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KINETICS OF INTERMIXING OF LIPIDS AND MIXING OF AQUEOUS CONTENTS DURING VESICLE FUSION

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The kinetics of Ca^{2+}-induced fusion of small unilamellar phosphatidylserine (PS) vesicles was investigated by continuous monitoring of the mixing of vesicle lipids and, in parallel, the mixing of aqueous contents. The release of vesicle contents during fusion to the external medium was also determined by monitoring the release of carboxyfluorescein and the release of the aqueous compounds which were employed to report the occurrence of fusion upon mixing of the intravesicular volumes. The results demonstrate that the initial rates of lipid mixing and the mixing of aqueous contents are very similar, and appear to be much faster than the initial rate of vesicle leakage. This suggests that the majority of the initial events during PS vesicle fusion represents the fusion of vesicles prior to leakage of vesicle contents. It is concluded that, under conditions where leakage of vesicle contents during fusion is limited, both fusion assays accurately reflect the occurrence of this process, thus indicating the usefulness of these assays as reliable, complementary tools in the study of fusion of (model) membranes.

Artificial lipid vesicles composed of acidic phospholipids, particularly phosphatidylserine (PS), are extensively used as a model system to explain the molecular mechanism(s) underlying membrane fusion [1,2]. To demonstrate the occurrence of genuine fusion between two lipid vesicles, two rigorous criteria should be met: (i) merging of the two lipid bilayers (and excluding mechanisms other than fusion) and (ii) concomitant coalescing of the aqueous intravesicular compartments. However, no previous study has compared the kinetics of intermixing of lipids with the kinetics of mixing of aqueous contents.

In this study the kinetics of both processes were compared in the case of Ca^{2+}-induced fusion of PS vesicles. The results show, in contrast to previous suggestions [3,4], that the initial rates for lipid mixing and mixing of aqueous contents are similar, suggesting that, after Ca^{2+} is added to pure PS vesicles, the initial rate of fusion exceeds that of vesicle rupture, and hence, reflects the fusion of (virtually) intact vesicles prior to leakage of vesicle contents.

Recently, we [5,6] described an assay for vesicle fusion, based on lipid intermixing, in which the energy transfer efficiency between two non-exchangeable [5–7] fluorescent lipid analogues, *N*-NBD-PE (donor) and *N*-Rh-PE (acceptor) is continuously monitored.

Assays based on the same principle have also been described by others [8,9]. However, fusion

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Abbreviations: PS, phosphatidylserine; Hepes, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid; *N*-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine)Rhodamine B sulfonyl phosphatidylethanolamine; Tb, terbium.

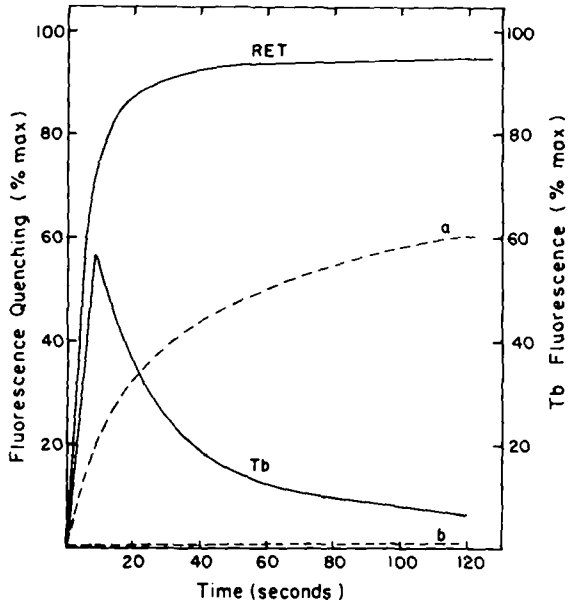

Fig. 1. Ca^{2+}-induced fusion of PS vesicles and release of vesicle contents. RET, kinetics of fusion between an equimolar mixture of *PS/N-NBD-PE (98:2)* and *PS/N-Rh-PE (98:2)* vesicles, induced by addition of 4 mM Ca^{2+}, as measured by the resonance energy transfer fusion assay [5,6]. Small unilamellar vesicles were generated in 0.1 M NaCl/0.01 M Hepes, pH 7.4, by ultrasonication under an argon atmosphere at 10°C [7]. Continuous monitoring of NBD-fluorescence ($\lambda_{ex} = 475$ nm, $\lambda_{em} = 530$ nm) was carried out with an American Instrument Co. spectrophotofluorimeter, using a Corning 3-68 cutoff filter, narrow band pass slits and crossed polarizers to minimize light scattering. Incubations were carried out at 24°C in 0.1 M NaCl/0.01 M Hepes/0.1 mM EDTA, pH 7.4, and the final lipid concentration was 125 μM. Tb, kinetics of fusion between an equimolar mixture of Tb^{3+}- and dipicolinic acid-containing vesicles. Vesicles were prepared by ultrasonication in 23 mM $TbCl_3$/150 mM citrate/10 mM Hepes, pH 7.4, and 150 mM dipicolinic acid/10 mM Hepes, pH 7.4, respectively. Non-entrapped material was removed by Sephadex G-75 filtration in 0.1 M NaCl/0.01 M Hepes/1.0 mM EDTA [10]. To remove external EDTA, the vesicle preparations were subsequently rechromatographed on Sephadex G-75 in 0.1 M NaCl/0.01 M Hepes, pH 7.4. Fusion was induced and monitored continuously as described above, except that the excitation of Tb fluorescence was at 276 nm and emission was detected at 545 nm. Tb^{3+} leakage during Ca^{2+}-induced fusion (dashed line, in the absence (a) and presence (b) of 0.1 mM EDTA) was determined as follows: 125 nmol Tb^{3+}-containing PS vesicles (separated from EDTA by filtration on Sephadex G-75) were mixed with 125 nmol unloaded PS vesicles in 2 ml 0.1 M NaCl/0.01 M Hepes, pH 7.4. Then excess dipicolinic acid (45 μM) was added, followed by the addition of 4 mM Ca^{2+}, and the fluorescence was monitored as above. Since Ca^{2+} interferes with the formation of the fluorescent complex of Tb^{3+} and dipicolinic acid [10], appropriate corrections were made to determine the total Tb-fluorescence by mixing free Tb^{3+} and dipicolinic acid and reading the fluorescence before and after addition of Ca^{2+}. Fluorescence quenching increased with increasing Ca^{2+} concentration and reached a value of approx. 55% in the presence of 4 mM Ca^{2+}. To simulate the leakage process and to determine the fluorescence quenching by Ca^{2+} under these conditions, increasing concentrations of free Tb^{3+} (up to the concentration of Tb^{3+} that would be present upon total release of Tb^{3+} from the vesicles) were mixed with a fixed excess of dipicolinic acid (45 μM). Fluorescence was read before and after addition of Ca^{2+}. Since no significant changes in fluorescence quenching occurred over the concentration range tested, concentration-dependent corrections were not made. The value of 100% Tb^{3+} fluorescence was determined in the presence of excess dipicolinic acid by lysis of the Tb^{3+}-containing vesicles (in the absence of EDTA) with 0.5% (w/v) cholate [10].

assays based on lipid mixing do not provide insight into the integrity of the vesicles during fusion and, in principle, the possibility exists that only fusion of membrane fragments is monitored [3,4]. To examine this possibility, the kinetics of vesicle fusion based on membrane lipid mixing were compared to those obtained for the coalescence of intravesicular compartments. The latter process was followed with the Tb/dipicolinic acid (DPA) assay [10,11].

The interpretation of fusion assays based solely on the coalescence of aqueous contents may be complicated by the possibility that the vesicle contents may react outside the vesicles due to vesicle leakage [12,13]. Therefore, proper control experiments [14] are required to exclude the possibilities that fusion assays based on the mixing of aqueous contents monitor a reaction of the contents (i) outside the vesicles after vesicle leakage or (ii) inside the vesicles, but facilitated by non-fusion related mechanisms (for example, by inward diffusion of initially entrapped compounds across the bilayer after vesicle leakage). In this report, these possibilities were examined in the case of the Tb^{3+}-fusion assay. The release of Tb^{3+} and dipicolinic acid upon Ca^{2+}-induced fusion was directly assayed by addition of non-entrapped Tb^{3+} or dipicolinic acid to vesicles containing entrapped dipicolinic acid and Tb^{3+}, respectively. Since carboxyfluorescein has been frequently used to determine the kinetics of vesicle leakage during fusion [10,15], it was also of interest to test whether carboxyfluorescein release accurately reflected the release of Tb^{3+} and dipicolinic acid.

Fig. 1 shows the kinetics of mixing of lipid molecules (RET), the kinetics of mixing of vesicle contents (Tb) and the release of Tb^{3+} (a) during Ca^{2+}-induced fusion of PS vesicles. Fusion was induced by the addition of 4 mM Ca^{2+}. As shown in Fig. 1, the rate of lipid mixing (determined by the resonance energy transfer assay) increased rapidly and reached its maximum after approx. 50 s. The initial kinetics of mixing of aqueous contents, as determined by the Tb^{3+} fusion assay, was slightly slower than that observed for lipid mixing and decreased after reaching its maximal level (after approx. 10 s). This decrease has been attributed to subsequent leakage and eventual dissociation of the $Tb(DPA)_3^{3-}$ complex by EDTA and Ca^{2+} in the medium [10]. In a separate experiment Tb^{3+} leakage during Ca^{2+}-induced fusion was determined by incubating the same concentration of Tb^{3+}-containing PS vesicles as used in the fusion assay in the presence of excess non-entrapped dipicolinic acid. The total lipid concentration was kept the same as in the fusion assay by adding unloaded PS vesicles. As shown in Fig. 1, leakage of Tb^{3+} (curve a) began immediately after addition of Ca^{2+}, but its initial kinetics appeared to be much slower than that of both fusion assays. A similar initial leakage rate was observed for carboxyfluorescein (see also below) although consistently higher leakage values were obtained (10–20%) at $t > 20$ s (not shown), which possibly resulted from a partial dequenching of the substantially decreased intravesicular carboxyfluorescein concentration after this time interval (due to leakage), in addition to its dilution into the medium.

Similar leakage experiments were performed to determine the leakage kinetics of dipicolinic acid by adding non-entrapped Tb^{3b} to dipicolinic acid containing vesicles. When free Tb^{3+} was added to dipicolinic acid-containing vesicles, only a slight 'background' fluorescence was detected (less than 2% of the total fluorescence level). However, upon addition of Ca^{2+} an almost identical kinetic pattern of $Tb(DPA)_3^{3-}$ fluorescence development was observed as for the fusion assay itself (not shown, cf. Fig. 1, curve Tb). This reaction was completely inhibited when 0.1 mM EDTA (final concentration) was added to the medium prior to the addition of Ca^{2+}. As indicated above, EDTA and Ca^{2+} prevent complex formation of $Tb(DPA)_3^{3-}$ by preferential binding to Tb^{3+} and dipicolinic acid, respectively (see also legend Fig. 1 and Ref. 10). This result excluded the possibility that free Tb^{3+} could interfere with the actual fusion assay by diffusing across the bilayer of the dipicolinic acid-containing vesicles during fusion after initial vesicle leakage. Furthermore, the possibility that complex formation inside the vesicles was prevented by substantial inward leakage of EDTA can also be excluded, since (i) the fusion assay is also performed in EDTA-containing medium (see legend of Fig. 1) and (ii) the initial kinetics of both fusion assays are very similar. Thus, these results further corroborate recent observations of Wilschut et al. [10]. In this context, it is relevant to note that from the data presented by the same authors (cf. Fig. 5 in Ref. 11) it can be inferred that a direct transfer of vesicle contents between aggregated vesicles can also be excluded.

The discrepancy in the initial leakage rates of Tb^{3+} and dipicolinic acid is not fully understood. However, a likely explanation for this observation could be that upon addition of the non-entrapped Tb^{3+}-citrate complex to the dipicolinic acid-containing vesicles, Tb^{3+} may be able to bind rapidly to the (negatively charged) PS bilayer (see Refs. 16 and 17), and, after addition of Ca^{2+}, allow a rapid reaction of Tb^{3+} with dipicolinic acid. However, in the presence of EDTA, Tb^{3+} is rapidly and effectively complexed which prevents the binding of the cation to the PS bilayer and, moreover, prevents the formation of the $Tb(DPA)_3^{3-}$ complex after vesicle leakage (see also below). Therefore, it is concluded that the appearance of fluorescence upon addition of free Tb^{3+} to dipicolinic acid-containing vesicles does not properly represent the leakage of dipicolinic acid from the vesicles.

As was also observed in the case of dipicolinic acid leakage, no fluorescent signal was observed when the Tb^{3+} leakage experiments were performed in medium containing 0.1 mM EDTA (curve b, Fig. 1). Leakage will occur under these conditions, but the complex formation between Tb^{3+} and dipicolinic acid was evidently prevented (Fig. 1, curve a vs. curve b). This experiment argues against the possibility that the observed increase in fluorescence during fusion resulted from the formation of $Tb(DPA)_3^{3-}$ complexes within or near

the vesicle aggregates but outside the vesicle bilayers, due to release of the vesicle contents to locally high concentrations of Tb^{3+}. Furthermore, addition of EDTA during the initial stages of the fusion between Tb- and dipicolinic acid-containing vesicles resulted in a complete fixation of the $Tb(DPA)_3^{3-}$ fluoresence intensity. Accordingly, when leakage of carboxyfluorescein was monitored, no additional enhancement of fluorescence was observed following addition of excess EDTA (not shown). Thus, these results suggest that (i) massive leakage of vesicle contents prior to fusion did not occur and/or (ii) any released Tb^{3+} (or dipicolinic acid) diffused rapidly from the site of leakage into the bulk solution, where EDTA (and Ca^{2+}) effectively prevented any $Tb(DPA)_3^{3-}$ complex formation outside the vesicles.

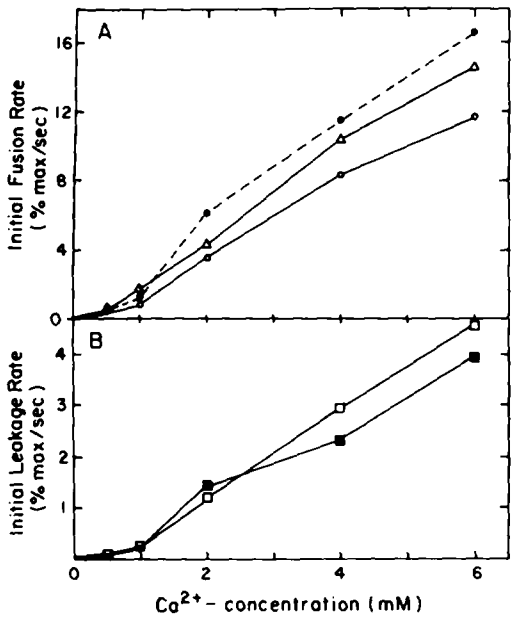

Fig. 2. Initial rate of PS vesicle fusion (A) and release of vesicle contents (B) as a function of the Ca^{2+} concentration. (A) Fusion was induced by addition of various concentrations of Ca^{2+} to an equimolar mixture of PS/*N*-NB-PE (98:2) and PS/*N*-Rh-PE (98:2) vesicles to monitor the fusion kinetics using the resonance energy transfer assay or to an equimolar mixture of Tb^{3+}- and dipicolinic acid-containing vesicles to monitor the fusion kinetics using the Tb^{3+} assay. Methods were as described in Fig. 1. The final lipid concentration was 125 μM. The initial rates of fusion were calculated from the tangents to the fluorescence curves (as plotted in Fig. 1) at $t = 0$ for the resonance energy transfer assay (△——△) and the Tb^{3+}-assay (○——○) and were plotted as a function of the Ca^{2+} concentration. Dashed line (●- - -●) shows the initial fusion rate as determined by the Tb^{3+}-assay, after correction for vesicle leakage (see text). (B). Tb^{3+} leakage was determined as described in Fig. 1. Vesicles containing 100 mM carboxyfluorescein were generated and the release of vesicle contents was monitored as described elsewhere [20]. The initial rates of Tb^{3+} leakage (■——■) and carboxyfluorescein leakage (□——□) were determined from the tangents to the fluorescence curves at $t = 0$, and were plotted as a function of the Ca^{2+} concentration. The concentration of Tb^{3+} vesicles (or carboxyfluorescein-containing vesicles) was the same as the concentration of Tb^{3+} vesicles present in the fusion assay. Total lipid concentration was adjusted to 125μM by addition of unloaded PS vesicles. All initial rates were recorded at fast chart speeds.

The initial rate of vesicle fusion was dependent on the Ca^{2+} concentration in the medium (Fig. 2A). As expected, the initial rate of leakage of vesicle contents also increased with increasing Ca^{2+} concentration (Fig. 2B), and similar initial rates were measured whether leakage was determined by carboxyfluorescein or Tb^{3+} release. Based on the ratio of the initial rate of fusion to that of the initial rate of release, it can be calculated (cf. Ref. 10) that during the initial fusion events the rate of release was approx. 25% of the rate of fusion as monitored by the Tb^{3+} fusion assay. The initial fusion rate, corrected for leakage, is also shown (Fig. 2A, dotted line), demonstrating that the initial fusion rates were very similar using both fusion assays.

From these results, it is concluded that the initial fusion event during PS-vesicle fusion is a genuine fusion event between essentially intact bilayers. It is unlikely that fusion of only membrane fragments (after lysis of the PS vesicles [3,4]) was monitored when using the resonance energy transfer assay, or that in the case of the Tb^{3+} assay, the occurrence of $Tb(DPA)_3^{3-}$ complex formation outside the vesicle bilayer, or after permeation of either dipicolinic acid or Tb^{3+} across the bilayer into Tb^{3+}- or dipicolinic acid-containing vesicles, respectively, was monitored, since no reaction was seen in the presence of EDTA. Instead, it appears that substantial vesicle fusion can occur prior to release of vesicle contents.

Because of (partial) release of vesicle contents during PS-vesicle fusion, the physiological relevance of this model has been questioned [3,4], since a number of fusion events in vivo are supposedly non-leaky. However, it should be noted that no evidence exists suggesting that pure PS

lipid phases are formed in membranes or are even involved in the induction of in vivo fusion events. In fact, this seems rather unlikely considering the concentration of PS in biological membranes [1] and its ability to separate into a pure PS phase when incorporated into mixed bilayers [7]. In this respect it is interesting to note that leakage is greatly reduced both in the fusion of mixed PS/PC vesicles [14,18] and in PA/PC vesicles (PA, phosphatidic acid) [19]. However, since PS and other acidic phospholipids can conceivably function as 'catalytic units' in the initiation of biological membrane fusion phenomena, the pure PS system may provide considerable information on the molecular events involved in membrane fusion, particularly the initial events. During these initial events, the PS-vesicle fusion system meets the requirements for genuine fusion. Moreover, the results indicate that the resonance energy transfer assay and the Tb^{3+}-assay may be very useful as complementary tools in the study of membrane fusion.

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