Biochimica et Biophysica Acta, 690 (1982) 297–301
Elsevier Biomedical Press

BBA Report

BBA 70055

Ca^{2+}-INDUCED FUSION OF CARDIOLIPIN/PHOSPHATIDYLCHOLINE VESICLES MONITORED BY MIXING OF AQUEOUS CONTENTS

JAN WILSCHUT, MARLEEN HOLSAPPEL and RITA JANSEN

Laboratory of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen (The Netherlands)

(Received March 16th, 1982)

Key words: Ca^{2+}; Vesicle fusion; Cardiolipin; Phosphatidylcholine

The kinetics of Ca^{2+}-induced fusion of large (0.1 μm) unilamellar cardiolipin/phosphatidylcholine (1:1) vesicles have been investigated by continuous monitoring of the mixing of the aqueous vesicle contents. In parallel, release of vesicle contents to the external medium has been followed. Initial fusion of the vesicles is non-leaky, release of vesicle contents being largely a secondary phenomenon. The minimal Ca^{2+} concentration required for fusion in this system is approx. 9 mM. At higher Ca^{2+} concentrations fusion is extremely fast, occurring on the time scale of seconds.

Although phospholipids in biological membranes are generally considered to be organized in a bimolecular leaflet structure, several functional capacities of membranes are difficult to reconcile with this concept. A prominent example is membrane fusion, which obviously requires the phospholipids to deviate, even if transiently, from a bilayer arrangement. Thus, phospholipids which can adopt non-bilayer conformations have been suggested to be involved in the formation of intermediary structures during membrane fusion [1–3]. Among these phospholipids are cardiolipin (CL) and phosphatidylethanolamine. In the absence of divalent cations beef-heart cardiolipin adopts the bilayer arrangement, but in the presence of Ca^{2+} or Mg^{2+} the hexagonal (H_{II}) phase is preferred, as detected by X-ray diffraction [4,5] or ^{31}P-NMR measurements [6]. In mixtures of cardiolipin and the bilayer-forming lipid phosphatidylcholine (PC), Ca^{2+} has been shown to produce an intermediate molecular organization, the lipidic particle, which is thought to represent an inverted micellar structure [7–9]. Furthermore, in CL/PC vesicles aggregated by addition of $CaCl_2$, lipidic particles have been observed at the site of contact between the vesicles [2]. Although there has been some debate as to the nature of the lipidic particles [10–13] their preferential localization between interacting vesicles suggests a possible involvement in bilayer fusion.

In the present paper we demonstrate the fusion of large unilamellar CL/PC vesicles as monitored by the Tb/dipicolinate assay for mixing of internal contents [14–16]. Briefly, $Tb(citrate)_3^{6-}$ is encapsulated in one population of vesicles and an excess of the anion of dipicolinic acid (DPA) in another. Fusion results in the fast formation of the fluorescent $Tb(DPA)_3^{3-}$ complex, which can be monitored continuously. The presence of EDTA and Ca^{2+} in the external medium effectively prevents the formation of the Tb/DPA complex outside the vesicles [15]. Release of vesicle contents is measured in separate experiments employing the dequenching of carboxyfluorescein (CF) upon dilution [14–17].

Large unilamellar vesicles were prepared in the desired aqueous media, from an equimolar mixture

Abbreviation: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

(phosphorus ratio, 2:1) of beef-heart cardiolipin (Sigma) and egg-yolk phosphatidylcholine (Sigma) by reverse-phase evaporation [18], and sized to 0.1 μm by successive extrusion [19] through 0.2 μm and 0.1 μm Unipore polycarbonate filters (Bio-Rad), as described before [15]. The vesicles were separated from non-encapsulated material by gel filtration on BioGel A 0.5 m (BioRad). Internal aqueous contents of the vesicles were as follows. Tb-vesicles: 5.0 mM $TbCl_3$ (Alfa), 50 mM sodium citrate; dipicolinate-vesicles: 50 mM sodium dipicolinate (Sigma), 20 mM NaCl; CF-vesicles: 50 mM carboxyfluorescein (sodium salt). Carboxyfluorescein was obtained from Eastman and purified by chromatography on Sephadex LH20 (Ref. 17). All aqueous media contained 2 mM L-histidine and 2 mM Tes, adjusted to a final pH of 7.4. The trapped volume of the vesicles was 3.5–4 l/mol of lipid, based both on the encapsulation of Tb, dipicolinate or carboxyfluorescein (see Ref. 15) and on that of 1 mM [^{14}C]sucrose, which in some experiments was added to the aqueous media as a marker for the encapsulated volume. Fluorescence measurements were performed with a Perkin Elmer MPF43 fluorimeter in a final volume of 2.0 ml 100 mM NaCl, 2 mM L-histidine, 2 mM Tes, 0.1 mM EDTA (pH 7.4), which was stirred magnetically and maintained at a temperature of 25°C. Routinely, the medium in the cuvette contained $CaCl_2$ and fusion was initiated by injection of a small aliquot of a concentrated vesicle suspension with a Hamilton Syringe. Final lipid concentration was 0.1 mM. The Tb/DPA complex was excited at 276 nm, fluorescence was measured at 545 nm with a cut-off filter (> 520 nm) between sample and monochromator. The fluorescence scale was calibrated [15] such that the intensity recorded during the fusion assay represented the percentage of the total amount of Tb, associated with dipicolinate. Carboxyfluorescein was excited at 430 nm and emission was measured at 520 nm. For calibration of the scale maximal fluorescence was determined by lysing 0.1 mM of the vesicles with 1% (v/v) Triton X-100.

Fig. 1A shows the kinetics of Tb fluorescence increase upon introduction of a 1:1 mixture of Tb- and DPA-vesicles into a medium containing various concentrations of Ca^{2+}. Very little fluorescence was seen at Ca^{2+} concentrations of 8 mM or less. At 9 mM or higher a significant development of Tb fluorescence was observed, the initial rate of which increased rapidly with the Ca^{2+} concentration (Fig. 2). Extremely high rates of fusion were attained at the higher Ca^{2+} concentrations. For example, at 20 mM Ca^{2+} the initial rate of Tb fluorescence increase was approx. 30%/s. With even higher Ca^{2+} concentrations the initial rate of fusion leveled off at a value which could not be

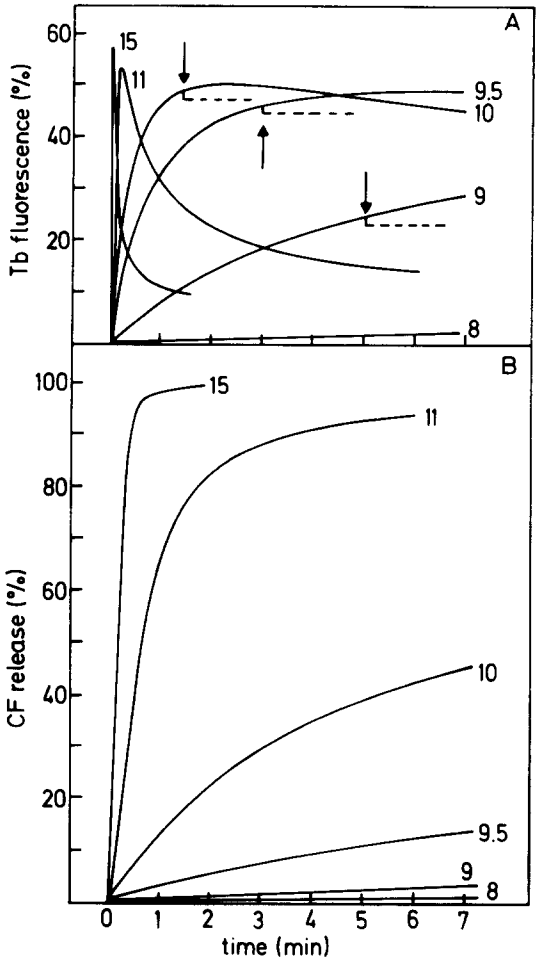

Fig. 1. Ca^{2+}-induced fusion of CL/PC vesicles. 0.1 ml of a vesicle suspension (concentration, 2.0 mM lipid phosphorus) was injected into a cuvette with 1.9 ml Ca^{2+}-containing medium. Final millimolar Ca^{2+} concentrations are indicated. Either Tb- and DPA-vesicles, premixed in a 1:1 ratio (A) or carboxyfluorescein-vesicles (B) were used. In some experiments a small aliquot of a concentrated EDTA solution was added to a 2-fold molar excess over Ca^{2+} (see arrows); the dashed lines represent the fluorescence intensity after correction for the dilution as a result of the addition of the EDTA solution.

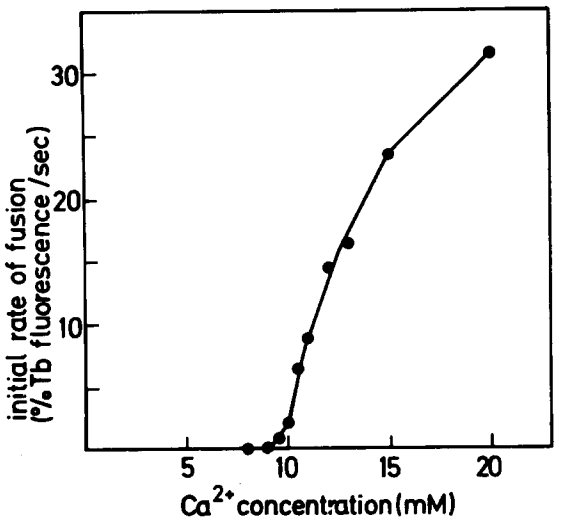

Fig. 2. Dependence of CL/PC vesicle fusion on the Ca^{2+} concentration. Initial rates of Tb fluorescence increase were determined from the tangents to the fluorescence curves. Conditions were as indicated in the legend to Fig. 1. At the higher rates of fusion fluorescence was recorded at high recorder chart speeds.

determined accurately due to instrumental limitations. It should be noted that these high fusion rates are achieved in relatively dilute vesicle suspensions. In more concentrated suspensions fusion will be even faster since the process is second-order with respect to the vesicle concentration (results not shown).

Addition of excess EDTA at various stages after the initiation of the fusion resulted in an immediate arrest of the process and in an almost complete fixation of the Tb fluorescence intensity (Fig. 1A, dashed lines), indicating an effective sequestration of the Tb/DPA complex from the external medium.

The threshold Ca^{2+} concentration required for fusion of CL/PC vesicles is dependent on the relative amounts of the two lipids in the vesicle bilayer. Like with vesicles containing phosphatidylserine rather than cardiolipin as the negatively charged lipid [20,21] the threshold value increases with increasing phosphatidylcholine content of the bilayer (results not shown).

The secondary decrease of the Tb fluorescence at the higher Ca^{2+} concentrations (Fig. 1A) presumably resulted from release of vesicle contents and subsequent quenching of the Tb fluorescence in the external medium. We confirmed this employing a direct assay for leakage based on the dilution-induced dequenching of carboxyfluorescein fluorescence [14–17]. The results are shown in Fig. 1B. At the lower Ca^{2+} concentrations very little release of carboxyfluorescein occurred, whereas at the higher concentrations, as expected from the Tb fluorescence curves, release was extensive. In all cases the initial rate of carboxyfluorescein release was very small as compared to the corresponding rate of Tb fluorescence increase. For example, at 9 mM Ca^{2+} the rate of Tb fluorescence increase was 8.5%/min and that of carboxyfluorescein release 0.4%/min. At 9.5 mM Ca^{2+} these values were 53%/min and 3%/min, respectively. In this respect it should be noted that during the first round of fusion in a 1 : 1 mixture of Tb- and DPA-vesicles only half of the fusion events is productive in terms of fluorescence, the probablity of Tb-DPA fusion being 0.5. Consequently, the initial rate of Tb fluorescence increase is an underestimate of the actual rate of fusion by a factor of 2. Corrected rates of fusion at 9 and 9.5 mM Ca^{2+} are therefore 17%/min and 106%/min, respectively. These values are approx. 40-fold higher than the corresponding rates of carboxyfluorescein release, indicating that initially

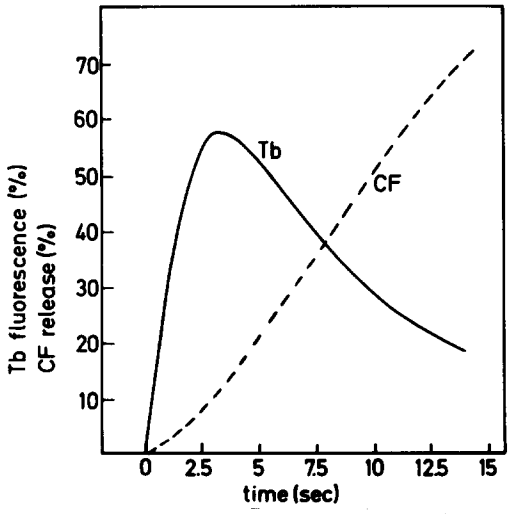

Fig. 3. Fusion of CL/PC vesicles at 15 mM Ca^{2+}. Conditions were as indicated in the legend to Fig. 1. Fluorescence was recorded at high recorder chart speed.

fusion is largely a non-leaky phenomenon. This also holds at the higher Ca^{2+} concentrations, as shown in Fig. 3, where Tb and carboxyfluorescein fluorescence curves at 15 mM Ca^{2+} are presented on an extended time scale. As with the Ca^{2+}-induced fusion of phosphatidylserine vesicles [14–16], leakage of contents appears to be a secondary process presumably related to collapse of the vesicles following their fusion.

The above results basically confirm the conclusion from previous freeze-fracture studies [2,10,13], that CL/PC vesicles fuse under the influence of Ca^{2+}. However, there is a major discrepancy with respect to the kinetics of the fusion process and the threshold Ca^{2+} concentration required. Verkleij et al. [2] incubated CL/PC vesicles with Ca^{2+} for 30 min at a high lipid concentration. At final Ca^{2+} concentrations as low as 2 mM they observed large vesicles and aggregated structures with lipidic particles at contact sites between the vesicles. These structures were proposed to represent fusion intermediates. Our present work demonstrates that fusion occurs at extremely fast rates even in dilute vesicle suspensions. This implies that the fusion even per se is a very rapid process and, consequently, that the period of time during which a fusion intermediate will exist must be extremely short. Therefore it would seem highly unlikely that fusion intermediates can be visualized at a significant frequency after incubation periods of many minutes. The structures observed presumably represent equilibrium states rather than dynamic intermediates.

With respect to the discrepancy in Ca^{2+} threshold concentration between the work of Verkleij et al. [2] and our results, we considered the possibility that in the freeze-fracture study, in order to avoid dilution of the vesicle suspension, $CaCl_2$ was added as a small aliquot of a concentrated solution. This would result in a transient exposure of a fraction of the vesicles to local Ca^{2+} concentrations that are higher than the final concentration, which in turn would result in aggregation and fusion of this fraction of the vesicles. To test the feasibility of this explanation we performed the experiment shown in Fig. 4. When, rather than addition of vesicles to a Ca^{2+}-containing medium, a small aliquot of a 0.4 M $CaCl_2$ solution was added to a dilute vesicle suspension, a rapid transient fusion process was observed. This also occurred under conditions where the final Ca^{2+} concentration was below the threshold value of 9 mM. This indicates that a fraction of the vesicles fused as a result of brief exposure to a locally high Ca^{2+} concentration. In view of the short period of time required for complete mixing of the solutions in the cuvette (< 1 s), the rate of fusion must have been very high, but not inconsistent with the fusion rates presented in Figs. 2 and 3. It should be noted that with more concentrated vesicle suspensions, such as those used in freeze-fracture studies, rates will be even higher since the fusion process is second-order with respect to the vesicle concentration, as mentioned above.

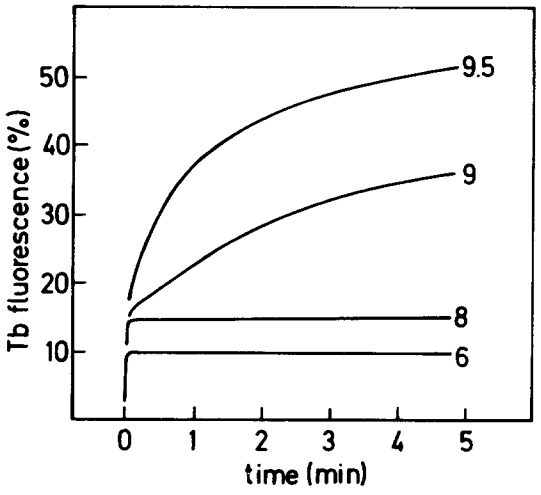

Fig. 4. Fusion of CL/PC vesicles upon addition of a concentrated $CaCl_2$ solution. A small aliquot of a 0.4 M $CaCl_2$ solution was injected into a cuvette containing a 1 : 1 mixture of Tb- and DPA-vesicles at a final lipid concentration of 0.1 mM (lipid phosphorus). Final millimolar Ca^{2+} concentrations are indicated.

In conclusion, our present results demonstrate that CL/PC (1 : 1) vesicles in dilute suspensions fuse at high rates in the presence of Ca^{2+} at concentrations of approximately 9 mM or higher. Fusion is essentially non-leaky initially, but release of vesicle contents does occur as a secondary process, presumably due to collapse and complete transformation of the vesicles. Our observation (Fig. 4) of rapid fusion during transient exposure of vesicles to locally high Ca^{2+} concentrations raises the possibility that the dimpled vesicles car-

rying lipidic particles, observed at low, final, Ca^{2+} concentrations (Ref. 2) were also formed as a result of an initial very brief exposure of part of the vesicles to high Ca^{2+} concentrations. This underlines that these structures, which were visualized after a subsequent incubation of 30 min, presumably represent equilibrium states rather than dynamic intermediates. This conclusion, however, does not exclude the possibility that lipidic particles or other hexagonal-like structures are involved in a dynamic intermediate structure during CL/PC vesicle fusion. Answering that question will require the application of fast-freezing techniques and freezing of the samples after very brief exposure to Ca^{2+}.

We wish to thank Dr. Gerrit Scherphof for his continuous interest in this project and for valuable advice during the preparation of the manuscript, Dr. Arie Verkleij (Institute of Molecular Biology, University of Utrecht) for stimulating discussions and Mrs. Karin van Wijk and Mrs. Rinske Kuperus for expert secretarial work. One of us (M.H.) was financially supported by the Koningin Wilhelmina Fonds (The Netherlands Cancer Foundation).

References

1 Cullis, P.R. and De Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420
2 Verkleij, A.J., Mombers, C., Gerritsen, W.J., Leunissen-Bijvelt, L. and Cullis, P.R. (1979) Biochim. Biophys. Acta 555, 358–361
3 Verkleij, A.J., Van Echteld, C.J.A., Gerritsen, W.J., Cullis, P.R. and De Kruijff, B. (1980) Biochim. Biophys. Acta 600, 620–624
4 Deamer, D.W., Leonard, R., Tardieu, A. and Branton, D. (1970) Biochim. Biophys. Acta 219, 47–60
5 Rand, R.P. and Sengupta, S. (1972) Biochim. Biophys. Acta 255, 484–492
6 Cullis, P.R., Verkleij, A.J. and Ververgaert, P.H.J.T. (1978) Biochim. Biophys. Acta 513, 11–20
7 Verkleij, A.J., Mombers, C., Leunissen-Bijvelt, J. and Ververgaert, P.H.J.T. (1979) Nature 279, 162–163
8 De Kruijff, B., Verkleij, A.J., Van Echteld, C.J.A., Gerritsen, W.J., Mombers, C., Noordam, P.C. and De Gier, J. (1979) Biochim. Biophys. Acta 555, 200–209
9 Van Venetië, R. and Verkleij, A.J. (1981) Biochim. Biophys. Acta 645, 262–269
10 Miller, R.G. (1980) Nature 287, 166–167
11 Hui, S.W. and Stewart, T.P. (1981) Nature 290, 427
12 Verkleij, A.J. and De Kruijff, B. (1981) Nature 290, 427–428
13 Rand, R.P., Reese, T.S. and Miller, R.G. (1981) Nature 293, 237–238
14 Wilschut, J. and Papahadjopoulos, D. (1979) Nature 281, 690–692
15 Wilschut, J., Düzgünes, N., Fraley, R. and Papahadjopoulos, D. (1980) Biochemistry 19, 6011–6021
16 Wilschut, J., Düzgünes, N. and Papahadjopoulos, D. (1981) Biochemistry 20, 3126–3133
17 Ralston, E., Hjelmeland, L.M., Klausner, R.D., Weinstein, J.N. and Blumenthal, R. (1981) Biochim. Biophys. Acta 649, 133–137
18 Szoka, F.C. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4194–4198
19 Szoka, F., Olsen, F., Heath, T., Vail, W., Mayhew, E. and Papahadjopoulos, D. (1980) Biochim. Biophys. Acta 601, 559–571
20 Düzgünes, N., Wilschut, J., Fraley, R. and Papahadjopoulos, D. (1981) Biochim. Biophys. Acta 642, 182–195
21 Düzgünes, N., Nir, S., Wilschut, J., Bentz, J., Newton, C., Portis, A. and Papahadjopoulos, D. (1981) J. Membrane Biol. 59, 115–125