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Interaction of clathrin with large unilamellar phospholipid vesicles at neutral pH. Lipid dependence and protein penetration

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The interaction of clathrin with large unilamellar vesicles of various lipid compositions has been examined at neutral pH. Clathrin induces leakage of contents of vesicles that contain the acidic phospholipid phosphatidylserine. Leakage is greatly enhanced by the presence of a relatively minor amount of cholesterol, but is inhibited by phosphatidylcholine. Resonance energy transfer measurements between tryptophan residues of the protein and a fluorescent lipid analog, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine incorporated into the liposomal bilayer, suggests a dynamic interaction of clathrin with the bilayer at neutral pH. This interaction includes a (partial) penetration of the protein into the lipid bilayer, as revealed by hydrophobic photoaffinity labeling with 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)-diazirine. The interaction of clathrin with lipid vesicles at neutral pH is inhibited when the protein is pretreated with trypsin or with the reducing agent dithiothreitol, suggesting that structural requirements govern clathrin–membrane interaction at these conditions. The physiological relevance of the present observations in light of vesiculation and endosomal maturation is discussed.

Introduction

Clathrin-coated vesicles are fulfilling important steps in intracellular transport processes, occurring during receptor-mediated endocytosis and protein transport in the biosynthetic pathway [1–3]. For example, specific receptors are continuously and/or constitutively cleared from the cell surface by membrane invagination at regions, coated by clathrin and other protein complexes at the inner membrane leaflet. Coated vesicles, exposing clathrin at the outer membrane leaflet facing the cytoplasm, are thought to loose their coat rapidly after their generation, mediated by an ATPase-uncoating enzyme [4]. The subsequent scenario of their fate involves membrane fusion, with each other and/or with other membrane vesicles, including endosomal compartments. The role of clathrin in these events as well as the molecular basis for the apparent requirement of clathrin removal, is unclear.

In a recent study we observed that during receptor-mediated endocytosis of transferrin in BHK-cells, clathrin can be found associated with Tf-containing vesicles that bypass tubular endosomal compartments [5]. These coated endosomal vesicles (CEVs) also must possess fusion activity, in spite of the presence of clathrin and in contrast to earlier suggestions that clathrin might exert a barrier function in fusion. In fact recent work in cell-free systems supports this view [6]. In this context, clathrin-induced fusion of artificial membranes has been demonstrated [7–9]. However, all studies referred to, show fusion to occur at a pH less than 6.0, i.e., a value too low for the vesicles to be confronted with in the cytoplasm.

It is generally accepted [3] that clathrin forms a three-legged pinwheel structure which is called 'triskelion'. Clathrin itself consists of a heavy chain (molecular mass 180 kDa) and two different, but related light chains (molecular masses 30 and 33 kDa). Together with accessory proteins, clathrin envelopes the lipid bilayers of coated vesicles shell-like wise [10], the terminal domains of clathrin heavy chains pointing inwards and forming an intermediate shell while the

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remaining parts of the molecule constitute the outer shell. The inner shell is thought to be comprised of accessory proteins. Accumulation of ligand-receptor complexes at coated pit regions, subsequent inward budding of these regions and potential disassembly of the coated structure suggests a highly dynamic interaction of clathrin with the lipid bilayer. Numerous aspects of this interaction have yet to be resolved. As indicated, a number of studies have focussed on the interaction of clathrin with lipid vesicles within a framework of the protein's capacity to induce membrane fusion. Since this event takes place at acidic pH, the biological relevance in the context of the dynamic interactions cited above remains questionable.

The goal of the present study was, therefore, to examine the interaction of clathrin with membranes at neutral pH, i.e., conditions more likely resembling physiological conditions. The interaction of clathrin with lipid vesicles was studied as a function of the lipid composition and was further characterized by examining the effects of clathrin interaction on membrane integrity and stability.

Materials and Methods

Materials

Phosphatidylserine (PS, bovine brain), phosphatidylcholine (PC, egg) and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) were obtained from Avanti Polar Lipids (Birmingham, AL). All other chemicals and reagents used in this study were of the highest grade available.

Preparation and purification of clathrin

Clathrin was prepared from coated vesicles, isolated according to the procedure described by Nandi et al. [11] with the following modifications. Typically, brains of seven pigs were processed within 2 h after slaughter. The meningues, blood vessels and white matter were removed. The brains were homogenized in 1.5 volume of a buffer, consisting of 0.1 M Mes, pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.8 mM DTT, 0.1 mM PMSF, 0.04% NaN_3. Cell debris was spun down at $20000 \times g$ for 50 min. The supernatant containing the coated vesicles was centrifuged at $140000 \times g$ for 1 h. The pellet was resuspended in homogenisation buffer and centrifuged at $10000 \times g$ for 10 min to remove aggregates. The vesicles were washed twice more. The final pellet was resuspended in 20 ml homogenisation buffer, and 6 ml of this suspension was loaded on a 5-ml cushion of homogenisation buffer in D_2O, containing 8% sucrose. The coated vesicles were pelleted again by centrifugation in a swing out rotor at $80000 \times g$ for 2 h.

The coated vesicles obtained in this way are not completely pure. Therefore an additional purification step was carried out, involving gel filtration on Sephacryl S-1000 (100 × 1 cm). The column was run at a flow rate of 20 ml/h and the coated vesicles eluted in a well-defined peak [12]. The fractions containing the coated vesicles, as revealed by A_{280} measurements, were pooled and collected by centrifugation at 140000 $\times g$ for 1 h. They were then resuspended in homogenisation buffer and stored in aliquots at 4°C at a final protein concentration of 10 mg/ml. From the coated vesicles thus obtained, clathrin was isolated and purified. This was done by mixing the vesicle suspension with an equal volume of 1 M Tris (pH 7.0) followed by an incubation for 30 min at 4°C. Uncoated vesicles and aggregates were removed by centrifugation in an airfuge for 1 h at $100000 \times g$. The clathrin-containing supernatant was divided in aliquots and stored at −80°C. Purity, as judged by polyacrylamide gel electrophoresis, was more than 80%, with only minor amounts of assembly proteins.

Preparation of LUV's and MLV's

Large unilamellar vesicles (LUV's) were prepared by reverse phase evaporation, as described by Szoka and Papahadjopoulos [13]. The vesicles were sized by filtration through 0.1 μm polycarbon filters, and their concentration was determined by a phosphorus assay. Carboxyfluorescein (CF)-containing LUV's were prepared as follows. The fluorescent dye was purified by hydrophobic gel filtration on an LH-20 Sephadex column (Pharmacia) as described by Ralston et al. [14]. For entrapment of a selfquenching concentration of CF, 20 mM CF was solubilized in 150 mM NaCl/10 mM Hepes (pH 7.4). Non-entrapped CF was removed by exclusion chromatography on a Sephadex G-100 column.

LUV's, labeled with *N*-NBD-PE, were prepared by mixing phospholipid and the fluorescent lipid analog (2 mol%) prior to vesicle preparation. For some experiments multilamellar vesicles (MLV's) were used. These vesicles were prepared by hydrating a dried lipid film with 150 mM NaCl/10 mM Hepes (pH 7.4) followed by vortexing for 5 min.

Lipid composition of the vesicles was as indicated in the legends.

Leakage experiments

Release of encapsulated CF, causing a concomitant relief of fluorescence selfquenching, was used to monitor clathrin-induced leakage of aqueous liposomal contents. In a typical experiment 20 nmol of CF-containing LUV's were diluted in 2 ml incubation buffer (150 mM NaCl/10 mM Hepes, pH 7.4 and 6.5; or 150 mM NaCl/10 mM acetate (pH 4.0)). The incubation temperature was 37°C. The reaction was initiated by adding clathrin, and fluorescence was monitored continuously (excitation and emission wavelengths were 480 nm and

520 nm, respectively). For calibration, the residual fluorescence of the CF-loaded vesicles was taken as the zero level. Fluorescence read after addition of Triton X-100 (1% v/v), corrected for sample dilution, was taken as 100% leakage.

Binding experiments

A procedure involving centrifugation and an assay based on resonance energy transfer were used to determine potential binding of clathrin to lipid vesicles. The former procedure was done with MLV's. One hundred nmoles of lipid were incubated with 50 μg clathrin in a NaCl/Hepes or NaCl/acetate buffer (depending on the desired pH) for 10 min at 37°C. Subsequently, the vesicles were centrifuged for 10 min in an Eppendorf centrifuge. In the supernatant fraction residual protein was determined, as a measure of the extent of binding.

Alternatively, binding of clathrin to the lipid vesicles was determined by an assay based on resonance energy transfer from tryptophan to NBD, attached to lipid in the LUV bilayers [9]. Binding can thus be monitored by following Trp fluorescence quenching by NBD. Unless indicated otherwise, 100 nmol of N-NBD-PE-labeled vesicles were suspended in the incubation buffer, adjusted to the desired pH at 37°C. Clathrin (30 μg) was added and fluorescence was monitored at excitation and emission wavelengths of 275 and 340 nm, respectively. Each run was corrected for background decay of Trp fluorescence monitored in the absence of LUV.

Photoaffinity labeling of clathrin

Liposomes were labeled with the hydrophobic photoaffinity label 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (TID), by incubating 100 nmol of lipid with 10 μCi TID in 1 ml of incubation buffer of the desired pH for 30 min at room temperature. The liposomes were then transferred to a new tube (allowing separation of nonincorporated probe by its attachment to the wall of the tube; cf. Ref. 15) and 50 μg clathrin was added. Subsequently, the mixture was incubated at 37°C for 10 min. The sample was then photoactivated by irradiation for 30 s with an Osram HBO 100 W/2 super-pressure mercury lamp at 10 cm, with a Schott Glass Technology WG-360 high-pass cut-off filter. Until activation, all handling was done in the dark. After activation, the mixture was transferred again to a new Eppendorf tube, the protein was precipitated [15] and loaded on 10% PAA gels. After electrophoresis, the gels were stained, dried and autoradiographed, by exposing the Kodak XAR-5 film for 1–2 weeks at −80°C.

Results

Release of vesicle contents

To reveal the interaction of clathrin with lipid vesicles, protein-induced leakage of CF, entrapped in the aqueous compartment of the vesicles, was monitored. As shown in Fig. 1, clathrin-induced release of CF at neutral pH is minor from vesicles consisting of PC only, increases substantially when the vesicles contain, in addition, a negatively charged lipid such as PS, but is greatly enhanced when besides PS, cholesterol is included. The rate of CF release from PS LUV was almost doubled (10.7%/min vs. 6%/min) when the same experiment was carried out at pH 6.5. By contrast, at those mild acidic conditions no enhancement of CF release from PC vesicles was observed. At pH 4.0, a lipid-dependent release was no longer observed and all contents leak out within two minutes, irrespective of the lipid composition (not shown). It should be noted that at pH 7.4, no clathrin-induced aggregation of the vesicles can be detected, implying that the release of contents occurs from separate, individual vesicles.

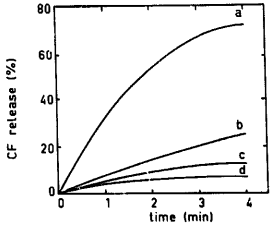

Fig. 1. Clathrin-induced leakage of liposomal contents as a function of lipid composition. CF-containing LUV's (20 nmol of lipid) were incubated with 15 μg clathrin in a final volume of 2 ml. The release of CF at 37°C (pH 7.4) was determined as described in Materials and Methods. Lipid composition was as follows: a, PS/cholesterol (95:5); b, PS; c, PS/PC (2:1); d, PC.

The release of CF from PS vesicles at neutral pH as a function of the protein concentration, is shown in Fig. 2. The results indicate that the initial rate of release is almost linearly dependent on the protein's concentration over a range of 0–60 μg. This suggests that at the experimental conditions (pH 7.4, 37°C) leakage is induced by monomeric interaction of clathrin with the lipid bilayer.

Clathrin-induced release of vesicle contents is temperature dependent. At pH 7.4, leakage is essentially negligible when the experiment was carried out at 2°C. With an increase of temperature, the protein's capacity to induce leakage also increased, though gradually. At 25°C, leakage amounted 3.3%/min. The rate of leakage further increased from 4.8%/min at 30°C up to 6%/min at 33°C. A remarkable enhancement of 1.7-fold (up to 10.2%/min) occurred between 33°C and

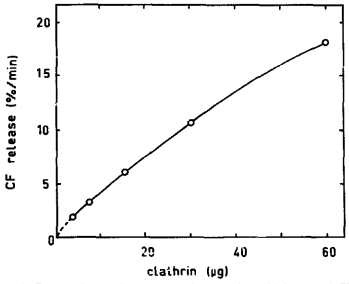

Fig. 2. Effect of clathrin concentration on the initial rate of CF release. Various amounts of clathrin were injected into a cuvette, containing 20 nmol of CF containing PS LUV. The initial rate of release of contents was determined from the tangent at $t = 0$ to curves as those in Fig. 1 and plotted as a function of the amount of clathrin. Release was monitored at pH 7.4, 37°C.

37°C. At the latter temperature a maximal rate of leakage was obtained.

To determine in some more detail the nature of the clathrin-induced release in terms of structural requirements of the protein, clathrin was pretreated with trypsin and the trypsinized protein was subsequently incubated with the vesicles. As shown in Fig. 3, trypsinization leads to a dramatic inhibition of clathrin's capacity to induce release of contents. Interestingly, the presence of the reducing agent dithiothreitol (DTT) in the medium caused an enhanced ability of the

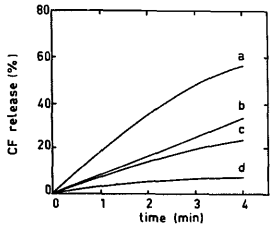

Fig. 3. Effect of DTT and trypsin on clathrin-induced leakage of CF from PS LUV. 15 μg clathrin was preincubated with 1–5 μg trypsin for 15 min at 37°C. The mixture was then added to the liposomes (20 nmoles of lipid) and release of CF was monitored as described (curve d). For DTT treatment, CF-containing vesicles were mixed into the incubation buffer that contained 5.0 (a) or 2.5 (b) mM DTT. Clathrin (15 μg) was added and CF release was then determined. The release occurring in the presence of clathrin only is also shown (curve c). All measurements were carried out at pH 7.4.

protein to release contents (Fig. 3). This implies that release of contents is not strictly regulated by the native conformation of the protein. Remarkably, when clathrin was preincubated with DTT for 10 min at 37°C, i.e., prior to addition to the vesicle mixture, leakage was inhibited. Relative to the kinetics of release observed for the control experiment (c in Fig. 3), the kinetics of release of DTT-pretreated clathrin were comparable to those obtained for trypsin treatment (not shown, cf. d in Fig. 3).

Interaction of clathrin with PS vesicles

To explain the observations presented above, it would seem reasonable to assume that binding of clathrin to the vesicle occurs, followed by bilayer perturbation and subsequently leading to release of contents.

To determine protein binding, two approaches were taken, one involving the direct determination of the net amount of bound clathrin to multilamellar vesicles by protein measurements and another, indirect procedure, relying on resonance energy transfer. Using the first approach no significant loss of protein from the supernatant could be detected at pH 7.4 nor at 6.5, after centrifugation of the multilamellar PS vesicles, in spite of the observed release of CF at those conditions (see above). At pH 4.0, massive release of contents occurs. However, at these conditions essentially all clathrin precipitated, precluding an accurate estimation of the amount of bound clathrin to the vesicles. To determine more accurately the potential interaction of clathrin, a more sensitive procedure was employed based on resonance energy transfer between Trp residues in the protein and NBD, linked to the headgroup of PE (*N*-NBD-PE). With this procedure, efficient interaction of clathrin with PS lipid vesicles at pH 4.0 could be demonstrated as reflected by quenching of Trp fluorescence by approx 44%. However, with PC vesicles as target membranes, energy transfer was only observed at pH values below 3.0. Thus at pH 4.0, when essentially all CF is lost within 2 min, no stable binding of clathrin to the PC vesicles could be detected. Both at pH 6.5 and 7.4, clathrin interaction with PS did occur, although to very limited extents as indicated by Trp fluorescence quenching of only 4.0 and 3.3%, respectively. Interaction of clathrin with PC vesicles at analogous conditions was virtually negligible (Fig. 4).

Penetration of clathrin in PS bilayers

To determine the mode of interaction of clathrin with the lipid bilayer, two possibilities were taken into account. First, it appeared possible that clathrin, by a predominant electrostatic interaction, could be capable of bilayer perturbation by inducing lipid phase separations. Alternatively, it seems possible that clathrin perturbs the bilayer by actual penetration. To test the

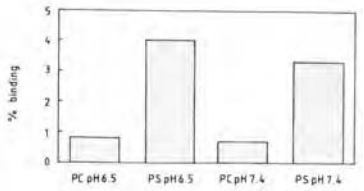

Fig. 4. pH and lipid-dependent binding of clathrin to LUV. PS or PC LUV (100 nmol) were incubated with clathrin (20 μg) at pH 6.5 or 7.4. The extent of binding was determined by resonance energy transfer, as described in Materials and Methods.

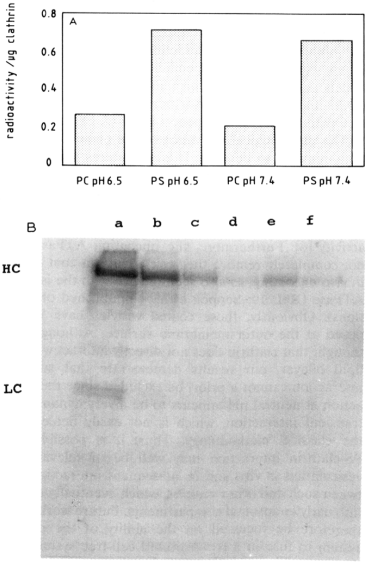

Fig. 5. Penetration of clathrin into lipid bilayers. (A) PS and PC LUV were labeled with ^{125}I-labeled TID. The vesicles (100 nmol) were subsequently incubated with clathrin (50 μg) at pH 7.4 or 6.5 (37°C) for 15 min and then irradiated. Clathrin-associated radioactivity was determined after gelelectrophoresis. (B) The corresponding autoradiogram is shown. For comparison, labeling occurring at pH 4.0 is also included. Lanes indicate: a, PS and b, PC at pH 4.0; c, PS and d, PC at pH 6.5; e, PS and f, PC at pH 7.4. HC and LC are clathrin heavy chain and light chain, respectively. Note the substantial labeling of LC, occurring with PS vesicles at pH 4.0 (a).

latter possibility, bilayers were prepared that were labeled with the hydrophobic, photoactivatable membrane probe TID. Penetration of protein will be revealed by the association of radiolabel with the protein after photoactivation of the hydrophobic photoaffinity label. As shown in Fig. 5A, clathrin labeling (i) is apparent after the protein's interaction with both PS and PC vesicles, (ii) is more pronounced with PS than PC vesicles and (iii) increases with decreasing pH. These observations are entirely consistent with the lipid species and pH dependence of CF release. Careful analysis of the autoradiograms shows that with PS vesicles and at pH 4.0, clathrin light chains become distinctly labeled (Fig. 5B). With PC vesicles under similar conditions light chain labeling was barely detectable in spite of substantial heavy chain labeling, comparable to that of PS vesicles. At both pH 6.5 and 7.4 only labeling of the clathrin heavy chain was seen, irrespective of the vesicle composition.

Discussion

In the work presented here we have demonstrated that significant interaction of clathrin coat protein can occur with lipid vesicles at neutral pH. The interaction was revealed by clathrin-induced membrane disruption, as monitored by leakage of aqueous liposomal contents, and was correlated with the ability of clathrin to actually penetrate into membranes, as demonstrated by hydrophobic photoaffinity labeling. In this respect, the latter experiment serves a dual purpose by also demonstrating that only clathrin penetrated and that the residual protein 'impurity' did not interfere with the interaction. The interaction of clathrin appears to depend on the presence of PS in the target membrane, is particularly facilitated by the presence of relatively minor amounts of cholesterol, and is inhibited by PC. These observations are typical for a protein interacting initially by electrostatic binding, accompanied or followed by a hydrophobic binding component that eventually leads to penetration. It is becoming increasingly clear that this scenario also applies to the mechanism of protein-induced membrane fusion, which might occur at appropriate conditions (Ref. 16; see below). Apart from its relevance in receptor-mediated endocytosis, clathrin is frequently used as a model for lipid–protein interactions [7–9;12,17]. The protein is acidic [17] and, hence, some of its residues are susceptible to protonation at low pH, which results in conformational changes, an increase in protein hydrophobicity and, below pH 6.0, in selfaggregation of the protein [9]. Therefore, the occurrence of fusion under such conditions is not unexpected [7,8]. However, it seems not very likely, given the extreme conditions, that these observations bear significant physiological relevance. Also, the local pH changes that would be required in

such a case (see Ref. 18) would act in a direction opposite to the H^+-ATPase pump that acidifies early endosomes.

A most remarkable observation in the present work is that the interaction of clathrin at neutral pH appears to be very dynamic. Stable binding, i.e., the association of clathrin with the bilayer, can only be determined after low pH incubations (with LUV at pH 4.0 in the case of PS, and at pH 2.7 for PC), representing conditions where the protein has undergone gross conformational changes and selfaggregation. At those conditions (pH 4.0) even the light chains of clathrin become substantially labeled when the protein interacts with PS vesicles. It is emphasized that such labeling barely occurs with PC vesicles and is virtually absent at higher pH values, where only heavy chain labeling is seen. Still, at neutral pH and in a lipid-dependent fashion, the protein does transiently penetrate, causing membrane perturbations as reflected by release of CF. Apparent 'instability' of the protein-lipid complex formed at neutral pH conditions may be correlated to the large size of the clathrin triskelion (650 kDa, Ref. 1) requiring multiple anchorage sites for stable interactions to occur. The latter may arise when at low pH, but not at neutral pH, the protein selfaggregates. In this regard it should be noted that spontaneous, partial removal of clathrin from coated vesicles takes place following an incubation at 37°C (pH 7.4) in the absence of any disrupting factor (Ref. 19; our unpublished observations). Within this context it is finally relevant to point out that increasing membrane curvature (SUV vs. LUV) appears to facilitate clathrin interaction, presumably due to enhanced penetration. Thus interaction with PC *SUV* occurs, albeit only at acidic pH [12,17,20], even causing such vesicles to fuse [7]. With PC *LUV* no fusion could be detected [8], which is consistent with the present observations, showing that significant binding of clathrin to PC LUV only occurs at pH values below 3.0.

The initial interaction of clathrin with the membrane surface depends on electrostatic interactions, involving positively charged groups in the protein [9,12]. Indeed the pH-dependent experiments indicate the preference of this type of interaction for monomeric clathrin as well. At pH 6.5 (where clathrin does not yet selfaggregate) the rate of CF release from PS vesicles is almost double the rate observed at pH 7.4. By contrast, only background leakage is seen for PC vesicles at either pH. Apparently, the native triskelion structure is rather important for the initial interaction to occur. Trypsinization leads to an inhibition of clathrin's ability to release vesicle contents at neutral pH. Such treatment has been shown to remove a fragment containing the terminal domain [21], found at the end of the clathrin heavy chain, which is known to be rich in positively charged lysine residues [22]. The anchoring function of this domain at physiological conditions has also been proposed [10,23]. The subsequent penetration event seems to be carefully orchestrated, occurring after initial binding of unperturbed clathrin to the bilayer. This may be inferred from the observation that prior treatment of clathrin with DTT inactivates the leakage capacity of the protein, analogous to trypsin, whereas DTT treatment during the interaction process boosts the leakage process. RET binding experiments showed that the latter was not due to an enhanced binding (not shown). Hence, the results lead then to the suggestion that structural constraints in the protein are relieved that give rise to optimization of the ensuing hydrophobic interaction step, involving penetration. Whether the hydrophobic interaction involves defined conformational changes, which can be induced by lowering the pH [24] or which, as suggested by the present work, might be triggered at neutral pH as a result of the initial electrostatic interaction, remains to be established. However, some specificity of clathrin penetration is suggested as the protein only penetrates after initial interaction, i.e., prior perturbation of the structure with DTT prevents penetration (cf. Fig. 3d).

The physiological relevance of the present observations remains to be seen. It is yet interesting to note, however, that several recent observations indicate that endocytosis-derived clathrin-coated vesicles may exist in cells for prolonged times after their formation [5] and that clathrin-coated vesicles can display fusion activity [6]. Furthermore, the uncoating ATPase may not completely remove the vesicle clathrin coat during in vivo uncoating, as also seen in vitro with the isolated ATPase (Ref. 19: Seppen et al., unpublished observations). Obviously, these coated vesicles have PS exposed at the outer membrane surface. Although it is thought that clathrin does not directly interact with the lipid bilayer, our results demonstrate that such an interaction cannot a priori be excluded since the interaction at neutral pH appears to be a very dynamic and transient interaction, which is not easily detected by the classical methodology. Thus, it is possible that PS-clathrin interaction may well be of relevance in vesiculation in vivo and in subsequent interactions between such and other vesicles, which eventually mature into early endosomal compartments. Future work might therefore be focussed on the ability of the present system to fuse in a reconstituted cell-free system, and the potential dependence of this process on cytosolic factors [6,25,26].

Acknowledgments

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