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RETENTION OF AQUEOUS CONTENTS DURING DIVALENT CATION-INDUCED FUSION OF PHOSPHOLIPID VESICLES

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The relative kinetics of intermixing and release of liposome aqueous contents during Ca^{2+}-induced membrane fusion has been investigated. Fusion was monitored by the Tb-dipicolinic acid (DPA) fluorescence assay. Release was followed by the relief of self-quenching of carboxyfluorescein or by Tb fluorescence, with essentially identical results. Fusion of large unilamellar vesicles (LUV) made of phosphatidylserine (PS) in 100 mM NaCl (pH 7.4) at 25°C was initially non-leaky, whereas the fusion of small unilamellar vesicles (SUV) was accompanied by partial release of contents. After several rounds of fusion, the internal aqueous space of the vesicles collapsed. The rate of intermixing of lipids, measured by a resonance energy transfer assay, and the rate of coalescence of aqueous contents during fusion were similar over a range of Ca^{2+} concentrations. Most of the aqueous contents were retained after the fusion of SUV (PS) in 5 mM NaCl and 1 mM Ca^{2+}. LUV made of a 1:1 mixture of *Bacillus subtilis* cardiolipin and dioleoylphosphatidylcholine went through about two rounds of fusion in the presence of Ca^{2+} at 10°C, with complete retention of contents. Similar results were obtained with vesicles composed of phosphatidate/PS/phosphatidylethanolamine/cholesterol (1:2:3:2) in the presence of Ca^{2+} and synexin at 25°C. These results emphasize the diversity of the relative kinetics of fusion and release in different phospholipid vesicle systems under various ionic conditions, and indicate that the initial events in the fusion of LUV are in general, non-leaky.

Introduction

Membrane fusion involves the momentary loss of integrity of two lipid bilayer structures during their close apposition. The mechanism of the reorganization process during membrane fusion is not known. This reorganization can render the membranes transiently leaky to the contents of the aqueous space enclosed by the membranes. The study of the leakage associated with membrane fusion is important for characterizing the mechanism of membrane fusion, as well as for obtaining estimates of leakage to be expected during the

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Abbreviations: SUV; Small unilamellar vesicles; LUV, Large unilamellar vesicles; PS, Phosphatidylserine; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PA, Phosphatidic acid; CL, Cardiolipin; CF, Carboxyfluorescein; DPA, Dipicolinic acid; *N*-NBD-PE: *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE: *N*-(lissamine)Rhodamine-B-sulfonylphosphatidylethanolamine; Tes, 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulfonic acid.

delivery of liposome-encapsulated substances to cultured cells.

It was shown previously that the Ca^{2+}-induced fusion of small unilamellar vesicles (SUV) composed of phosphatidylserine (PS) is accompanied by the release of about 10% of the aqueous contents per fusion event during the initial stages of the process [1,2]. On the other hand, the initial fusion events between large unilamellar vesicles (LUV) composed of PS are relatively non-leaky [2]. These events correspond to the fusion of dimers and trimers of aggregated vesicles. At later times during the fusion reaction, with both SUV and LUV, the aqueous contents are completely released, particularly in the presence of high Ca^{2+} concentrations. Presumably, this is the result of the collapse of the internal aqueous space of the vesicles, eventually leading to the formation of cochleate lipid cylinders [3]. Vesicles composed of PS mixed with phosphatidylcholine (PC) or phosphatidylethanolamine (PE) release their contents to varying degrees in the course of fusion, but again the initial fusion events are not leaky [4]. In this report, we examine further the kinetics of the leakage associated with membrane fusion in a number of phospholipid vesicle systems.

In the studies mentioned above, fusion was followed with the Tb-dipicolinic acid (DPA) method for monitoring mixing of aqueous vesicle contents [1,2]. Leakage was followed in parallel experiments, employing the dequenching of carboxyfluorescein (CF) upon dilution [5–7]. The kinetics of CF release has also been employed by Nir et al. [8] to correct the fusion kinetics of PS vesicles, as determined with the Tb-DPA assay, for leakage of vesicle contents. It may be argued, however, that it is inappropriate to use compounds of different charge and size to study the relative kinetics of mixing and release of vesicle contents. Therefore, in the present paper, we compare the release of CF during vesicle fusion with that of Tb, one of the components involved in the fusion assay. It is shown that both compounds reveal similar kinetics of release in a variety of vesicle systems and that in several such systems, fusion is not accompanied by any measurable release of contents. Moreover, it is demonstrated that under conditions where the vesicles are heavily aggregated, their released aqueous contents are not readily trapped within intervesicular spaces in the vesicle aggregates, but diffuse rapidly into the bulk medium. To corroborate the evidence for membrane fusion, we also present data on the close correspondence between the rate of mixing of aqueous vesicle contents (measured by the Tb-DPA assay) and the rate of lipid mixing (measured by a resonance energy transfer assay, Ref. 9) during the fusion of PS SUV. A preliminary account of our observations has been presented elsewhere [10].

Materials and Methods

Materials

Phosphatidylserine (PS) was prepared from bovine brain as described previously [11,12] or purchased from Avanti Polar Lipids (Birmingham, AL). Phosphatidic acid (PA), egg phosphatidylethanolamine (PE), egg phosphatidylcholine (PC), *N*-(7-nitro-2,1,3-benzoaxadiazol-4-yl)PE (*N*-NBD-PE) and *N*-(lissamine)Rhodamine-B-sulfonylPE (*N*-Rh-PE) were obtained from Avanti. Dioleoylphosphatidylcholine (DOPC) and nitrilotriacetate (Na salt) were purchased from Sigma (St. Louis, MO). Cholesterol was also obtained from Sigma and recrystallized twice. Cardiolipin (CL) from *Bacillus subtilis* [13] was a generous gift of Drs. I. Vasilenko and A.J. Verkleij (University of Utrecht, The Netherlands). CF (Eastman Kodak, Rochester, NY) was purified by chromatography on Sephadex LH-20 (Pharmacia, Piscataway, NJ). Synexin was prepared as described by Hong et al. [14].The source and purity of other chemicals used in this study were as reported previously [2].

Preparation of vesicles

Large unilamellar vesicles (LUV) were prepared by reverse-phase evaporation [15] with some modifications [2,16] and sized to an average diameter of 0.1 μm by extrusion [17] through Unipore polycarbonate filters (Bio-Rad). Vesicles were made in the following aqueous media: (i) 2.5 mM $TbCl_3$, 50 mM sodium citrate and buffer (2.0 mM each of L-histidine and Tes at a final pH of 7.4. In some experiments, 2.5 mM $TbCl_3$/50 mM sodium nitrilotriacetate/5 mM Tes was used with essentially identical results; (ii) 50 mM DPA (sodium salt), 20 mM NaCl and buffer (pH 7.4); (iii) 50 mM CF

(sodium salt) and buffer (pH 7.4).

Small unilamellar vesicles (SUV) were prepared by sonication and ultracentrifugation as described previously [2,18] and contained one of the following: (i) 15 mM $TbCl_3$, 150 mM sodium citrate and buffer (pH 7.4); (ii) 150 mM DPA (sodium salt) and buffer (pH 7.4); (iii) 100 mM CF (sodium salt) and buffer (pH 7.4).

Nonencapsulated material was eliminated by gel filtration on Sephadex G-75 (Pharmacia) as described in detail by Wilschut et al. [2].

Tb-DPA fusion assay

Measurements were carried out with a 1 : 1 mixture of Tb- and DPA-vesicles in 100 mM NaCl, 0.1 mM EDTA and buffer (pH 7.4) at a final lipid concentration of 50 μM, as determined by phosphate analysis [19], unless indicated otherwise. The temperature was 25°C, except for the experiment shown in Fig. 6. Fluorescence was measured in an SLM 4000 or a Perkin-Elmer MPF 43 fluorometer. Excitation of the Tb-DPA complex was at 276 nm and emission at 545 nm with a Corning 3-68 cut-off filter (permitting light at wavelengths greater than 530 nm) between sample and monochromator to eliminate interference from light scattering. 100% Tb fluorescence was determined in the absence of EDTA after lysing 25 μM Tb-vesicles with 0.5% (w/v) sodium cholate in the presence of excess free DPA (20 μM) and, if necessary, sonicating for 5 min.

Carboxyfluorescein and Tb release

Release of CF was determined by following the relief of self-quenching of the dye upon dilution. Excitation was at 430 nm with LUV and at 493 nm with SUV. Emission was measured through a Corning 3-68 cut-off filter. 100% CF fluorescence was determined after lysing the vesicles with 0.1% (v/v) Triton X-100. The lipid concentration in the release experiments was the same as in the fusion experiments (50 μM) and the medium was 100 mM NaCl, 0.1 mM EDTA and buffer (pH 7.4).

Tb release was determined with Tb-vesicles (50 μM lipid) in 100 mM NaCl and buffer (pH 7.4), in the presence of 20 μM DPA and in the absence of EDTA. 100% fluorescence was determined by lysing the vesicles with cholate (0.5%, w/v) in the presence of Ca^{2+} at the concentration to be used in the experiment.

Resonance energy transfer assay for lipid mixing

The resonance energy transfer assay has been described in detail by Hoekstra [9]. The fluorescence donor (*N*-NBD-PE) and acceptor (*N*-Rh-PE) were in separate SUV (PS) populations (PS/fluorescent lipid, 98 : 2). Ca^{2+}-induced fusion of vesicles results in intermixing of the lipids which brings the donor and acceptor in close proximity, causing fluorescence quenching of *N*-NBD-PE by *N*-Rh-PE. NBD fluorescence was followed as a measure of the extent of fusion. Measurements were carried out in a Perkin Elmer MPF43 fluorimeter at an excitation wavelength of 475 nm and an emission wavelength of 530 nm with narrow slits and crossed polarizers to minimize effects of light scattering. The two types of vesicle were present at a ratio of 1 : 1, and at a total lipid concentration of 50 μM in 100 mM NaCl, 0.1 mM EDTA and buffer (pH 7.4) at 25°C.

Results

CF and Tb release during PS vesicle fusion

Fig. 1 shows the time-course of fusion and release of aqueous contents of LUV (PS) in the presence of Ca^{2+}. Fusion was monitored with the Tb-DPA assay, registering the coalescence of the internal vesicle volumes. In parallel experiments, release was followed employing the dequenching of CF upon dilution. In the presence of 2 mM Ca^{2+}, no appreciable release was detectable within 2 min after initiation of the reaction. At that time, a significant degree of mixing of contents had occurred (Fig. 1). In another set of parallel experiments, we followed the release of Tb, one of the components involved in the fusion assay, in the presence of DPA in the external medium. A very close correspondence between the kinetics of CF release and the kinetics of Tb release was observed (Fig. 1).

With increasing Ca^{2+} concentrations both the rate of fusion and the rate of release increased. When fusion was induced by addition of Ca^{2+} to a concentration of 3 mM (Fig. 1) or 5 mM (not shown), leakage became apparent only after 60 s and 15 s, respectively. During these time intervals,

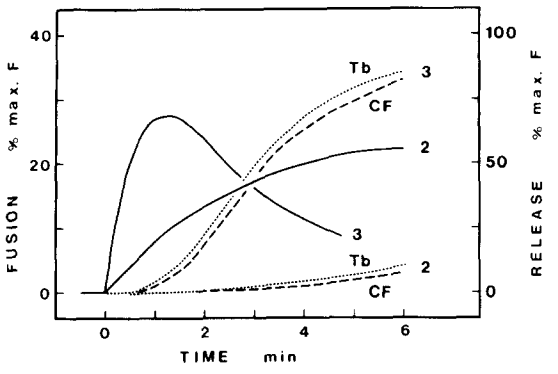

Fig. 1. Fusion of PS LUV and release of CF and of Tb into the medium. $CaCl_2$ was added at $t = 0$ to the indicated final concentrations (mM). Solid lines, mixing of vesicle contents; dashed lines, release of CF; dotted lines, release of Tb.

the fusion signal had attained a value of approx. 25% of the maximal fluorescence, which would correspond to the fusion of about 50% of the vesicles, since the coalescence of the contents of Tb (or DPA) vesicles with one another does not result in fluorescence. These results demonstrate that the initial events of Ca^{2+}-induced fusion of LUV (PS) are essentially nonleaky, and they corroborate previous observations [2].

Ca^{2+}-induced fusion of SUV (PS) is a more leaky process than that of LUV (PS). Wilschut et al. [2], using CF as a measure of release, demonstrated that initially, each fusion event between SUV is accompanied by some 10% release of aqueous contents. We have now compared the release of CF with that of Tb. Fig. 2 shows that leakage

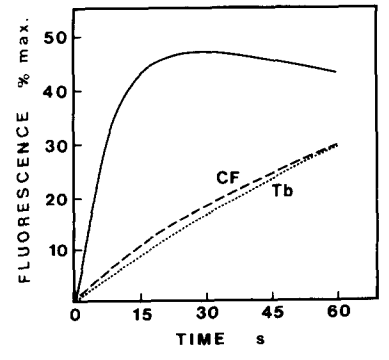

Fig. 2. Fusion of PS SUV and release of CF and of Tb into the medium. $CaCl_2$ was added at $t = 0$ to a final concentration of 1.5 mM. Solid line, mixing of vesicle contents; dashed line, release of CF; dotted line, release of Tb.

from SUV started immediately after addition of Ca^{2+}, although at a much lower rate than the rate of mixing of vesicle contents. As with the LUV (PS), the two release assays gave similar results (Fig. 2).

Release of vesicle contents within vesicle aggregates

The two release assays mentioned above monitor leakage of vesicle contents to the bulk medium. It is possible that within large aggregates of vesicles, the aqueous contents leak rapidly into inter-vesicular spaces and subsequently diffuse into the bulk medium by a slower process. In such a case, the fluorescence monitored by the fusion assay could be an overestimate of the degree of coalescence of encapsulated aqueous volumes due to a contribution of local release to the signal. To investigate this possibility, we considered a condition in which vesicles aggregate, but fuse only to a limited extent, while vesicle contents are released in a delayed process. This condition is achieved with LUV (PS) in the presence of a mixture of Ca^{2+} and Mg^{2+}. Mg^{2+} induces aggregation of LUV (PS), but no fusion or release of contents, and it inhibits the Ca^{2+}-induced fusion (but not the aggregation) of the vesicles [20]. In Fig. 3, it is shown that with 10 mM Mg^{2+} and 4 mM Ca^{2+}, very little Tb fluorescence was generated in the LUV (PS) system, although the vesicles aggregated immediately after the addition of the cations. Under these

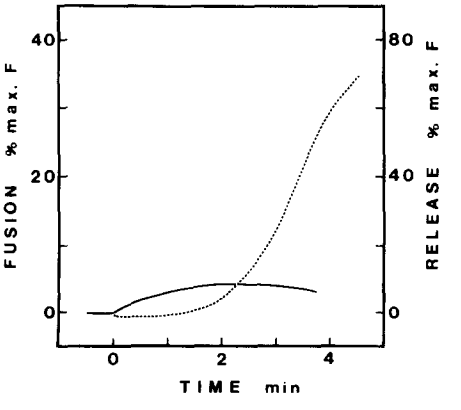

Fig. 3. Mixing and release of vesicle contents during aggregation of LUV (PS) in the presence of Ca^{2+} and Mg^{2+}. A mixture of $CaCl_2$ and $MgCl_2$ was added at $t = 0$ to final concentrations of 4.0 mM Ca^{2+} and 10 mM Mg^{2+}. Solid line, mixing of vesicle contents; dotted line, release of CF.

conditions, substantial leakage of vesicle contents was observed after a lag phase of about 2 min. This release process, however, does not represent slow diffusion into the bulk medium of vesicle contents initially released rapidly into intervesicular spaces, since (i) the leakage process is delayed, but, once started, it does not proceed at a slow rate, and (ii) in the parallel fusion experiment initial rapid release into intervesicular spaces would have resulted in a pronounced Tb fluorescence signal, which was not observed. It appears that released vesicle contents diffuse rapidly from the site of leakage into the bulk medium where they are diluted and are thus prevented from forming the Tb-DPA complex due to the presence of EDTA and Ca^{2+}.

Mixing of aqueous contents vs. lipid mixing

Since vesicle fusion not only involves the mixing of aqueous vesicle contents but, in addition, the mixing of bilayer lipids, it should be possible to compare the kinetics of both processes. If the Tb-DPA assay properly reflects the kinetics of fusion the rate of Tb fluorescence development should correspond to the rate of lipid mixing. We determined the rates of both processes in the SUV (PS) system at various Ca^{2+} concentrations, lipid mixing being monitored with the resonance energy transfer assay as described by Hoekstra [9]. In Fig. 4, it is shown that there is indeed a fair agreement between the rate of mixing of aqueous volumes and that of lipid mixing. The rate of fusion as measured with the resonance energy transfer assay was slightly faster than the rate observed with the Tb-DPA assay.

Nonleaky fusion systems

It is evident from the above results (Fig. 3) that aqueous contents released from vesicles during aggregation and fusion are not readily trapped within inter-vesicular spaces but escape rapidly to the bulk medium. In this section, we present data on a number of phospholipid vesicle systems showing very little, if any, release of aqueous contents during fusion. Consequently, sustained levels of Tb fluorescence are observed during the fusion assay.

As mentioned above, the Ca^{2+}-induced fusion of SUV (PS) in a 100 mM NaCl medium (pH 7.4), is a relatively leaky process, leading eventually to the complete release of contents. However, this does not occur when SUV (PS) are induced to fuse in the presence of low Ca^{2+} concentrations in media of low ionic strength. Fig. 5 shows the time-course of fusion of SUV (PS) induced by 1.0 mM Ca^{2+} in 5 mM NaCl (solid line) and the

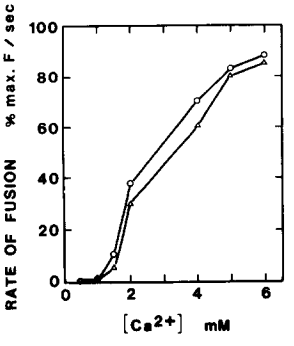

Fig. 4. The initial rate of fusion of PS SUV monitored by the Tb-DPA assay and the lipid mixing assay. Fusion was induced by addition of various concentrations of Ca^{2+} to a 1 : 1 mixture of PS/*N*-NBD-PE and PS/*N*-Rh-PE vesicles to monitor the fusion kinetics using the resonance energy transfer assay or to a 1 : 1 mixture of Tb- and DPA-vesicles composed of pure PS to monitor the fusion kinetics using the Tb-DPA assay. The final lipid concentration was 50 μM. Fluorescence was recorded at high chart speeds and the initial rates were calculated from the tangents to the fluorescence curves at $t = 0$. Circles, resonance energy transfer assay; triangles, Tb-DPA assay.

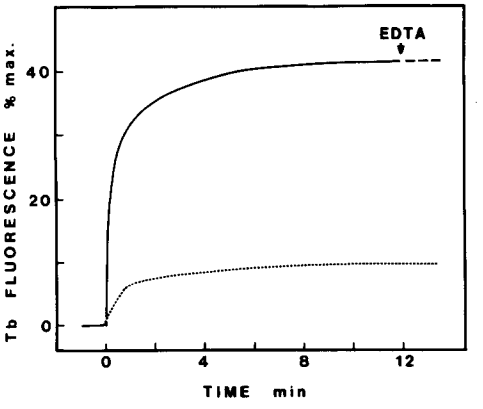

Fig. 5. Fusion and release of contents of PS SUV in a medium containing 5.0 mM NaCl. $CaCl_2$ was added at $t = 0$ to a final concentration of 1.0 mM. The medium also contained buffer (pH 7.4) and 0.1 mM EDTA. Solid line, mixing of vesicle contents; dotted line, release of CF. Arrow, addition of EDTA to a final concentration of 2.0 mM; dashed line represents the fluorescence intensity corrected for the dilution resulting from the addition of EDTA.

leakage of CF during the fusion process (dashed line). As described elsewhere [18], the threshold Ca^{2+} concentration required for fusion of SUV made of PS (or PS/PC mixtures) is reduced when the NaCl concentration in the medium is reduced, because of the higher binding of Ca^{2+} to PS under these conditions. In the fusion assay, the Tb fluorescence reached a level of slightly over 40% (Fig. 5), which could be maintained for 1 h (not shown). Addition of excess EDTA resulted in the fixation of the fluorescence level (Fig. 5), indicating a complete sequestration of the Tb-DPA complex from the external medium. The release of CF, monitored in a parallel experiment, was considerably slower than fusion and stopped at about 10% as the fusion reached a plateau.

Ca^{2+}-induced fusion of vesicles composed of an equimolar mixture of beef-heart CL and egg PC has been demonstrated by freeze-fracture electron microscopy [21–23]. Wilschut et al. [24] have monitored the kinetics of this process utilizing the Tb-DPA fusion assay. At a Ca^{2+} concentration of 10 mM, the vesicles fuse and maintain a high level of Tb fluorescence for a few minutes. However, release of vesicle contents does occur and eventually the vesicles collapse. This collapse occurs more rapidly at Ca^{2+} concentrations higher than 10 mM. Here we show the kinetics of the fusion of vesicles made of an equimolar mixture of CL from *B. subtilis* and dioleoylphosphatidylcholine (DOPC). This CL species is highly saturated, whereas the bovine-heart CL, containing about 90% linoleic acid, is unsaturated [13]. At room temperature, the vesicles containing the bacterial CL showed essentially the same fusion and leakage behavior as vesicles containing the bovine-heart CL (data not shown). However, at 10°C, the rate of release dropped to negligible values (Fig. 6). As a consequence, in the parallel fusion experiment, a high level of Tb fluorescence was reached (Fig. 6) and was maintained for at least 1 h (not shown). Addition of excess EDTA when the maximal Tb fluorescence was reached resulted in the fixation of the fluorescence intensity, demonstrating the complete sequestration of the Tb-DPA complex from the external medium (Fig. 6).

The cytoplasmic water-soluble protein, synexin, enhances the initial rate of Ca^{2+}-induced fusion in a number of phospholipid vesicle systems and

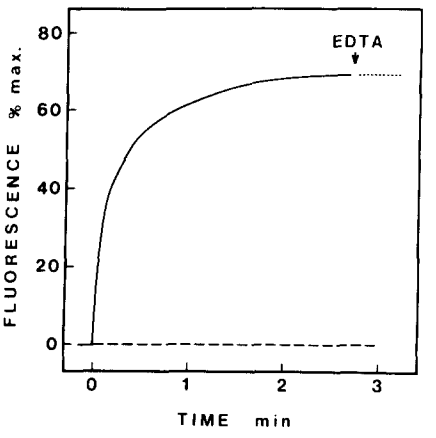

Fig. 6. Fusion and release of contents of CL/DOPC LUV at 10°C. Vesicles, composed of an equimolar mixture (phosphorus ratio, 2:1) of CL from *B. subtilis* and DOPC, were present at a concentration of 100 μM (lipid phosphorus). $CaCl_2$ was added at $t = 0$ to a final concentration of 10 mM. Solid line, mixing of vesicle contents; dashed line, release of CF. Arrow, addition of EDTA to a final concentration of 20 mM; the dotted line represents the fluorescence intensity corrected for the dilution resulting from the addition of EDTA.

reduces the threshold concentration necessary to initiate fusion [14,25–27]. In several of these vesicle systems, the enhancement of fusion is accompanied by a corresponding increase in the leakage of contents, although the initial fusion events are nonleaky. However, essentially nonleaky fusion

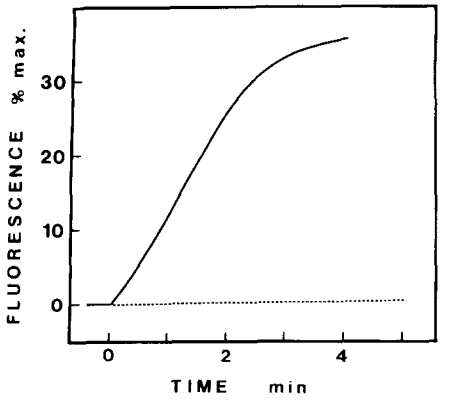

Fig. 7. Fusion of PA/PS/PE/cholesterol (1:2:3:2) LUV in the presence of synexin. Synexin and $MgCl_2$ were added to the vesicles to final concentrations of 6 μg/ml and 1.5 mM, respectively. At $t = 0$ fusion was initiated by addition of $CaCl_2$ to a final concentration of 1.5 mM. Solid line, mixing of vesicle contents, dotted line, release of Tb.

was seen with vesicles composed of PA/PS/PE/cholesterol (1 : 2 : 3 : 2), as shown in Fig. 7.

Discussion

Leakage of different encapsulated compounds

A significant result of the present study is the close correspondence between the kinetics of leakage of Tb and of CF from LUV (PS) and SUV (PS) (Figs. 1 and 2). The kinetics of the leakage process are apparently not determined to a major extent by the charge and size of the molecules involved, CF and the chelation complexes of Tb being different in both respects. Therefore, the use of CF seems to be an appropriate, convenient and representative way of monitoring the release of vesicle contents during fusion. It should be noted that measurements of Tb leakage may also involve the entry of DPA into the vesicle interior.

As indicated by the data in Fig. 3, the observed kinetics of release represent leakage from the vesicles proper, since there is no evidence for a slow diffusion from vesicle aggregates following rapid leakage into intervesicular spaces. This observation indicates that aqueous internal contents, once released from the vesicles, escape rapidly to the bulk medium even when the vesicles are aggregated. The encapsulated molecules, once outside the vesicles, would be expected to diffuse away at a rate much greater than the timescale of our measurements because of their high diffusion coefficients. The absence of any increase in Tb fluorescence during the release of contents in this experiment (Fig. 3) also indicates that the formation of the Tb-DPA complex in fusion experiments is not the result of the release of contents to locally high concentrations within vesicle aggregates. Furthermore, in many other vesicle systems membrane fusion proceeeds initially without the formation of large aggregates.

The possibility that the fluorescence signal in the fusion assay may be an overestimate of the extent of coalescence of internal vesicle volumes due to local release within vesicle aggregates has also been investigated in an earlier study [2] by terminating the Ca^{2+}-induced aggregation and fusion of PS vesicles with excess EDTA. Addition of EDTA during the initial stages of the fusion process results in a complete fixation of the Tb fluorescence intensity. Accordingly, when leakage of CF is monitored, no additional enhancement of fluorescence is observed following addition of excess EDTA. Moreover, the initial rate of LUV (PS) fusion is the same with 0.1 mM or 10 mM EDTA in the external medium (in either case in the presence of an excess of 10 mM Ca^{2+}). This observation also argues against a contribution of local release to the fluorescence signal, since under the conditions of the experiment 10 mM EDTA would have been sufficient to largely quench the Tb-DPA complex formation, even if occurring in intervesicular spaces within vesicle aggregates [2].

Finally, the kinetic analysis of the fusion of SUV (PS) in 100 mM Na^+ and 1.25 to 2.0 mM Ca^{2+} has shown that this fusion process is essentially rate-limited by aggregation; the initial fusion events thus involve only dimer and trimer aggregates [8,28,29]. This configuration is not likely to create an intervesicular space separated from the bulk medium.

Recently, Kendall and MacDonald [30] have used the reaction of EDTA with a Co^{2+}-calcein complex as a fusion assay and have claimed that the contents of SUV (PS) leak more quickly than they can intermix with each other in the presence of Ca^{2+}. The fluorescence reaction used for this assay is extremely slow compared to the Tb-DPA reaction. The reactants would thus be expected to leak into the medium before being able to generate fluorescence within fusing vesicles. Other aspects of this assay are under investigation in our laboratory.

Mixing of aqueous contents and of membrane components

The results in Fig. 4 show that there is a close correspondence between the kinetics of lipid mixing and of the mixing of aqueous vesicle contents during Ca^{2+}-induced fusion of SUV (PS), corroborating earlier results [31]. This correspondence would not have been observed if the data obtained with the Tb-DPA assay represented an overestimate of the extent of fusion due to a contribution of release within vesicle aggregates. The slightly slower rate of fusion observed with the Tb-DPA assay may be ascribed to the partial quenching of Tb fluorescence due to leakage of vesicle contents

and thus to an underestimation of the rate of fusion. A correction factor may be introduced to compensate for this initial leakage for the quantitative kinetic analysis of fusion [8,28,29]. The kinetics of the fusion of phosphatidylglycerol vesicles detected by the lipid mixing and Tb-DPA assays are also very similar [32,33].

Relative kinetics of fusion and leakage of contents in various phospholipid vesicles

Phospholipid vesicle systems vary considerably in the extent to which fusion is accompanied by leakage of internal contents. SUV (PS) release a small fraction of their contents even during the initial stages of the fusion process, whereas LUV (PS) undergo essentially nonleaky fusion. With either type of vesicle, massive release of internal contents represents a secondary process after considerable fusion has occurred. We propose that this secondary release process is the result of collapse of the fused vesicles, which would have a higher surface to internal volume ratio than the unfused vesicles. We speculate that the interior monolayers at opposite ends of the vesicle may interact with one another, squeezing the aqueous contents out, as a result of Ca^{2+} entry into the internal aqueous space of the fused vesicle during the actual reorganization of the fusing bilayer.

The leakiness of the initial stages of the SUV (PS) could be ascribed to the possibility that during fusion the relief of the strain and of the quantitative asymmetry of the highly curved bilayer of SUV leads to a major reorganization of lipids. This may result in a transient increase in the permeability of the bilayer. It is also possible that the area of initial contact between aggregated SUV is not large enough to accommodate a nonleaky fusion process, whereas in the LUV the contact area is likely to be larger and may constitute a more efficient seal around the site of destabilization.

At low Ca^{2+} concentrations and under conditions of low ionic strength, the fusion process of SUV (PS) ceases after a few rounds (Fig. 5) and as a result the vesicles do not collapse. The level of Tb fluorescence reached in the fusion assay (42%) and that of CF release in the parallel leakage experiment (10%) suggest that on the average, only one round of fusion has occurred. Theoretically, in a completely nonleaky system, one round of fusion would result in 50% Tb fluorescence, since only half of the fusion events in a 1 : 1 mixture of Tb- and DPA-vesicles are productive in terms of Tb-DPA complex formation. The low level of leakage (10%) observed after the fusion has gone to completion shows that only a limited fraction of the internal volume is released during fusion of SUV (PS). The retention of contents following an initial round of fusion may be related to the increase in vesicle size, which in turn renders the vesicles resistant to further fusion, since LUV have a higher threshold for divalent cations than do SUV [2] and in some cases are completely resistant to fusion [4,20]. It is known that the repulsive free energy of interaction between vesicles increases with the radius of the vesicles [29]. The product of the fusion of two SUV will thus be resistant to fusion with other such vesicles under the particular ionic conditions.

Completely nonleaky fusion was observed at 10°C with vesicles made of an equimolar mixture of CL from *B. subtilis* and DOPC (Fig. 6). The fusion process continues until the level of Tb fluorescence reaches 70%, suggesting that about two rounds of fusion occur on the average. In a 1 : 1 mixture of Tb- and DPA-vesicles, two rounds of fusion would result in 75% Tb fluorescence. In contrast, at room temperature, the vesicles continue to fuse, in the course of which they release part of their contents and eventually collapse. Freeze-fracture electron microscopic observations confirmed the difference between the final fusion product at 10°C and room temperature. At the lower temperature, the vesicles increased in size but remained as intact, aggregated vesicular structures, whereas at room temperature the final product consisted of extended collapsed sheets (Wilschut, J. and Verkleij, A.J., unpublished data).

The fusion of PA/PS/PE/cholesterol vesicles in the presence of Ca^{2+} and synexin is another example of tightly sealed membrane fusion. In this system, the well-known effect of cholesterol in reducing the permeability of phospholipid bilayers [34,35] is probably also functional during the momentary disruption of bilayer integrity in the fusion reaction. Retention of contents during fusion has been observed in other systems. For example, SUV made of PA/PC (1 : 2) retain most of their

contents while undergoing fusion [36]. The leakage associated with the fusion of PS/PC vesicles is also slower than the fusion reaction [18,37]. Finally, no CF or Tb leakage has been detected in the Ca^{2+}-induced fusion of LUV made of phosphatidylglycerol [33].

The absence of leakage during fusion may be explained by a variety of factors, such as: (i) the resistance of these membranes to formation of tightly packed intermembrane complexes, preventing the collapse of the internal aqueous space; (ii) the resistance to phase separation of the acidic lipid from the vesicle-stabilizing zwitterionic lipid in the presence of Ca^{2+}; and (iii) the maintenance of a low permeability to Ca^{2+}, preventing the entry of Ca^{2+} into the internal aqueous compartment. Further studies are needed in order to understand the relative importance of each of these possibilities.

Physiological relevance

The possible relationship between phospholipid vesicle fusion and membrane fusion in biological systems has been discussed previously [25,27,38, 39]. We should emphasize here that the relevance of the information gathered on the fusion behavior of phospholipid vesicles to that of biological membranes arises particularly from observations of early events upon addition of divalent cations, that is, when the vesicles are primarily in the form of dimers. In the majority of vesicle systems studied so far, the initial events of fusion are essentially nonleaky. Later events, such as collapse of the fused vesicles and accompanying leakage of contents, are interesting from the viewpoint of the properties of phospholipid vesicles, but are not particularly relevant for the characteristics of biological membrane fusion. Fusion in biological systems usually represents a single event, taking place between two distinct membranes, such as in sperm-egg fusion, the cortical reaction, neurotransmitter release and myoblast fusion. In secretory phenomena where Ca^{2+} plays an important role in stimulus-secretion coupling [40,41], the entry of Ca^{2+} into the cell or its mobilization from intracellular pools is a transient event, followed by its immediate sequestration or dilution after the stimulus is removed. An analogous situation is obtained in the phospholipid vesicle system when Ca^{2+} is chelated by EDTA at early stages of fusion, when essentially no leakage has occurred.

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