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Fusion of negatively charged liposomes with clathrin-uncoated vesicles

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The interaction of lipid vesicles with uncoated vesicles from bovine brain has been studied by fluorescence energy transfer between fluorescent lipid analogs (NBD-PE, Rh-DOPE), by loss of fluorescence self-quenching (NBD-PE, carboxyfluorescein) and by freeze-fracture electron microscopy. The fluorescence techniques monitor the mixing of membranous lipids and the induced release of encapsulated material. The results demonstrate a mixing of the negatively charged lipid (PA, PS) vesicles with the uncoated vesicles. In parallel with the lipid mixing a release of intravesicularly encapsulated material takes place. Lipid vesicles composed of zwitterionic lipids (PC, DOPC, PC: PE) do not specifically interact with uncoated vesicles. The electron micrographs reveal single fusion events. Studies on the kinetics are consistent with a fusional mechanism of the negatively charged lipid vesicles with uncoated vesicles.

Introduction

Fusion of exogenous and endogenous material to plasma membranes or intracellular membranes is a fundamental process of cellular life. With the advent of liposomal or vesicular bilayers it was hoped to understand the physiological events involved in fusional processes from studies on model membranes. However, even these studies on model membranes turned out to be rather complex and fusion was neither well demonstrated nor well separated from lipid transfer (for comparison on a kinetic basis of both phenomena see Ref. 1). Studies on model membranes currently experience a renaissance with respect to the potential both of liposomal drug encapsulation and gene transfer.

One fundamental prerequisite for the induction of fusion in model membranes was found in defect structures within the lipid bilayer [2]. The action of bivalent or polyvalent cations on bilayers composed of negatively charged lipids (PS, PA) was also considered with respect to the creation of phase separations leading to membranous fusion [3,4]. Especially the Ca^{2+} effect was discussed with the formation of tightly packed and less hydrated

Abbreviations: PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; PS, phosphatidylserine; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; Rh-DOPE, *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine; Tris, tris(hydroxymethyl) aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Mes, 4-morpholineethanesulfonic acid.

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structures of the lipid bilayer [5]. These organized structures could be responsible for the initiation of fusion events. However, unphysiological high Ca^{2+} concentrations are needed to promote fusion in model systems. On the other hand studies on viral fusion to a variety of cells reveal a highly specific role of fusogenic proteins [6–11]. Fusion mediated by electric fields has become a widespread method in biotechnology [12]. The role of an osmotic pressure on fusion of biological membranes has recently been hypothesized [13]. It is further conceivable that the fusion of vesicle-like particles with intracellular organelles or membranes should be highly specific.

The endocytotic uptake of material through the formation of coated pits and coated vesicles inside the cell has been studied to a great extent [14,15]. However, the molecular events which are responsible for the loosening of the coat and the further fate of the uncoated vesicles are still not very clear. It has been recently shown from this laboratory that in in vivo experiments a DNA encapsulated in targeted liposomes can be found in the clathrin-coated vesicles of liver cells shortly after the intravenous administration into rats [16]. Previously it has been suggested that the coated and uncoated vesicles may transfer their internal cargo from one intracellular compartment to the other possibly by fusion [17]. Based on a fluorescence assay it was shown that uncoated ('stripped') vesicles fuse to lysosomes and that the clathrin coat seems to inhibit the fusion observed for the stripped vesicles [18]. First studies in cell-free systems reveal a possible fusion of uncoated vesicles to endosomes [19]. For a better understanding of the possible fusion events of uncoated vesicles with various intracellular compartments, we here report on the interaction of bilayer vesicles composed of phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PC) with uncoated vesicles at neutral pH. The results obtained so far show that vesicles of negatively charged lipids (PS, PA) readily fuse with uncoated vesicles. As consequence of fusion a release of the vesicle content is observed. In the accompanying communication [51] these studies are extended to the interaction of negatively charged lipid vesicles with nuclear membranes. The results of both studies further serve to open pathways for possible combinations of an extra- and intracellular targeting of material.

Experimental Procedures

Materials

Phospholipids were obtained from Lipid Products, Surrey, U.K. (phosphatidylserine from bovine brain, PS), and Sigma, St. Louis, U.S.A. (phosphatidylcholine (PC) from egg yolk, phosphatidic acid (PA) from egg yolk lecithin, L-α-phosphatidylethanolamine (PE) from *Escherichia coli*, L-α-dioleoylphosphatidylcholine (DOPC)). *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine (Rh-DOPE) were from Avanti Polar-Lipids Inc., Birmingham (AL), U.S.A. The lipids were dissolved in chloroform and stored under argon at −70 °C.

5,6-Carboxyfluorescein from Eastman Kodak, U.S.A., was recrystallized from ethanol and subsequently purified on LH-20 according to Rolston et al. [20]. Fluram (Fluorescamine) was obtained from Serva Feinbiochemica, Heidelberg, F.R.G. Methylviologen (MV), 4-morpholineethanesulfonic acid (Mes). Ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), tris(hydroxymethyl)aminomethane were purchased from Sigma, St. Louis, U.S.A. Inorganic salts were from E. Merck, Darmstadt, F.R.G. and deuterium oxide was from Aldrich Chemicals Milwaukee, U.S.A. Sephacryl S-1000, Sepharose 4B, G-25 and LH-20 were from Pharmacia, Uppsala, Sweden.

Methods

Coated vesicles from fresh bovine brain were prepared in 0.1 M Mes, 1 mM EGTA, 0.5 mM $MgCl_2$ and 0.02% NaN_3 according to Pearse [21] with modifications described by Nandi et al. [22]. Electron micrographs by negative staining indicated that a homogeneous fraction of coated vesicles was eluted from a S-1000 column following the peak at the void volume which mostly contained aggregates.

Uncoated vesicles were obtained by dialysing the coated vesicles against 0.02 M Tris, 0.02% NaN_3 (pH 8.20) for 24 h or more [23,24]. The resultant suspension was centrifuged 10 min at

low speed to remove any aggregates and then two times for 1 h at 36000 rpm (70 Ti rotor) in a Beckman L8-65B centrifuge. The final pellet was homogenized in Tris buffer (pH 8.20) followed by a second low-speed centrifugation. The supernatant containing the uncoated vesicles was used for the experiments. The protein concentration of the uncoated vesicles was determined from the absorbance at 280 nm in 6 M guanidine hydrochloride assuming $A^{1\%}_{280\,nm} = 10.9$ [25].

Small unilamellar vesicles were prepared by a bath sonication of the lipid dispersion under nitrogen. The lipid concentration was usually 0.8 mg in 0.5 ml buffer (0.02 M Tris, 0.02% NaN_3 (pH 7.20) or 0.02 M sodium phosphate, 0.02% NaN_3 (pH 7.33)). For the phosphatidylethanolamine samples a PE : PC ratio of 1 : 1 was used.

Fluorescent spectra were recorded in thermostated cuvettes in a FICA 55 MK II spectral fluorometer using the ratio mode. Stopped-flow experiments were performed using a Sigma-mixing apparatus and a fluorescence detection system described elsewhere [26].

Labeling

For the staining of lipid bilayers the fluorescent lipid analogs dissolved in chloroform were pipetted to the lipids according to the molar ratio desired. The chloroform was evaporated under vacuum and the aqueous solvent was added. The dispersion was sonified in a bath sonifier under nitrogen. The procedure ensures a homogeneous distribution of the lipids without any interference from organic solvents.

In cases where the aqueous intravesicular milieu contained the marker (carboxyfluorescein) the aqueous lipid dispersion was sonified in the presence of the marker. The external marker molecules were subsequently removed by exclusion chromatography on G-25 or Sepharose 4B-Cl. Uncoated vesicles were labeled by a procedure which avoids contact with organic phases (usually up to 1% of the aqueous solvent). A dry film of the fluorescent molecules was deposited on the wall of small test tubes and the uncoated vesicles were added under vigorous stirring. The solution was incubated for 1 h at 37 °C and subsequently layered on a S-1000 gel column. The stained uncoated vesicles eluted after the void volume containing mostly aggregates. The elution profiles measured by the absorbances at 280 nm (absorption of the aromatic amino acids), at 463 nm (absorption of NBD) and at 545 m (absorption of Rh) are identical. In some cases a second peak close to the bed-volume appeared consisting of protein (residual clathrin) or free rhodamine. Extensive attempts to label the uncoated vesicles with both NBD-PE and Rh-DOPE did not show any incorporation of NBD-PE and Rh-DOPE at concentrations which allow clear resonance energy transfer experiments. This may be due to the preferential incorporation of NBD-PE. Thus for the present studies the uncoated vesicles were only labeled with NBD-PE under self-quenching concentrations for the fusion experiments. Fluorescamine (Fluram) binds covalently to amino groups and forms a fluorescent product [27]. The labeling strategy was similar as above except that the separation step of the free fluram can be avoided because fluram competitively reacts with water to a non-fluorescent compound [28].

Freeze-fracture electron microscopy

Solutions of vesicles were cryoprotected in 30% glycerol. 1 μl drops of the solution were deposited on gold supports, then quick-frozen in Freon 22 cooled with liquid nitrogen and stored in liquid nitrogen. Specimens were fractured in a Balzers 301 freeze-etching unit at $-130\,°C$ in a vacuum of 10^{-4} Pa. The fracture surfaces were shadowed with carbon-platinum evaporated at an angle of 45°. The replicas were strenghtened by evaporation of carbon at an angle of 90°. The replicas were then cleaned with ethanol, rinsed in distilled water and collected on 300 mesh copper grids. Observations were carried out in a Siemens Elmiscop 102 electron microscope.

Fusion assays

Two principally different fusion assays were used which are based on the mixing of the lipid bilayers or membranous components and on the mixing or release of the aqueous intravesicular compartments, respectively. The mixing of the lipid or membrane components was followed by induced changes of the fluorescence energy transfer between a fluorescent donor and acceptor or by the loss of self-quenching of fluorescent molecules

embedded within the membrane. The lipid analogs NBD-PE and Rh-DOPE served as donor-acceptor pair [29], in addition NBD-PE shows self-quenching at high concentrations (see below) [30].

The mixing of a free and of a membrane containing donor-acceptor pairs dilutes the donor and acceptor molecules and thus increases the average distance between the donor-acceptor pair. Consequently the resonance condition for the radiationless energy transfer (RET, resonance energy transfer) is reduced as the resonance energy transfer falls off according to r^{-6} where r is the distance between donor and acceptor molecules [31]. In addition to the commonly used fluorescent lipid analogs NBD-PE (donor) and Rh-DOPE (acceptor) we took advantage of the combination tryptophan (endogenous) - flurescamine. Flurescamine becomes fluorescent by covalently binding to amino groups and acts as acceptor of the tryptophan fluorescence. In the latter case the mixing of the flurescamine labeled with unlabeled membranes destroys the favourable energy transfer due to a rearrangement of the membranous components. Resonance energy transfer between NBD- and Rh-carrying lipid analogs was recently demonstrated in a microscope on a cellular level [32]. Major contributions of the fluorescence quenching originating from membrane proteins can be neglected.

In the case of self-quenching the mixing of a free and of a membrane containing e.g. NBD-PE under self-quenching concentrations leads to a dequenching as consequence of the dilution of the fluorescent molecules within the newly formed membranes. A dequenching also results by the mixing of the aqueous intravesicular compartments where only one vesicle species carried e.g. carboxyfluorescein at self-quenching concentration [33]. The leak of carboxyfluorescein during the fusion event was proven by the extravesicular addition of methylviologen which is an effective quencher of the carboxyfluorescein fluorescence (Lawaczeck et al., to be published).

Results

Fig. 1 demonstrates the self-quenching properties of NBD-PE embedded in PS-vesicular bilayers. A constant NBD-PE concentration was

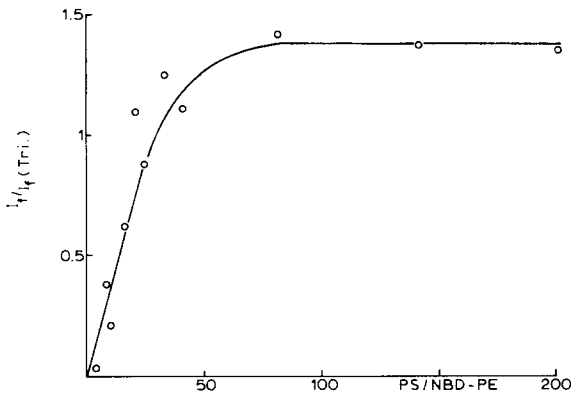

Fig. 1. Self-quenching of NBD-PE embedded in PS vesicles. At constant amount of NBD-PE the PS/NBD-PE ratio is varied. Ratio of the fluorescence intensities I_f/I_f(Tri.) (excitation 445 nm, emission 525 nm) before and after adding Triton X-100 (final concentration 1%) as function of the PS/NBP-PE ratio (w/w). No correction of the quantum yield for the Triton containing samples is performed. Room temperature.

sonified in the presence of various amounts of PS. The NBD-fluorescence (excitation 445 nm, emission 525 nm) was read before and after adding Triton X-100 (no correction for the different fluorescence quantum yields of NBD-PE in lipid bilayers and Triton aggregates have been made). The ratio of both fluorescence intensities versus the PS/NBD-PE ratio shows that there was no self-quenching at low NBD-concentration. However, the self-quenching rapidly increased at higher NBD-concentrations. A high self-quenching was observed when neighbor NBD-PE molecules were surrounded by only one layer of PS molecules or less. A homogeneous NBD-PE distribution within both halves of the PS bilayer was assumed and an asymmetry was not considered. NBD-PE labeled uncoated vesicles at self-quenching concentrations were mixed with vesicles composed of two negatively charged (PS, PA) and two zwitterionic lipids (PC, PC : PE). As pure PE does not form single-bilayer vesicles a 1 : 1 mixture of PC : PE was used throughout the experiments. The fluorescence spectra of uncoated vesicles in Fig. 2 were recorded before and after the mixing with equal amounts of the respective vesicles. Fig. 2 also contains a spectrum of the uncoated vesicles after adding Triton X-100 and a representative light scattering from a lipid vesicle

solution. All spectra were taken of dilute solutions at high magnification under identical conditions. It is evident from Fig. 2 that only the negatively charged lipid vesicles led to a dequenching of the NBD fluorescence. If one compares the intensity ratio of the labeled uncoated vesicles before and after adding Triton X-100 with values from Fig. 1 the initial NBD-labeling amounts up to 8–10% assuming a homogeneous incorporation of the NBD-PE molecules into the outer leaflet of the uncoated-vesicle membrane.

For the uncoated vesicle-PS (UV-PS) system stopped-flow measurements on the dequenching kinetics were performed. A typical result of these experiments is shown in Fig. 3 where the fluorescence intensity versus the time after mixing of the labeled uncoated vesicles with PS vesicles is plotted. From a series of such measurements the initial slopes of the dequenching were calculated as function of the stoichiometric PS concentration, and a linear relationship is observed by plotting the initial slopes versus the PS concentration. This linear behaviour is consistent with a bi-particle mechanism as rate-limiting step favouring fusion over lipid exchange.

According to the procedure outlined in Ref. 1 the initial fusion events can kinetically be modelled by the following reaction scheme giving rise to the

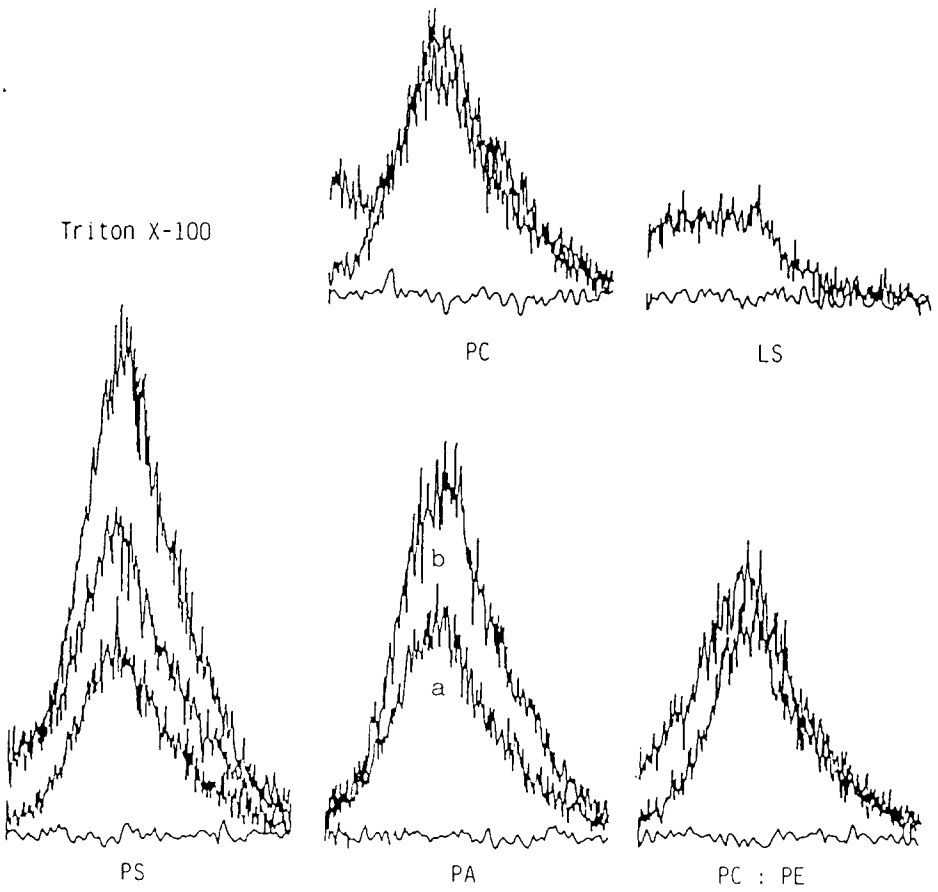

Fig. 2. Self-quenching experiments on the fusion of lipid vesicles with uncoated vesicles. The uncoated vesicles were labeled with NBD-PE under self-quenching concentrations. 200 μl labeled uncoated vesicles were added to 300 μl buffer (0.02 M Tris, 0.02% NaN_3 (pH 7.20)) (spectra a), to these samples 50 μl vesicles as indicated were added (spectra b) followed by the addition of Triton X-100 (final concentration 0.2%). Excitation 445 nm, emission from 470 to 600 nm. LS corresponds to the light scattering measured for a PC-containing sample. Room temperature.

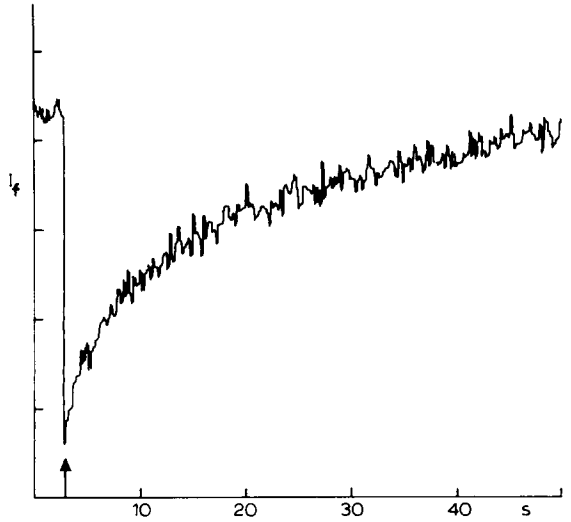

Fig. 3. Stopped-flow measurements on the fusion-kinetics. NBD-PE labeled uncoated vesicles (at self-quenching NBD-concentration) are mixed with PS vesicles. Fluorescence intensity I_f as function of time in pre-trigger mode. At the time indicated by the arrow the two solutions are rapidly mixed. Excitation 445 nm, emission by 505 nm cut-off filter. 20 °C. Same buffer as indicated in Fig. 2.

reaction rates of Eqn. 1

$$UV_1 + PS \xrightarrow{k_{1s}} UV_2$$

$$UV_2 + PS \xrightarrow{k_{2s}} UV_3; \quad UV_1 + UV_2 \xrightarrow{k_{12}} UV_{12}$$

$$UV_3 + PS \xrightarrow{k_{3s}} UV_4; \quad UV_1 + UV_3 \xrightarrow{k_{13}} UV_{13}$$

In the time-range considered (seconds to minutes) there is no aggregation of the UV under the experimental conditions chosen. Fusion among the UV is also not evident from the freeze-fracture electron micrographs. The same holds for the PS vesicles. Thus the steps $UV_1 + UV_1 \rightarrow UV_{11}$ and $PS + PS \rightarrow PS_2$ can be omitted (these steps would further not lead to changes of the fluorescence intensities).

$$\frac{d[UV_1]}{dt} = -k_{1s}[UV_1][PS] - k_{12}[UV_1][UV_2] - k_{13}[UV_1][UV_3] - \ldots$$

$$\frac{d[PS]}{dt} = -k_{1s}[UV_1][PS] - k_{2s}[UV_2][PS] - \ldots \quad (1)$$

$$\frac{d[UV_2]}{dt} = k_{1s}[UV_1][PS] - \ldots$$

$[UV_i]$, [PS] and k_i denote the vesicle concentrations and rate constants, respectively. Assuming that NBD fluoresces with the quantum yields Φ_1 and Φ_2 (with $\Phi_1 < \Phi_2$) in the species UV_1 and UV_2, respectively, the increase of the fluorescence signal follows from

$$I_f(t) = \Phi_1[UV_1] + \Phi_2[UV_2] + \ldots \quad (2)$$

At $t = 0$ only PS vesicles and UV_1 are present therefore the initial slope of the fluorescence time-dependence is given by (at $t = 0$: $UV_1 = UV_1^\circ$ and $PS = PS^\circ$)

$$\left[\frac{dI_f(t)}{dt}\right]_{t=0} = \Phi_1\left[\frac{d[UV_1]}{dt}\right]_{t=0} + \Phi_2\left[\frac{d[UV_2]}{dt}\right]_{t=0}$$

$$= -\Phi_1 k_{1s}[UV_1^\circ][PS^\circ] + \Phi_2 k_{1s}[UV_1^\circ][PS^\circ]$$

$$= (\Phi_2 - \Phi_1) k_{1s}[UV_1^\circ][PS^\circ] \quad (3)$$

This linear relation between the initial slopes and the PS vesicle concentration agrees well with the experimental findings from Fig. 4.

The results obtained by labeling the uncoated vesicles are consistent with results where NBD-PE and Rh-DOPE were incorporated into the four lipid vesicles regarded, and resonance energy

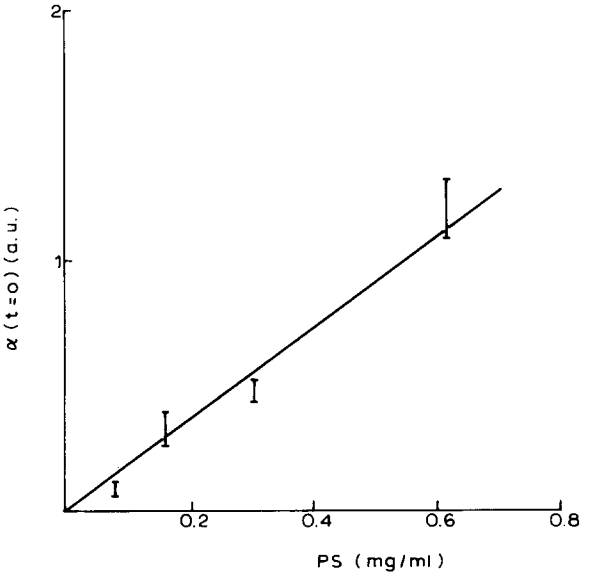

Fig. 4. Initial slopes of the fluorescence intensity, α ($t = 0$), from stopped-flow measurements such as indicated in Fig. 3, as function of the stoichiometric PS concentration (the bars indicate the ranges of experimental scatter).

transfer studies were performed. Examples of the resonance energy transfer studies are summarized in Fig. 5. Again only the negatively charged lipid vesicles led to a pronounced reduction of the resonance energy transfer conditions pointing to a mixing of the negatively charged lipid bilayer with the uncoated-vesicle membrane. PC, DOPC and PC : PE had no effect on the resonance energy transfer spectra. In the pH range studied (pH 7.5 to 5.2) there was no marked pH effect of the fusion tendency.

In addition to the experiments concerned with the mixing of lipids the mixing of the intravesicular contents was followed. Carboxyfluorescein was intravesicularly incorporated at self-quenching concentrations so that the fusion with empty particles should lead to a dequenching manifested by an increase of the fluorescence intensity. From the lipid systems studied (PC : PE, PC, DOPC, PA and PS) again only PA and PS led to the dequenching of the intravesicularly encapsulated carboxyfluorescein if uncoated vesicles were ad-

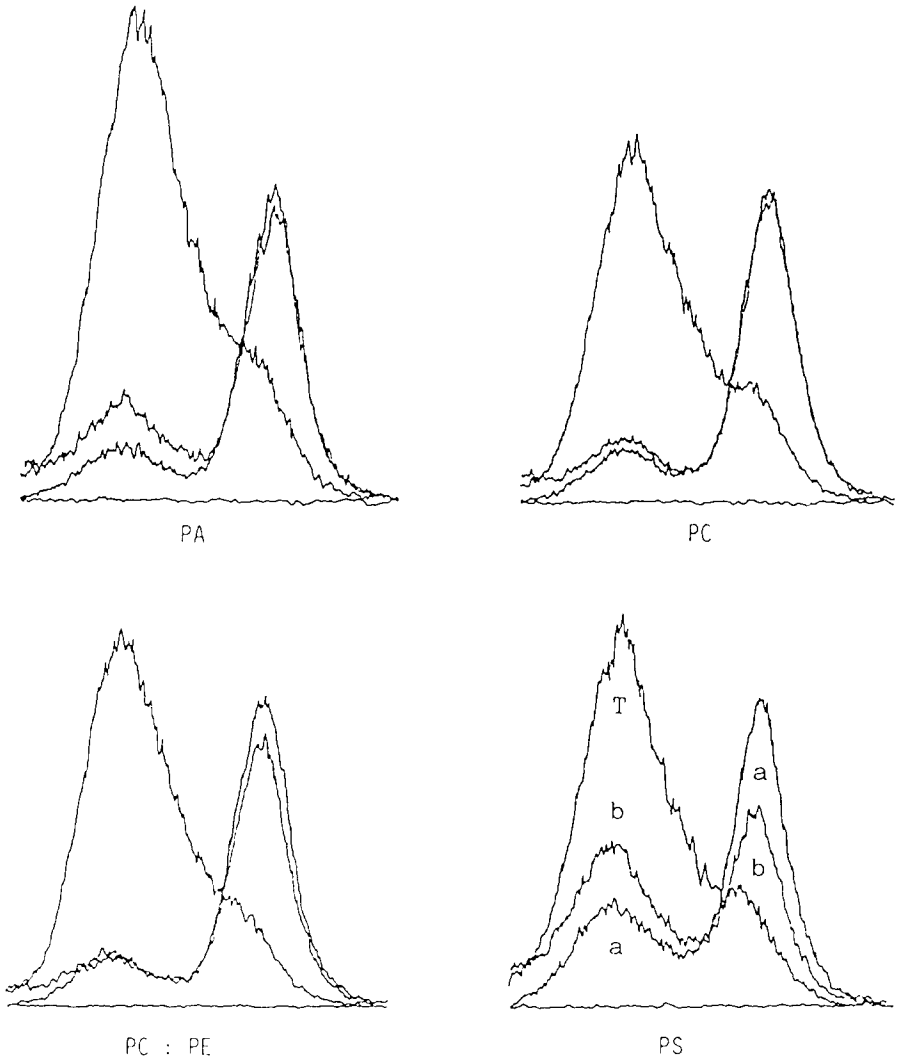

Fig. 5. Resonance energy transfer (RET) experiments for four sets of NBD-PE/Rh-DOPE containing vesicles. All samples contain 10 μl vesicles in 0.5 ml 20 mM Tris pH 7.2 (spectra a), to these samples 50 μl uncoated vesicles are added (spectra b) followed by the addition of Triton X-100 (concentration 0.2%) (spectra T). Excitation 445 nm, emission from 470 to 650 nm. The peaks at 520 and 585 nm corresponds to the NBD- and Rh-fluorescence, respectively. Room temperature.

ded. For PA the results are shown in Fig. 6 where the time-dependence and the fluorescence spectra at various stages of the experiment are summarized: (a) before any manipulation, (b) as consequence of the addition of uncoated vesicles and (c) after adding Triton to (b). To a new sample methylviologen, a potent quencher of the carboxyfluorescein fluorescence, was given (d), followed by the application of uncoated vesicles (e). The final addition of Triton led to spectra between those of (a) and (e). The experiment in the absence of methylviologen demonstrates a time-dependent dequenching of the carboxyfluorescein fluorescence with a reaction half-time equivalent to the half-time of the experiment from Fig. 3. However, it should be remembered that according to the bi-particle mechanism the half-times depend on

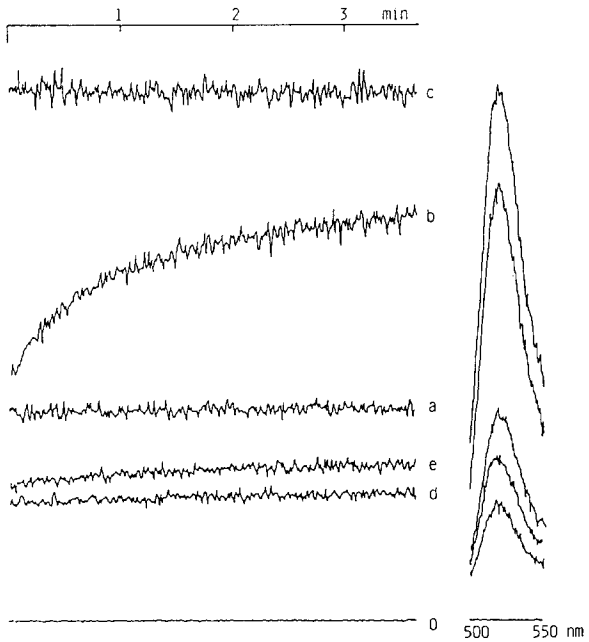

Fig. 6. Fusion experiment by dequenching of intravesicular carboxyfluorescein. Spectra (right, excitation 488 nm, emission 500 to 550 nm) and time-dependence (left, excitation 488, emission 520 nm) as function of the experimental manipulation. (a) 10 μl PS vesicle with carboxyfluorescein (0.2 M) intravesicularly incorporated in 0.5 ml 0.02 M Tris (pH 7.20); (b) same as (a)+20 μl uncoated vesicles; (c) same as (b)+ Triton X-100 (final concentration 0.2%); (d) new sample as in (a)+methylviologen (final concentration 3.3 mM); (e) same as (d)+20 μl uncoated vesicles (like in (b)). The addition of Triton to sample (e) shows a fluorescence between the spectra (a) and (e); (0) zero-line. Room temperature.

the concentrations of the reacting species. The experiments in the presence of methylviologen revealed that there is some residual carboxyfluorescein in the extravesicular milieu which was quenched by the extravesicularly added methylviologen, and further that the vesicles stayed intact after the methylviologen addition and were almost not permeable to methylviologen or carboxyfluorescein. The addition of both methylviologen and uncoated vesicles indicated that the carboxyfluorescein molecules became accessible to the external methylviologen resulting in the observed fluorescence quenching of carboxyfluorescein by methylviologen. The membranes therefore became leaky or the extravesicular methylviologen could enter into the intravesicular compartment as consequence of the fusion event.

The results of the spectroscopic studies were supported by freeze-fracture electron micrographs. The electron micrographs revealed sizes with diameters in the range form 8 to 110 nm (mean 46 nm) and 80 to 140 nm (mean 110 nm) for PS vesicles and uncoated vesicles, respectively. Usually some particles (probably proteins) could be seen on the fracture surface of the uncoated vesicles. The fusion between PS vesicles and uncoated vesicles is evident from Fig. 7 which shows single fusional events. For that purpose dilute solutions of PS vesicles and uncoated vesicles were mixed and allowed to fuse for short times (about 30 s) before they were quick-frozen to liquid nitrogen temperatures. From the values of the diameters of the PS vesicles and the uncoated vesicles one can state that Fig. 7 shows PS vesicles in association or in an early fusion state with uncoated vesicles. The initial aggregation and morphological changes of PS vesicles induced by Ca^{2+} ions [34] corresponds to the adhesion states of Fig. 7.

Discussion

The results of the preceding sections show that negatively charged vesicles composed of PA or PS fuse with uncoated vesicles. Fusion at neutral pH is manifested by lipid mixing, by freeze-fracture electron micrographs and complemented by experiments on the release of the intracellular content. The fusion experiments were carried out at

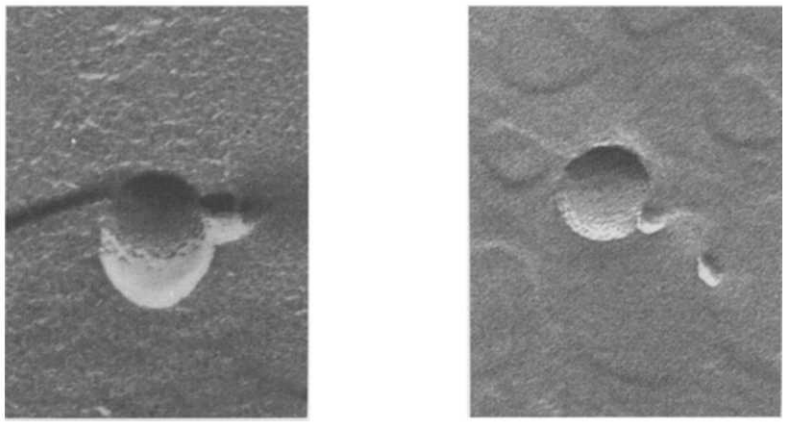

Fig. 7. Two typical examples of the freeze-fracture electron micrographs demonstrating fusion of PS vesicles with uncoated vesicles. Dilute solutions of PS vesicles were mixed with uncoated vesicles (both in 30% glycerol) and allowed to fuse for about 30 s. The samples were then quick-frozen and handled as described under Experimental Procedures. The diameters are: left, 45 and 115 nm and right, 20 and 80 nm, respectively. Samples of pure PS vesicles and of pure uncoated vesicles do not show associated vesicles under the same experimental conditions.

room temperature where the lipid bilayers used and the membranes were in their fluid state. In the majority of cases small unilamellar lipid vesicles as prepared by bath-sonication were used. As expected we did not observe any difference in the behaviour of egg PC and DOPC. Lipid-exchange [35,36] can be excluded as a major source for the observed lipid mixing because the kinetics should differ from the fusion kinetics observed in Fig. 4 [1]. In addition it was shown that headgroup labeled phospholipids with long fatty acyl chains are not exchanged between lipid vesicles or cells [29,30,37–39]. It was further demonstrated that fluorescent lipid analogs do not diffuse from one to a neighboring epithelial cell [40].

The kinetics of the fusion of PS vesicles with uncoated vesicles are consistent with a bi-particle mechanism (analog to a bi-molecular mechanism in ordinary kinetics). The freeze fracture electron micrographs reveal single fusion events and thus support the results from the spectroscopic studies. Thus there is convincing evidence that the underlying mechanism is a vesicle-vesicle fusion. As a consequence of the fusion step, the vesicular bilayer, possibly on the regions of the bi-particle contact, opens so that the intravesicular content comes in contact with the extravesicular medium. This is clearly demonstrated through the quenching of the intravesicular carboxyfluorescein fluorescence by extravesicular methylviologen. The leakiness of the liposomes or vesicles during the fusion event has been a matter of great attention and has led to differing interpretations (see, for example, Refs. 41 and 42).

Though contradictory in respect to the fatty acid specificity, clathrin, the major coat forming protein of coated vesicles, induces fusion among PC-lipid vesicles at low pH [43,44]. Lysozyme covalently bound to liposomes leads to fusion of these liposomes with erythrocyte membranes [45] at low pH. The polycation-dependent fusion as function of the pH has been described [46,47], and fusion seems to be initiated by a bilayer phase separation. Altstiel and Branton [18] showed that at neutral pH uncoated vesicles fuse four times more rapidly than coated vesicles with lysosomes indicating that the coat, predomiantly clathrin, prevents the fusion of coated vesicles with lysosomes.

Fusion is generally considered as a consecutive series of processes ranging from different stages of aggregation, i.e., flocculation and the more tightly packed coagulation, to the final fusion products (see, for example, Ref. 48). This intuitive picture is certainly adequate to describe the fusion of particles of comparable size, and in that respect the kinetics of fusion depends on the rate-limiting step. The collision-mediated aggregation is a second order process while the further reaction to the fused product can be of first order. The fusion

observed between uncoated vesicles and negatively charged vesicles is consistent with a second order process being the rate-limiting step. For the calcium induced fusion of PS-containing vesicles also the aggregational step is considered to be rate-limiting [29]. The fusion of influenza viruses to cardiolipin liposomes was also analysed in terms of a second-order aggregation followed by a first-order fusion process of the aggregates [49]. In the present case fusion is observed around neutral pH, room temperature, in the absence of measurable osmotic gradients and in the absence of bi- or polyvalent cations. The fusion tendency of the uncoated vesicles to the vesicles regarded arises from the negatively charged headgroups of the lipid bilayer. There is evidence that both endosomes and lysosomes carry negative charges [50] with endosomes being less charged than lysosomes. Thus the intracellular fusion of the uncoated vesicles with endosomes or lysosomes might be triggered in a directional way by the negatively charged surfaces of the target cells (endosomes, lysosomes) in a similar manner as the fusion described above between uncoated vesicles and vesicles composed of negatively charged lipid molecules.

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References

1 Lawaczeck, R. (1978) J. Coloid Interface Sci. 66, 247–256
2 Lawaczeck, R., Kainosho, M., Girardet, J.-L. and Chan, S.I. (1975) Nature 256, 584–586
3 Ohnishi, S. and Ito, T. (1974) Biochemistry 13, 881–887
4 Papahadjopoulos, D., Portis, A. and Pangborn, W. (1978) Ann. N.Y. Acad. Sci. 308, 50–66
5 Portis, A., Newton, C., Pangborn, W. and Papahadjopoulos, D. (1979) Biochemistry 18, 780–790
6 Marsh, M. (1984) Biochem. J. 218, 1–10
7 Strittmatter, W.J., Couch, C.B. and Mundy, D.I. (1985) Role of Proteins in the Fusion of Biological Membranes in Membrane Fluidity in Biology, Vol. 4, pp. 259–291, Academic Press, London, New York
8 White, J. and Helenius, A. (1980) Proc. Natl. Acad. Sci. USA 77, 3273–3277
9 Haywood, A.M. and Boyer, B.P. (1985) Proc. Natl. Acad. Sci. USA 82, 4611–4615
10 Citovsky, V. and Loyter, A. (1985) J. Biol. Chem. 260, 12072–12077
11 Citovsky, V., Blumenthal, R. and Loyter, A. (1985) FEBS Lett. 193, 135–140
12 Zimmermann, U., Vienken, J., Pilwat, G. and Arnold, W.M. (1984) Cell fusion (Ciba Foundation Symposium Vol. 103), pp. 60–85, Pitman Books, London
13 Lucy, J.A. and Ahkong, Q.F. (1986) FEBS Lett. 199, 1–11
14 Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1979) Nature 29, 679–685
15 Wileman, T., Harding, C. and Stahl, P. (1985) Biochem. J. 232, 1–14
16 Nandi, P.K., Legrand, A. and Nicolau, C. (1986) J. Biol. Chem. 261, 16722–16726
17 Pearse, B.M.F. and Bretscher, M. (1981) Annu. Rev. Biochem. 50, 85–101
18 Altstiel, L. and Branton, D. (1983) Cell 32, 921–929
19 Davey, J., Hurtley, S.M. and Warren, G. (1985) Cell 43, 643–652
20 Ralston, E., Hjelmeland, L.M., Klausner, R.D., Weinstein, J.N. and Blumenthal, R. (1981) Biochim. Biophys. Acta 649, 133–137
21 Pearse, B.M.F. (1975) J. Mol. Biol. 97, 93–98
22 Nandi, P.K., Irace, G., Van Jaarsveld, P.P., Lippoldt, R.E. and Edelhoch, H. (1982) Proc. Natl. Acad. Sci. USA 79, 5881–5885
23 Irace, G., Lippoldt, R.E., Edelhoch, H. and Nandi, P.K. (1982) Biochemistry 21, 5764–5769
24 Nossal, R., Weiss, G.H., Nandi, P.K., Lippoldt, R.E. and Edelhoch, H. (1983) Arch. Biochem. Biophys. 226, 593–603
25 Prasad, K., Alfsen, A., Lippoldt, R.E., Nandi, P.K. and Edelhoch, H. (1984) Arch. Biochem. Biophys. 235, 403–410
26 Pitterich, H. and Lawaczeck, R. (1985) Biochim. Biophys. Acta 821, 233–242
27 DeBernardo, S., Weigele, M., Toome, V., Manhart, K., Leimgruber, W., Böhlen, P., Stein, S. and Udenfriend, S. (1974) Arch. Biochem. Biophys. 163, 390–399
28 Stein, S., Böhlen, P. and Udenfriend, S. (1974) Arch. Biochem. Biophys. 163, 400–403
29 Struck, D.K., Hoekstra, D. and Pagano, R.E. (1981) Biochemistry 20, 4093–4099
30 Nichols, J.W. and Pagano, R.E. (1981) Biochemistry 20, 2783–2789
31 Förster, T. (1959) Discuss. Faraday Soc. 27, 7–17
32 Uster, P.S. and Pagano, R.E. (1986) J. Cell Biol. 103, 1221–1234
33 Weinstein, J.N., Yoshikami, S., Henkart, P., Blumenthal, R. and Hagins, W.A. (1977) Science 195, 489–491
34 Rand, R.P., Kachar, B. and Reese, T.S. (1985) Biophys. J. 47, 483–489
35 Martin, F.J. and MacDonald, R.C. (1976) Biochemistry 15, 321–327
36 Lawaczeck, R., Blackman, R. and Kainosho, M. (1977) Biochim. Biophys. Acta 468, 411–422

37 Struck, D.K. and Pagano, R.E. (1980) J. Biol. Chem. 255, 5404–5410
38 Wojcieszyn, J.W., Schlegel, R.A., Lumley-Sapanski, K. and Jacobson, K.A. (1983) J. Cell Biol. 96, 151–159
39 Arvinte, T. and Hildenbrand, K. (1984) Biochim. Biophys. Acta 775, 86–94
40 Van Meer, G., Gumbiner, B. and Simons, K. (1986) Nature 322, 639–641
41 Kendall, D.A. and MacDonald, R.C. (1982) J. Biol. Chem. 257, 13892–13895
42 Morris, S.J., Gibson, C.C., Smith, P.D., Greif, P.C., Stirk, C.W., Bradley, D., Haynes, D.H. and Blumenthal, R. (1985) J. Biol. Chem. 260, 4122–4127
43 Blumenthal, R., Henkart, M. and Steer, C.J. (1983) J. Biol. Chem. 258, 3409–3415
44 Hong, K., Yoshimura, T. and Papahadjopoulos, D. (1985) FEBS Lett. 191, 17–23
45 Arvinte, T., Hildenbrand, K., Wahl, P. and Nicolau, C. (1986) Proc. Natl. Acad. Sci. USA 83, 962–966
46 Wang, C.-Y. and Huang, L. (1984) Biochemistry 23, 4409–4416
47 Uster, P.S. and Deamer, D.W. (1985) Biochemistry 24, 1–8
48 Wu, P.-S., Tin, G.W., Baldeschwieler, J.D., Shen, T.Y. and Ponpipom, M.M. (1981) Proc. Natl. Acad. Sci. USA 78, 6211–6215
49 Nir, S., Stegmann, T. and Wilschut, J. (1986) Biochemistry 25, 257–266
50 Helenius, A., Mellman, I., Wall, D. and Hubbard, A. (1983) Trends Biochem. Sci. 8, 245–250
51 Lawaczeck, R., Nandi, P.K. and Nicolau, C. (1987) Biochim. Biophys. Acta 903, 123–131