was then removed by washing with phosphate buffer, before elution with a 10 mM–0.5 M phosphate gradient.

Protein content was determined according to the method of Bradford [12], or by UV absorption at 280 nm under denaturing conditions (guanidinium chloride) [25]. Both methods gave identical results.

2.3. Fluorescence anisotropy measurements

With this technique [13–16] membrane fluidity is interpreted in terms of hindrance to the rotation of a fluorescent rod-shaped probe, here DPH, embedded in the membrane bilayer. The parameter measured, r_s, is steady-state fluorescence anisotropy, i.e., under continuous illumination:

$$r_s = (I_V - GI_H)/(I_V + 2GI_H)$$

where I_V and I_H stand for the emitted light components respectively parallel and perpendicular to the vertically polarized excitation beam, and G is a correction factor for the photomultiplier's balance. r_s basically ranges from 0.0 to 0.4, the higher values denoting the higher structural lipidic order.

Vesicle samples were incubated for several hours at 4°C, under moderate stirring, with DPH (Molecular Probes) from a 2.5 mM stock solution in tetrahydrofuran, for a final probe to lipid molar fraction of 0.4%. r_s measurements were performed at room temperature with a SLM 8000 spectropolarofluorimeter set at 360 nm for excitation and 435 nm for emission. All measurements were corrected for scattered excitation light contribution [17]. For a relevant interpretation in terms of membrane fluidity parallel fluorescence lifetime measurements are required [16,18]. These were done with a SLM 48000 phase and modulation spectrofluorometer as described in [19].

2.4. PyPC excimerization spectroscopy

The term *excimer* refers to collisional complexes between an excited molecule and the same molecule at the ground-state. Pyrene and derivatives display a remarkable tendency to form excimers, depending on the medium fluidity [20]: the higher the fluidity, the greater the probability of excimer formation. Thus, in membranes, measuring ϕ_E/ϕ_M, the excimer to monomer quantum yield ratio or its equivalent, the corresponding fluorescence intensity ratio I_E/I_M at emission wavelength maxima, enables membrane fluidity levels to be evaluated. In this study we used a phospholipidic pyrene derivative, PyPC, anchored in the outer vesicle membrane leaflet. The evolution of the ratio I_E/I_M for this probe was followed as a function of temperature in vesicle membranes. Because of incorporation difficulties in coated vesicles the assays were performed only on uncoated vesicles, and the influence of the coat was examined by further addition of the coat proteins. PyPC (Molecular Probes) from a 0.3 mM stock solution in ethanol was added to vesicle sample to give a final probe to lipid ratio of 1%. After 30 min incubation the excess probe was removed by extensive centrifugation and washing, and the labelled vesicles were re-suspended. Fluorescence spectra were taken with a Jobin-Yvon JY-3C spectrofluorimeter (excitation wavelength 342 nm; monomer emission 395 nm; excimer 478 nm; bandwidth 5 nm). The temperature was regulated ($\pm 0.2°$) with a circulating water bath. All operations were performed under nitrogen atmosphere to prevent quenching effects of oxygen.

2.5. Protein coat reconstitution procedures

Crude coat extract, purified clathrin or adaptors, and clathrin-adaptor mixtures were stored in 0.5 M Tris (pH 7.0) to prevent aggregation [21]. When required, these components were added to the uncoated vesicle suspensions and reconstitution could take place after the Tris had been replaced with Mes buffer, 10 mM for DPH and 100 mM for PyPC assays [10] by

dialysis. Uncoated control vesicles were treated in the same way with protein-free Tris.

3. Results

3.1. Vesicle and coat protein preparation yields and purity levels

Coated vesicles were obtained with a very satisfactory yield of 120 mg total protein per preparation, from three bovine brains. Coat proteins could be extracted without membrane contamination, contrary to previously reported methods [22], thanks to the double sequence of dissociation with 1 M Tris (pH 7) and centrifugation at $100\,000 \times g$. Purification of the coat proteins from the same preparation gave 20 mg clathrin and 3 mg of the adaptor AP2 (endocytic vesicles). No appreciable amounts of AP1 (Golgi vesicles) were detected. It should be mentioned that we tested another method, reported for dissociating only clathrin, which consists in raising the buffer pH up to 8.5 [23–25]; from our observations, with this method, 30% of the clathrin actually remained associated, while up to 30% of the adaptors were removed.

3.2. DPH fluorescence anisotropy compared in coated and uncoated vesicles

Typical DPH fluorescence anisotropy values were $r_s = 0.237 \pm 0.005$ in coated vesicles and 0.199 ± 0.005 in uncoated vesicles, both in the Mes preparation buffer. No significant difference in fluorescence lifetime was observed between the two sample types: with DPH in tetrahydrofuran as a reference ($\tau = 6.7$ ns) [26] the average lifetime was 7.5 in coated and 7.7 ± 0.1 ns in uncoated vesicles. These results thus denote a high lipid order in coated vesicle membranes, and a marked fluidization in the absence of the coat.

Identical results were obtained when coated labelled vesicles were re-measured after dissociation of the coat with 1 M Tris. It is noteworthy that the interpretation of the present results as expressing a higher lipid order in coated vesicles than in uncoated ones, could match earlier data on pyrene excimerization [27].

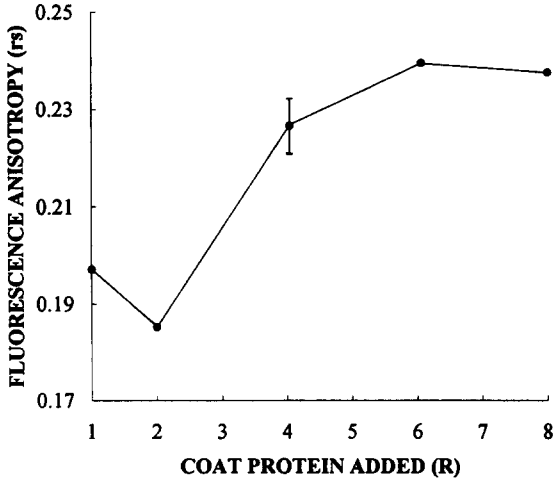

Fig. 1. The effect on DPH fluorescence anisotropy (r_s) in the vesicle membrane of adding coat proteins to uncoated vesicles. The bulk coat proteins from coated vesicles were added to DPH-labelled uncoated vesicles. The concentration of the added proteins is expressed in units of protein to the lipid ratio, which is set at 1 in native coated vesicles. Error bars represent S.D. from three separate determinations.

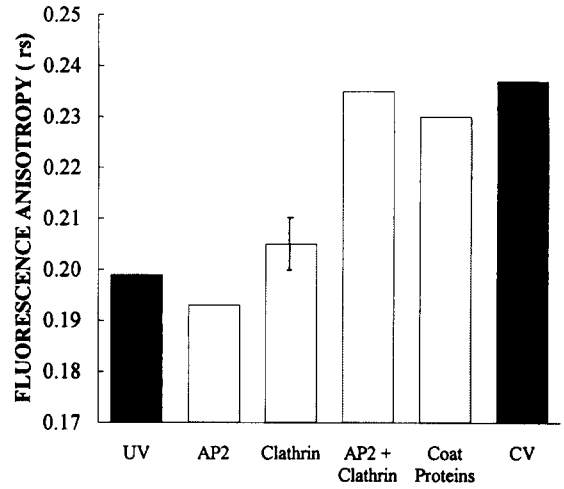

Fig. 2. DPH fluorescence anisotropy (r_s) values compared in native coated and uncoated vesicles and in pre-labelled uncoated vesicles after treatment with separate purified coat proteins. The added protein/lipid ratios were $R = 4$ and $R = 8$ ($R = 1$ in coated vesicles). The results were the same for both protein concentrations. Error bars denote typical S.D. values from six determinations.

3.3. Coat reconstitution experiments

When purified bulk coat proteins (membrane-free) were added to DPH pre-labelled uncoated vesicles, the fluorescence anisotropy increased in a protein concentration dependent manner (Fig. 1). The value of r_s in native coated vesicles was re-established when the concentration of added proteins was four times that of the native coat ($R = 4$). On the other hand, adding of either clathrin or AP2 adaptor separately (Fig. 2), up to $R = 8$, had no appreciable effect, whereas the addition of a 1:1 clathrin and AP2 mixture, at $R = 8$, also re-established the anisotropy value in native coated vesicles.

Similar effects were observed with PyPC excimerization (Fig. 3). Adding purified clathrin or AP2 separately ($R = 4$ and 8) did not affect I_E/I_M in uncoated vesicles. However, adding bulk coat proteins or an equivalent clathrin-AP2 ($R = 4$) mixture considerably reduced this ratio.

4. Discussion

All the results of this study are consistent with a strong influence of the clathrin coat on the membrane fluidity of endocytic vesicles. More precisely, the presence of the coat (i) increases the membrane lipid order and hinders rotational diffusion (DPH data), and (ii) reduces translational movements (PyPC data). From a quantitative point of view, DPH fluorescence anisotropy in uncoated vesicles (0.199) corresponds to typical values in cell plasma membrane (see for instance [28]), but the presence of the coat raises r_s to an unusually high value (0.237); the effect is considerable (19%). These observations are to be compared with those of a recent study in L929 cultured cells [29]. Fluorescence anisotropy was monitored in early endocytosis in these cells with TMA-DPH as a probe. Initially, its value was strikingly high, but then rapidly decreased to the same value as in the bulk plasma membrane. The effect was attributed to the uncoating process of coated vesicles, which displayed similar kinetics.

Our data in reconstitution experiments indicate that clathrin or the adaptor AP2 alone did not work and that a synergic effect was required for membrane rigidification. This suggests that the formation of clathrin baskets was necessary for the effect. It should be mentioned, by the way, that these reconstitution assays were performed under pH and saline conditions favouring self-association in so-called 'empty cages'; this competing effect probably accounts for the rather high concentrations of added proteins required to complete the membrane fluidity change.

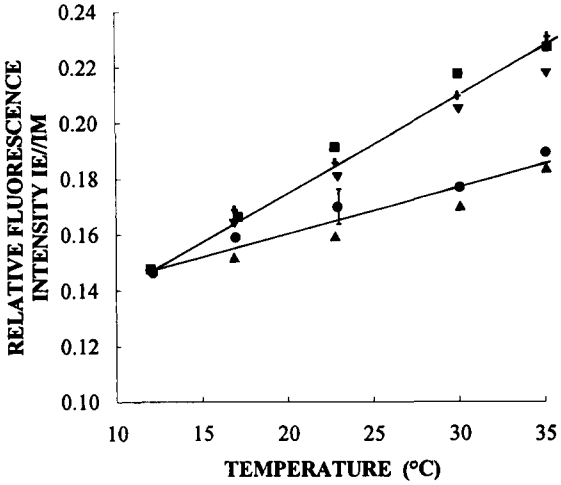

Fig. 3. Evolution of I_E / I_M, the ratio of PyPC excimer to monomer fluorescence intensity as a function of temperature in prelabelled-uncoated vesicles after different types of treatment with coat proteins: (■) native uncoated vesicles; the addition of AP2 (▼); the addition of clathrin alone (+); purified clathrin+AP2 (1:1) (▲); bulk coat protein (●). The concentration of the proteins added was $R = 8$, with R: protein to lipid ratio, taken as 1 in coated vesicles. The error bar denotes the typical S.D. from four separate determinations.

Another remark concerns probe localization: while DPH can occupy any hydrophobic region of the membrane, PyPC is thought to remain associated with the outer leaflet. On the contrary, TMA-DPH, the fluorescence probe used in the above quoted study in L929 cells [29] is found in the inner leaflet of endocytic vesicle membranes. Thus, the decrease in membrane fluidity induced by the presence of the coat, concerns not only the part of the membrane in contact with the coat, but the whole membrane. This would suggest an alternative interpretation: either the effect only results from interactions between coat proteins and the phospholipid heads of the vesicle membrane outer leafltet, which would then presuppose strong interdigitation (trans-bilayer interactions) [30,31] with the inner leaflet. Or, the coat proteins interact with membrane integral proteins so as to build a constraining network between the phospholipids, this network being itself rigidified by the clathrin baskets. Actually, the contribution of both effects may be assumed a priori.

The striking difference in membrane fluidity between coated and uncoated endocytic vesicles raises a fundamental question: is this difference nothing else than a trivial consequence of molecular structure, or is the phenomenon itself involved in endocytosis mechanisms? One might speculate, for instance, that the high lipid order could be responsible for trapping receptors in coated pits, which would be consistent with the fact that coated pits are formed prior to receptor accumulation. In the same way, could it not be suggested that this membrane rigidification might regulate some membrane protein activities, like that of H^+-ATPases or of fusions proteins? Whatever the case may be, the present experimental data should open up new avenues for exploring these questions.

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