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COMPARISON OF TWO LIPOSOME FUSION ASSAYS MONITORING THE INTERMIXING OF AQUEOUS CONTENTS AND OF MEMBRANE COMPONENTS

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Divalent cation-induced fusion of large unilamellar vesicles (approx. 0.1 μm diameter) made of phosphatidylserine (PS) or phosphatidylglycerol (PG) has been studied. Intermixing of aqueous contents during fusion was followed by the Tb/dipicolinic acid fluorescence assay, and intermixing of membrane components by resonance energy transfer between fluorescent lipid probes. Both assays gave identical threshold concentrations for Ca^{2+}, which were 2 mM for PS and 15 mM for PG. The dependencies of the initial rate of fusion on the concentration of PG vesicles determined by either assay were identical, the order of this dependence being 1.2 in the concentration range of 5–200 μM lipid. For PS liposomes, this order was found to be 1.5 in the fluorescent lipid assay. No leakage of contents was detected during the fusion of PG vesicles. Mg^{2+} inhibited the Ca^{2+}-induced fusion of PS vesicles, but did not cause any fusion by itself, consistent with previous results with the Tb/dipicolinic acid assay.

Introduction

The fusion of phospholipid vesicles induced by divalent metal ions has been studied by a variety of assays. Membrane fusion involves the complete intermixing of the internal aqueous contents and membrane components of two fusing vesicles. The Tb/dipicolinic acid assay for the intermixing of vesicular aqueous contents has been used extensively to monitor the fusion behavior of numerous phospholipid vesicle systems [1–3]. Resonance energy transfer between two fluorescent lipid probes has also been utilized to follow the intermixing of membrane components [4,5]. In this communication, we present a detailed analysis of the kinetics of fusion of large unilamellar phosphatidylserine (PS) and phosphatidylglycerol (PG) vesicles as monitored by these two techniques. We have found that, in general, there is a close correspondence between the results obtained by both assays. However, some differences exist, since these assays monitor different molecular events occurring during fusion.

In the Tb/dipicolinic acid assay terbium citrate is encapsulated in one population of vesicles and sodium dipicolinate in another. Intermixing of the aqueous contents results in the formation of the highly fluorescent $Tb(dipicolinate)_3^{3-}$ complex. Contents which may leak into the external medium are prevented from interacting by the presence of Ca^{2+} and EDTA in the medium [1]. In the fluorescent lipid assay, N-NBD-PE and N-Rh-PE are

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Abbreviations: PS, phosphatidylserine; PG, phosphatidylglycerol; N-NBD-PE; *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; N-Rh-PE, *N*-(lissamine)Rhodamine B sulfonylphosphatidylethanolamine or *N*-(tetramethyl rhodamine)phosphatidylethanolamine; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

incorporated into the membrane of one population of vesicles, whereby the fluorescence of NBD is quenched by resonance energy transfer to Rhodamine. Fusion of the labelled vesicles with unlabelled vesicles results in the dilution of the fluorescent phospholipids in the membrane of the unlabelled vesicles, thus reducing the efficiency of energy transfer between the groups, which results in an increase in NBD fluorescence [4].

The initial events in the fusion of large (approx. 0.1 μm diameter), PS vesicles have been shown previously to be essentially non-leaky, although eventually the internal contents are released into the medium as a result of the collapse of the internal aqueous space of the fusion products [1]. With PG vesicles. however, we have discovered that the internal contents are completely retained during fusion. Our preliminary results have been reported previously [6].

Experimental procedures

Materials

Phosphatidylserine (PS, bovine brain), phosphatidylglycerol (PG, prepared by transphosphatidylation of egg phosphatidylcholine), and N-NBD-PE were purchased from Avanti Polar Lipids (Birmingham, AL). N-Rh-PE, prepared from lissamine Rhodamine B sulfonyl chloride, was a generous gift from Dr. Walter Shaw of Avanti Polar Lipids. N-Rh-PE was also prepared as follows. 100 μmol dimyristoylphosphatidylethanolamine was incubated overnight with 120 μmol tetramethylrhodamine isothiocyanate (Research Organics) in chloroform/methanol (2:1) in the presence of 500 μmol of triethylamine (Pierce) at room temperature. N-Rh-PE was then purified by silicic acid chromatography and preparative thin-layer chromatography. $TbCl_3$ was obtained from Alpha (Danvers, MA), dipicolinic acid, L-histidine, and Tes from Sigma (St. Louis, MO), carboxyfluorescein from Eastman-Kodak (Rochester, NY). Carboxyfluorescein was purified by chromatography on Sephadex LH-20 (Pharmacia, Piscataway, NJ) in water at pH 7.4.

Preparation of phospholipid vesicles

Large unilamellar vesicles containing Tb, dipicolinic acid or carboxyfluorescein were prepared by the reverse phase evaporation technique as described by Szoka and Papahadjopoulos [7] with some modification [1], and contained the following aqueous media: (i) 2.5 mM $TbCl_3$, 50 mM sodium citrate and buffer (2 mM each of L-histidine and Tes at a final pH of 7.4); (ii) 50 mM sodium dipicolinate, 20 mM NaCl and buffer; (iii) 50 mM carboxyfluorescein (Na^+ salt) and buffer. Vesicles were freed of unencapsulated material by gel filtration in Sephadex G-75.

Large unilamellar vesicles to be used in the fluorescent phospholipid assay were also prepared by the reverse-phase evaporation technique using a solution comprising 100 mM NaCl/2 mM L-histidine/2 mM Tes/0.1 mM EDTA (pH 7.4). Fluorescently labelled vesicles were prepared containing 98 mol% non-fluorescent phospholipids (PS or PG) with 1 mol% each of N-NBD-PE and N-Rh-PE.

All phospholipid vesicles were sized by extrusion under N_2 through polycarbonate membranes of 0.1 μm pore diameter in a Millipore filtration apparatus.

Fluorescence measurements

Fluorescence emission was monitored using an SLM-4000 spectrofluorimeter and readings recorded on an Houston Instruments chart recorder. A stirring apparatus located beneath the spectrofluorimeter cuvette holder enabled constant mixing of cuvette contents. All Ca^{2+} additions were made by a syringe while constantly monitoring fluorescence emission. Excitation band slits were kept at 8 mm for all measurements, while emission band slits were 4 mm. All measurements were made at 25°C.

100% fluorescence calibration for the Tb/dipicolinic acid assay was accomplished according to the method of Wilschut et al. [1] using 1% sodium cholate to lyse the vesicles in the presence of excess dipicolinic acid (20 μM, pH 7.4). Calibration of the fluorescent phospholipid assay was accomplished using the appropriate concentrations of labelled and unlabelled vesicles in a solution of Triton X-100 (1% final concentration). This treatment eliminated energy transfer and the resulting fluorescence emission at 530 nm was set to 100%.

Vesicle-vesicle fusion and release of contents

Fusion between vesicle populations for both assays was initiated by the addition of Ca^{2+} to the indicated final concentrations. Rates of fusion between vesicle mixtures were measured by following changes in fluorescence intensity at 491 nm (Tb/dipicolinic acid assay, $\lambda_{ex} = 276$ nm) or 530 nm (fluorescent phospholipid assay, $\lambda_{ex} = 450$ nm) as a function of time. Alternatively, for the Tb/dipicolinic acid assay, emission through a Corning 3-68 cut-off filter (above 530 nm) was monitored.

Leakage of vesicle contents was measured by two different techniques. Using carboxyfluorescein-containing vesicles, the fluorescence of carboxyfluorescein was monitored during fusion ($\lambda_{ex} = 430$ nm, $\lambda_{em} = 520$ nm) as it was released into the medium and was no longer self-quenched. Alternatively, vesicle leakage was monitored by following the increase in fluorescence at 491 nm ($\lambda_{ex} = 276$ nm) as Tb-vesicles were induced to fuse in the presence of 20 μM free dipicolinic acid. Calibration for both techniques was performed by lysing the carboxyfluorescein or Tb-vesicles with 0.1% Triton X-100 or 1% cholate, respectively.

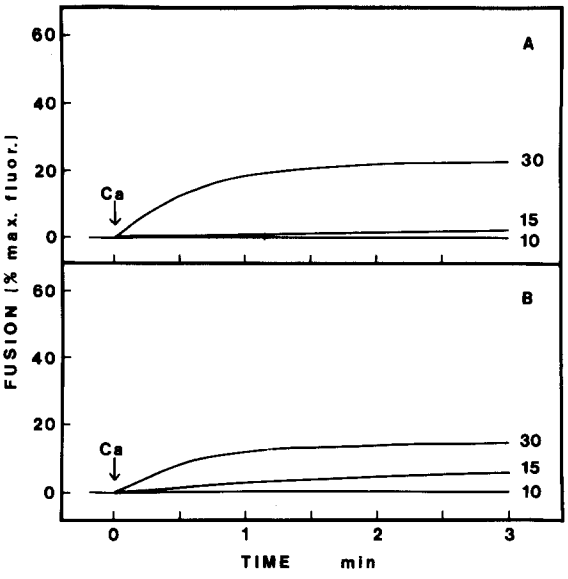


Fig. 1. Time-course for the Ca^{2+}-induced fusion of large unilamellar phosphatidylglycerol vesicles. (A) 25 μM Tb-vesicles and 25 μM dipicolinic acid-vesicles were suspended in 2.0 ml of NaCl buffer containing 0.1 μM EDTA. (B) 25 μM N-NBD-PE/N-Rh-PE-vesicles and 25 μM unlabelled vesicles were suspended in 2.0 ml of NaCl buffer containing 0.1 mM EDTA. In both A and B, Ca^{2+} at the indicated millimolar concentrations was added at time zero and the fluorescence increased was recorded as described in Experimental procedures.

Results

The time-course of the Ca^{2+}-induced fusion of PG vesicles monitored by both fluorescent assays is shown in Fig. 1. At Ca^{2+} concentrations of 10 mM and below, no fusion could be detected using either fluorescence technique. As the Ca^{2+} concentration was increased above this threshold value, both the rate and extent of liposome fusion increased in both assays, yielding a maximum extent of fusion after approx. 3 min. Moreover, this maximum extent of fusion (expressed as the percent of maximum fluorescence) remained essentially constant with time in both assays, indicating that PG liposome fusion is virtually non-leaky, since leakage of the Tb^{3+}-dipicolinic acid complex from a fused liposome pair into the surrounding medium would result in its dissolution due to the presence of Ca^{2+} and EDTA. Using 30 mM Ca^{2+} (the highest concentration tested) the Tb and fluorescent phospholipid assays yielded maximum values for PG liposome fusion of 23 and 14%, respectively. While differences in the extent of fusion for a given Ca^{2+} concentration do exist between the two techniques, these differences are due to the manner in which each assay was calibrated. The fluorescent phospholipid assay was calibrated by the dispersal of the N-NBD-PE and N-Rh-PE using Triton X-100 and designating the resulting fluorescence as 100%. When labelled and unlabelled vesicles are induced to fuse by Ca^{2+} addition, however, the relief of NBD fluorescence quenching will occur only to the extent of the dilution of fluorophores in the membrane of the fused vesicles. Since a limited amount of unlabelled vesicles were used in the assay (equimolar to the labelled vesicles) 100% fluorescence as obtained by Triton X-100 treatment cannot be reached in this system. In experiments in which a 1 : 9 mixture of labelled to unlabelled PG vesicles was used, higher levels of fluorescence were indeed obtained upon Ca^{2+} addition (data not shown). We have chosen not to use the 1 : 9 mixture in our experiments since the sensitivity of the assay would not permit accurate measurements at low fluorescent vesicle concentrations. In this study, we

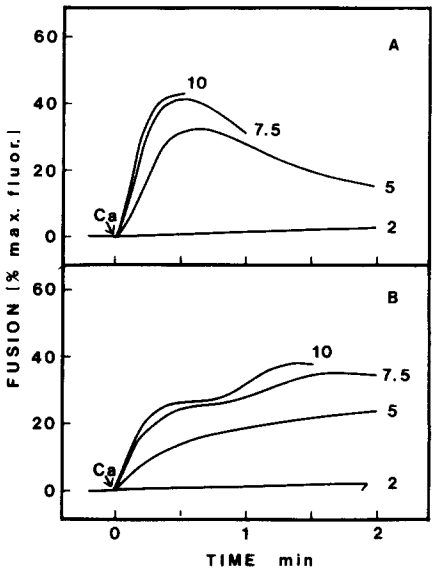


Fig. 2. Time-course for the Ca^{2+}-induced fusion of large unilamellar phosphatidylserine vesicles. (A) Tb/dipicolinic acid assay; (B) fluorescent phospholipid assay. Experimental conditions were as in Fig. 1.

focused on the relative initial rates of fusion as the Ca^{2+} or vesicle concentrations were varied and did not attempt to quantitate the extent of fusion in absolute terms. A more extensive comparison of the two assays using the mass action kinetic analysis developed by Nir et al. [8] is in progress.

Fig. 2 shows the time course of PS vesicle fusion induced by various concentrations of Ca^{2+}, as detected by both fluorescent assays. For this vesicle type, Ca^{2+} concentrations below 1 mM failed to cause fusion with either assay (data not shown), which is consistent with earlier investigations [1]. Again, both the rate and extent of liposome fusion were dependent upon Ca^{2+} concentration. Using the Tb/dipicolinic acid assay with PS vesicles, the fluorescence decreased after reaching a maximum for Ca^{2+} concentrations of 5 mM and above (Fig. 2A). This behavior, which has been observed previously [1,3,8–10] was explained by the entry of Ca^{2+} or EDTA into the aqueous interior of the vesicles or by the leakage of the Tb/dipicolinic acid complex into the external medium. This would result from vesicle collapse following extensive fusion, with subsequent formation of cochleate lipid cylinders [11].

As mentioned earlier, the maintenance of the maximal level of fluorescence after the fusion of

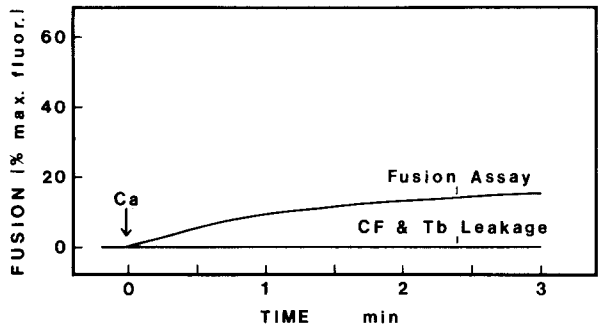
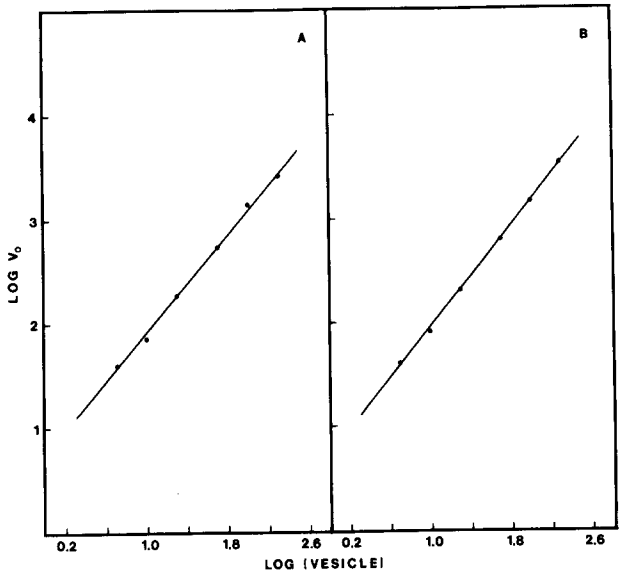


Fig. 3. Time-course for the Ca^{2+}-induced fusion of PG vesicles and release of vesicle contents. Conditions for fusion measurements were as in Fig. 1A. Experiemental conditions for monitoring the release of vesicle contents were as described in Experimental procedures. CF, carboxyfluorescein.

PG vesicles as monitored by the Tb/dipicolinic acid assay indicates that PG vesicle fusion is nonleaky. To confirm this interpretation, PG vesicle leakage during fusion was monitored using two separate techniques. One of these techniques involved the use of PG vesicles containing carboxyfluorescein. Upon fusion of these vesicles by the addition of Ca^{2+}, no increase in carboxyfluorescein fluorescence was observed, indicating that all the carboxyfluorescein remained inside the vesicles in a self-quenched state (Fig. 3). The second technique involved the fusion of PG vesicles with encapsulated Tb in a medium which contained 20 μM dipicolinic acid and no EDTA. Upon addition of Ca^{2+}, no increase in Tb fluorescence could be detected, again indicating that PG vesicle fusion occurs without leakage of internal aqueous contents (Fig. 3).

In order to compare the two fluorescence techniques in their ability to measure rates of fusion, a kinetic analysis of liposome fusion was performed with both fluorescent assays. Using PG vesicles, initial rates of fusion were measured for various vesicle concentrations between 5 and 200 μM phospholipid while keeping the Ca^{2+} concentration constant at 20 mM. The logarithm of the initial rate of fusion, log V_0, is plotted against the logarithm of vesicle concentration in Fig. 4. Both fluorescent techniques yield a straight line with virtually identical slopes and intercepts. Thus, an excellent agreement exists between the two assays concerning the kinetics of PG vesicle fusion.



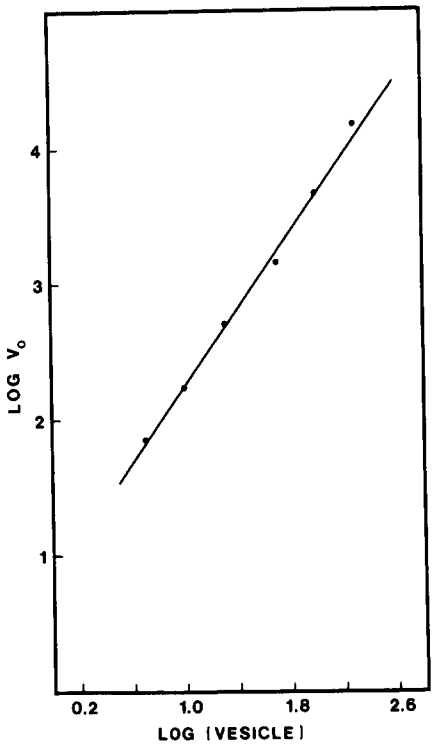


Fig. 4. Plot of the logarithm of initial rate of fusion (log V_0) versus logarithm of phosphatidylglycerol vesicle concentration. (A) Tb/dipicolinic acid assay; (B) fluoresent phospholipid assay. Vesicle concentrations were varied between 5 and 200 μM of phospholipid. Ca^{2+} concentration was kept constant at 20 mM.

Fig. 5. Plot of the logarithm of initial rate of fusion (log V_0) versus the logarithm of phosphatidylserine vesicle concentration using the fluorescent phospholipid assay. Experimental conditions were as in Fig. 4.

The relation between vesicle concentration and the initial rate of fusion of large unilamellar PS vesicles has been previously investigated using the Tb/dipicolinic acid assay [1]. For vesicle concentrations below 50 μM, a plot of log V_0 vs. log vesicle concentration yields a straight line with a slope of 1.8. Above 50 μM vesicles, this relation is linear, with a slope of 1.1. Using the fluorescent phospholipid assay, we obtained a straight line with a slope of 1.5 for the entire vesicle concentration range (5–200 μM) (Fig. 5).

TABLE I

EFFECT OF Mg^{2+} ON THE INITIAL RATE OF Ca^{2+}-INDUCED FUSION OF PS VESICLES AS MONITORED BY THE FLUORESCENT PHOSPHOLIPID ASSAY

Experimental conditions were as in Fig. 1b.

$[Ca^{2+}]$ (mM)	$[Mg^{2+}]$ (mM)	Initial rate of fusion (% maximum fluorescence per min)
5	0	48±2
5	10	20±2
0	10	0

The fluorescent lipid probes used in this study do not undergo exchange between membranes as indicated by the absence of any fluorescence increase when labelled and unlabelled vesicles are incubated in 100 mM NaCl (data not shown). It was important to establish, however, that such an exchange does not take place when the vesicles are in an aggregated but non-fusing state. It was shown previously by use of the Tb/dipicolinic acid assay and freeze-fracture electron microscopy [12] that large PS vesicles aggregate in the presence of threshold concentrations of Mg^{2+} without concomitant fusion. We therefore examined the same system using the fluorescent phospholipid assay. No transfer of labelled lipids occurred during

Mg^{2+}-induced aggregation of PS vesicles (data not shown; however, see table I). This observation lends further support to the reliability of this assay.

Using the Tb/dipicolinic acid assay, Wilschut et al. [12] showed that Mg^{2+} inhibited the Ca^{2+} induced fusion of PS vesicles. Although fusion was initially non-leaky, the internal contents of these vesicles were released rapidly approx. 2 min after the onset of fusion. The possibility was raised that this release could be associated with a type of fusion which did not lead to the intermixing of aqueous contents. Thus it was of interest to investigate this phenomenon using the fluorescent phospholipid assay. Table I shows that the presence of Mg^{2+} inhibits the initial rate of vesicle fusion induced by 5 mM Ca^{2+} as detected by the lipid mixing assay, in accordance with the observations using the Tb/dipicolinic acid assay. The time-course of the fusion of PS vesicles in the presence of 4 mM Ca^{2+} and 10 mM Mg^{2+} is shown in Fig. 6. The fluorescence decreased after the initial rise, most likely as a result of the massive aggregation of the vesicles. This explanation is substantiated by the fact that additions of EDTA (equimolar to the cation) at various times after cation addition increased the level of fluorescence (dotted lines). It is also possible, however, that the decrease in fluorescence arises from the phase separation of the fluorescent lipids, resulting in the close apposition of the fluorophores. The fluorescence intensity obtained (70% max fluorescence) upon addition of EDTA 5 min after the induction of fusion by Ca^{2+} and Mg^{2+} indicates that the extent of fusion increased after 2 min

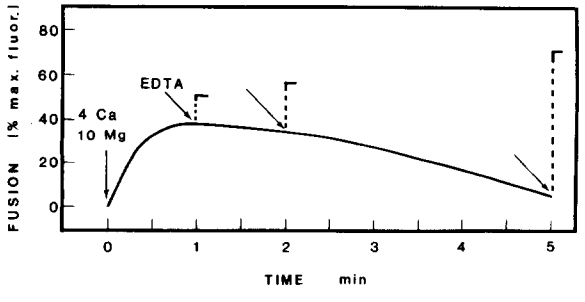


Fig. 6. Time-course of fusion of PS vesicles induced by 4 mM Ca^{2+} and 10 mM Mg^{2+} monitored by the fluorescent phospholipid assay. 14 mM EDTA was added at the times indicated by the arrows. The dotted lines show the resulting fluorescence.

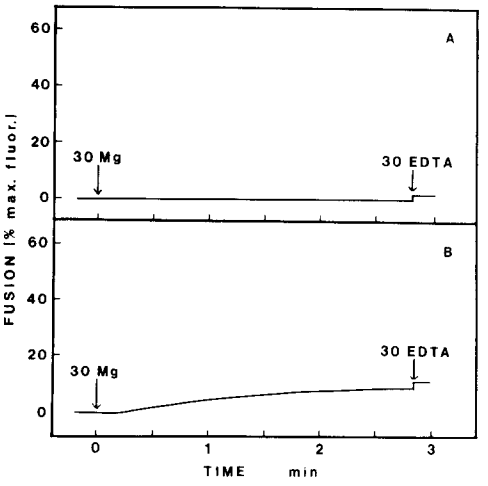


Fig. 7. The effect of Mg^{2+} on phosphatidylglycerol vesicles monitored by: (A) Tb/dipicolinic acid assay; (B) fluorescent phospholipid assay. 30 mM Mg^{2+} was added at time zero.

(where the fluorescence was 55% max fluorescence), although the fluorescence signal before EDTA addition was decreasing. This observation therefore suggests that in this system membrane fusion continues in a manner which does not lead to intermixing of contents but which results in the collapse of the internal aqueous space of the vesicles [12].

When Mg^{2+} was added to PG vesicles, the two assays produced differing results (Fig. 7). An apparent fusion was detected by the fluorescent phospholipid assay (Fig. 7b) in the presence of 30 mM Mg^{2+}. The Tb/dipicolinic acid assay, however, revealed no intermixing of aqueous contents (Fig. 7a) although the vesicles aggregated as indicated by the decrease in the 90° light-scattering signal (data not shown). Control experiments showed that Mg^{2+} did not alter the fluorescence of the labeled vesicles when no unlabeled vesicles were present. A possible explanation for this discrepancy between the two fusion assays is that Mg^{2+} induces the aggregation of large unilamellar PG vesicles with subsequent outer-monolayer intermixing. This intermixing of the closely apposed outer monolayers would lead to a dilution of the fluorescent phospholipids resulting in an increase in N-NBD-PE fluorescence. The internal aqueous contents of the vesicles, however, would remain separated if bilayer integrity is not lost and thus

the Tb/dipicolinic acid assay would not indicate vesicle fusion. Another possibility is the exchange of the fluorescent probes across the apposed part of the bilayers of labelled and unlabelled vesicles. However, this alternative explanation appears less likely, since such an exchange was not observed with PS vesicles aggregated in the presence of Mg^{2+}.

Discussion

The results presented above demonstrate that large (0.1 μm diameter) unilamellar vesicles composed of PS or PG undergo fusion at characteristic threshold Ca^{2+} concentrations. Fusion involves not only the intermixing of the internal aqueous contents but also the intermixing of the membrane components, as shown by the two independent assays. The threshold concentration of Ca^{2+} at which fusion occurs on a time-scale of seconds is considerably higher for PG than for PS vesicles. This observation is consistent with the lower binding constant of Ca^{2+} to PG than to PS, since the extent of ion binding to the membrane appears to be one of the determinants of fusion [9,13]. Lau et al. [14] have determined the 1 : 1 intrinsic association constant of Ca^{2+} to PG to be 8.5 M^{-1}, whereas the constant for Ca^{2+} binding to PS is 12 M^{-1} [15]. Fusion of multilamellar dimyristoylphosphatidylglycerol and dipalmitoylphosphatidylglycerol vesicles with each other in the presence of 10 mM Ca^{2+} and on a time-scale of minutes has also been observed by means of differential scanning calorimetry [16]. It should be pointed out that the concentration of vesicles used in the present study is 20-fold less than that used for the calorimetry experiments. The use of high concentrations of multilamellar vesicles, lipids with saturated acyl chains, high temperatures of incubation, and the time-scale of observation in the study of Papahadjopoulos et al. [16] render a direct comparison between the two sets of experiments difficult.

Comparison of the two fusion assays used in this study reveals similarities as well as some discrepancies. Both assays show essentially similar dependencies of the fusion of PS and PG vesicles on the Ca^{2+} concentration. The inhibition of Ca^{2+}-induced fusion of PS vesicles by Mg^{2+} detected by the Tb/dipicolinic acid assay [12] is also observed with the fluorescent lipid assay.

A further correspondence of the assays is found in the Mg^{2+}-induced aggregation of PS vesicles. The Tb/dipicolinic acid assay, as well as electron microscopy, has shown that large PS vesicles aggregate but do not fuse in the presence of Mg^{2+} above a threshold concentration [12]. The fluorescent lipid assay likewise shows no fusion in this case, also indicating that the probe molecules do not transfer from the labelled to the unlabelled vesicles.

The vesicle concentration dependence of the initial rate of fusion of PG vesicles shows that the fusion reaction is less than second order with respect to the vesicle concentration. This order is 1.18 for the Tb/dipicolinic acid assay and 1.21 for the fluorescent lipid assay, showing the consistency of both assays in this system. This finding may be interpreted as follows. The overall fusion reaction can be separated into two steps, the first being aggregation, the second being the intermixing of internal contents and membrane components.

$$m \text{ vesicles} \xrightarrow{k_1} \underset{m \geq 2}{\text{aggregate}} \xrightarrow{k_2} \text{fusion product}$$

During the aggregation step, addition of Ca^{2+} promotes the formation of an aggregate of m vesicles, where $m \geq 2$, with a rate constant k_1. Subsequent fusion of the vesicle membranes in this aggregate with a rate constant k_2 results in the intermixing of both the internal contents and the membranes of vesicles. Thus, in both fusion assays, only the second step is recorded. The overall order of the fusion reaction, as measured by the assays, with respect to vesicle concentration is therefore dependent upon the relative rates of the two steps. Considering that the minimum number of vesicles that will aggregate to fuse is two ($m \geq 2$), a rate-determining aggregation step will yield an overall order of at least two. Likewise, an order less than two would imply that the fusion step has become rate-limiting with respect to aggregation.

For PS vesicles we have found an order of 1.5 throughout the vesicle concentration range studied, in contrast to orders of 1.8 and 1.1 at low and high vesicle concentration ranges, respectively, determined by the Tb/dipicolinic acid assay [1]. A possible source for this difference is the fact that

the two assays monitor two different events during membrane fusion whose rates may vary depending on the type of phospholipid or divalent cation. Indeed, the time course of fusion as detected by the two assays (Fig. 2) reveals slightly different kinetics in the case of PS vesicles. The intermixing of contents starts with a slow rate which reaches a maximum with time, whereas the intermixing of lipids appears to start at the maximum rate.

In summary, the two assays described in this paper detect complementary aspects of membrane fusion and may be utilized in conjunction to characterize the molecular mechanisms of membrane fusion. The fluorescent phospholipid assay appears to be especially well suited to the study of phospholipid vesicle systems where fusion is extremely leaky to encapsulated compounds, such as the fusion of phosphatidate vesicles by Ca^{2+} at pH 8.4 [17].

Note added in proof: (Received September 21st, 1983)

Recently, the initial rates of fusion of small unilamellar PS vesicles as determined by the Tb assay and an alternative lipid mixing assay have revealed similar dependencies on the Ca^{2+} concentration [18,19].

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