Biochimica et Biophysica Acta, 689 (1982) 165–169
Elsevier Biomedical Press

BBA Report

BBA 70046

EARLY EVENTS IN CALCIUM-INDUCED LIPOSOME FUSION

DOUGLAS C. MILLER * and GERHARD P. DAHL

Department of Physiology and Biophysics, University of Miami School of Medicine, Miami, FL 33101 (U.S.A.)

(Received December 31st, 1981)

Key words: Membrane fusion; Liposome; Phosphatidylserine; Ca^{2+} dependence; (Stopped flow/rapid freeze technique)

Calcium-induced interaction of liposomes composed of pure phosphatidylserine (PS) has been studied using a rapid-mixing, rapid-freeze device. Freeze-fracture electron microscopy of this material revealed that liposomes react very rapidly after addition of calcium ions. After only 10 ms (the resolution of the technique) vesicle fusion was apparent. At the same time, however, vesicles also collapsed, and appeared as aggregates of flattened membranes. This may explain controversies which have arisen over vesicle fusion studied with more indirect methods.

Membrane fusion, which is an important event in numerous cellular functions, e.g. exocytosis, is at present poorly understood due to the complexity of cells. Consequently, a variety of model systems have been introduced for evaluating molecular mechanisms of the fusion process [1,2]. A highly simplified system which has been extensively studied is the fusion of liposomes composed of pure phosphatidylserine (PS) [3–9]. However even in this simple system the evidence for membrane fusion has been largely circumstantial and hence controversial. The concept of PS fusion has been challenged critically by Ginsberg [10] on the basis of mixing of vesicle contents with the extravesicular medium. Although progress has been made via a new fusion assay (mixing of contents) which demonstrates fusion occurs more rapidly than leakage [7–9], the possibility that this result was due to a higher threshold for detection of leakage than for fusion leaves persisting doubt about the reliability of this system. Similar parallelism of fusion and leakage were found in another assay using mixing of contents of vesicles formed from soybean phospholipids [11] although the leakage rate was probably smaller than the fusion rate [12] **. In an effort to get a more direct visualization of the type of membrane interaction involved in the PS system we have found with combined stopped-flow and fast-freezing techniques that in fact a fusion process can be observed.

Small unilamellar liposomes composed of pure PS, when interacted with calcium, form complex structures first described by Papahadjopoulos et al. [4] and termed 'cochleate cylinders' (Figs. 2a and 2b). It was suggested that these were formed by fusion of the vesicles, which then flattened out to form planar membrane sheets which subsequently rolled up to form spiral configurations [4,5]. Alternative interpretations would be simple molecular diffusion of phospholipids or, as proposed by Ginsberg [10], lysis of the vesicles with subsequent recombination of the fragments into

* Present address (to which all correspondence should be sent): Department of Pathology, Massachusetts General Hospital, Boston, MA 02114, U.S.A.

** Furthermore a third such mixing of contents assay, published in abstract form while this report was in review, was reported to show immediate leakage without fusion of PS vesicles [13].

larger structures. This proposal was based on the observed loss of liposome contents and failure of uptake of external medium into liposomes upon exposure to calcium, which suggested vesicle lysis upon exposure to calcium [10]. True fusion would require that the two limiting membranes of two liposomes merge and form a single membrane enclosing a single compartment without exposing the aqueous contents of the vesicles to the external medium. That this in fact occurs has been suggested by a new assay based on terbium (Tb) fluorescence [7–9]. However leakage of vesicle contents was observed, albeit subsequent to the increased Tb fluorescence. It remains unclear whether the time delay, which is crucial to the interpretation, is real or is inherent to the detection system.

In an attempt to elucidate the actual mechanism(s) of membrane interaction in such experiments we have analyzed the morphology of liposome-liposome interactions at very short (≤ 10 ms) incubations of liposomes in Ca^{2+} solutions. To accomplish this we have developed a rapid mixing/rapid freezing device (Fig. 1).

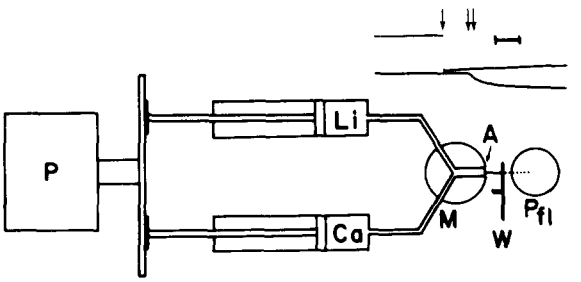

Fig. 1. Rapid mixing device. Liposome suspensions were placed in syringe Li, 22 mM Ca^{2+} (or Mg^{2+}) solutions in syringe Ca. Upon activation of the pneumatic cylinder (Festo Type XYD 50-140 B), P, the two solutions were forced into mixing chamber M and then expelled from its aperture, A (Siemens EM 50 μm aperture), into a pool of melting propane, P_{fl}. The stream of the liposome-Ca^{2+} mixture was broken into droplets prior to contacting the propane by a rotating spoked wheel, W. The frozen droplets were further processed for freeze-fracturing in a Balzers spray-freezing apparatus. The timing of the mixing and freezing was tested by placing salt solutions through the syringes and measuring the times of passage of the solution fronts past various points in the system; as shown in the insert, passage of the solution front was indicated by grounding of current loops (arrow and double arrow, bar = 10 ms). The minimum time between entrance into the mixing chamber and contact to the propane was <10 ms. In some experiments a second chamber was added after the first, and the Ca^{2+}-liposome mixture was mixed in the second chamber with 100 mM EGTA (or EDTA) solution contained in a third syringe. These mixtures were not frozen in propane but were collected at room temperature and frozen in Freon conventionally.

Small unilamellar liposomes were prepared by intense sonication of preparations of large unilamellar or oligolamellar liposomes formed by the reverse phase evaporation technique [14]. Phospholipids were obtained from Avanti Polar Lipids, Inc. The appearance of pure PS vesicles produced in this manner is illustrated in Fig. 2a. Exposure to 10 mM Ca^{2+} for 5 min prior to freezing produces large aggregates of vesicles, as shown in Fig. 2b. In these aggregates many complex membrane forms are apparent, including both flattened membrane sheets and cochleate forms. No mechanism can be inferred from these aggregates, since several modifications of membrane structures could occur over a period as long as 5 min. This complex morphology is less apparent at shorter incubation times. After 130 ms of incubation aggregates are small, and cochleate cylinders are virtually absent (not shown). With incubation times as short as 10 ms (Fig. 2d) or 27 ms (Fig. 2e) twin vesicles can be observed. This is strong evidence for a true membrane fusion process taking place. Although twin vesicles were frequently observed, they were not the exclusive form of membrane interaction observed at such short incubation times; small aggregates with flattened membranes presumably consisting both of membranes of collapsed vesicles and of apposed portions of adjacent vesicles could be found at the same time (Fig. 2c).

Even with short incubation times aggregation and flattening make quantitative analysis difficult. To obtain quantitative data calcium incubation was stopped, at various times, by addition of the Ca^{2+} chelators EGTA or EDTA. The addition of Ca^{2+} chelators introduces a second modification of liposome morphology. It has been shown that addition of chelator following Ca^{2+} incubation results in the formation of large unilamellar vesicles which take up external aqueous medium [4]. The average size of the vesicles, as determined by measurements of membrane area per vesicle exposed on freeze-fracture replicas, increases as a function of Ca^{2+} concentration (at equal exposure times) and as a function of incubation time (at equal Ca^{2+} concentration) as shown in Table I. Magnesium is also effective in inducing these size

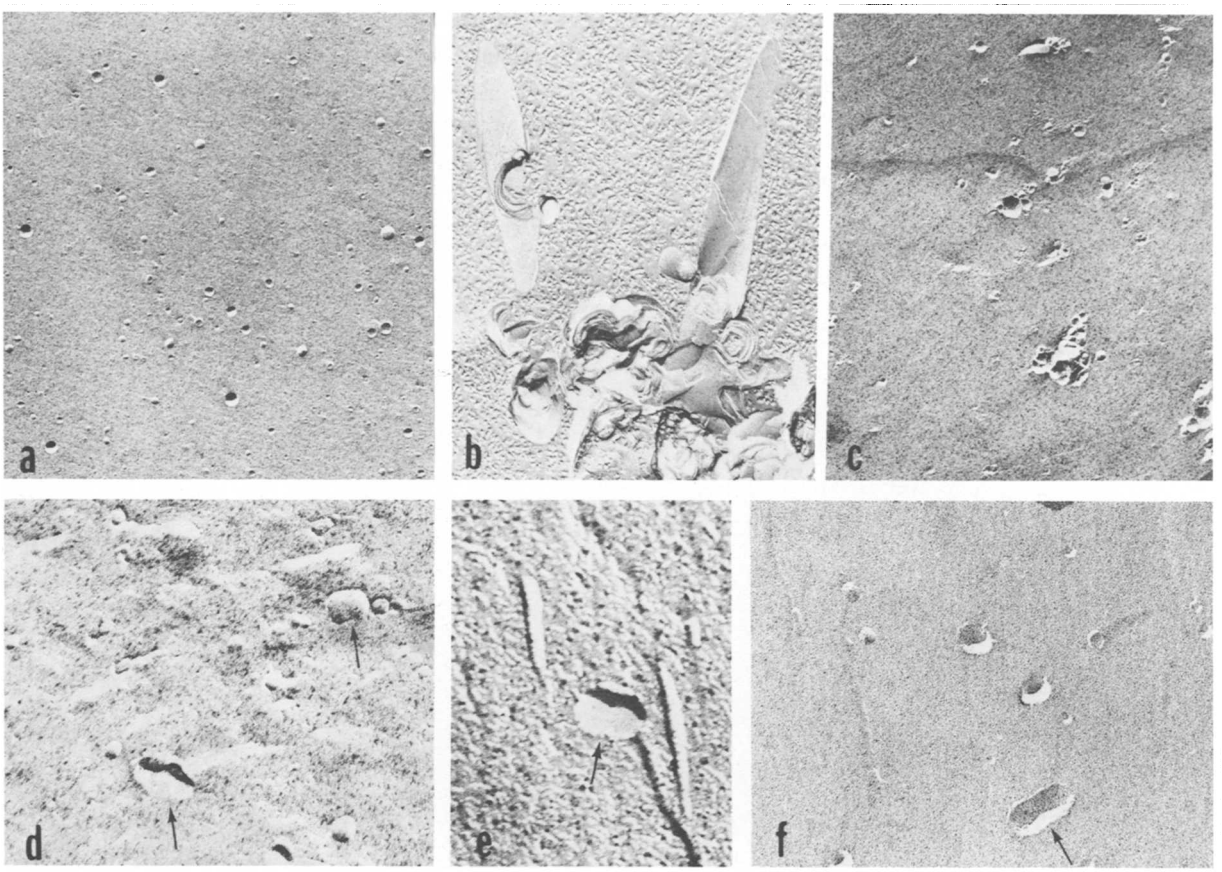

Fig. 2. Freeze-fracture electron micrographs of liposomes. (a) Control preparation of PS liposomes without Ca^{2+} incubation. (b) 5 min Ca^{2+} incubation produces extensive aggregation and flattening, with formation of cochleate forms. (c) Small PS vesicle aggregate with flattening after 10 ms of Ca^{2+} incubation and rapid freezing. (d) and (e). Twin vesicles formed after just 10 ms (d) or 27 ms (e) of incubation in Ca^{2+} followed by rapid freezing in melting propane. (f) Liposomes formed of a mixture reproducing the lipid stoichiometry of the adrenal chromaffin granule membrane but excepting lysophosphatidylcholine, incubated in Ca^{2+} for 10 ms. These vesicles typically are not flattened, and twin vesicles (arrow) are present. Magnifications: (a–c) ×24000, (d) ×82500, (e) ×105000, (f) ×49500.

changes, but higher concentrations are required. Despite the secondary rearrangement of membrane structure caused by EGTA (or EDTA) treatment the observed increase in size is a good indirect measure of calcium-induced structural changes. The data obtained by the rapid freezing technique indicate that membrane fusion is a primary event resulting from the liposome-calcium interaction. With extremely short incubation times (10 ms) the average size of the liposomes has increased considerably, indicating that the fusion process is much more rapid (on the order of ms) than expected from other studies which relied on more indirect assays [3–8], although fast interaction was indicated, too [9].

Similar experiments with phospholipid mixtures provided clearer evidence for membrane fusion, because liposomes composed of complex mixtures of phospholipid classes do not exhibit flattening when aggregated in the absence of Ca^{2+} chelators. Liposomes formed from whole lipid extracts of adrenal chromaffin granule membranes formed twin vesicles and underwent a progressive increase in size when mixed with calcium [1,15]. We have

TABLE I

The values represent means of the mean values of membrane area/vesicle for 100–1200 vesicles from three experiments done with a single liposome preparation for each set. Standard deviations for each experiment were considerable, especially for those with a fusion effect. The area of replica scanned for each experimental condition was 190 μm^2.

Time of interaction	Divalent ion concentration	Number of vesicles/ area of replica	Membrane area/vesicles
min	Ca^{2+} (mM)	*N*	nm^2
–	0	1256±372	2425± 948
5	0.1	1046±333	2691± 765
5	1.0	960±708	3246± 957
5	2.5	911±744	3734± 2328
5	5.0	747±323	4783± 1430
5	10.0	131±102	53540±40270
min	Mg^{2+} (mM)	*N*	nm^2
5	0.1	902±509	2304± 388
5	1.0	1274±719	2367± 299
5	2.5	1203±499	1898± 918
5	5.0	990±104	2333± 814
5	10.0	506±470	5340± 4285
ms	Ca^{2+} (mM)	*N*	nm^2
–	0	512±461	2499± 452
10	10	289±260	8692± 2203
27	10	180± 81	16201±11560
130	10	173±115	33782± 7240

obtained similar results with liposomes composed of phospholipids from a variety of sources, some synthetic, mixed to reconstitute the stoichiometry of the chromaffin granule membrane *.

It then became of interest to examine the behavior of liposomes formed of similar mixtures of phospholipids but with particular components omitted, in order to determine whether any one type of phospholipid was essential to the fusion process. In particular, it was of interest to omit lysophosphatidylcholine from the mixture, since it has been suggested that lysophosphatidylcholine may be fusogenic [17], and lysophosphatidylcholine is present in substantial amounts in chromaffin granule membranes.

Liposomes formed from such a mixture also exhibited fusion, as illustrated in Fig. 2f, which shows a twin vesicle, bounded by a single membrane. A series of such experiments showed that both the time dependence and ionic dependence of fusion of liposomes composed of the complete chromaffin granule membrane mixture but with lysophosphatidylcholine omitted was similar to those of liposomes of pure PS.

Thus the results of experiments using lipid mixtures are in many respects identical to those using PS, with the one major difference being the appearance of flattened structures along with twin vesicles in PS experiments where no chelator is used subsequent to calcium addition. Although this suggests that flattening follows sequentially

* While this paper was in review, an abstract appeared reporting that fusion on a time scale of seconds has been demonstrated, using an alternative fast-freezing technique, with large vesicles of phosphatidylserine-phosphatidylethanolamine mixtures and of cardiolipin-phosphatidylcholine mixtures [16].

from fusion the time resolution of our system was insufficient to positively identify this sequence. Therefore, alternatively flattening might result from an independent pathway of liposome-calcium interaction paralleling the fusion process. In contrast, when mixtures of phospholipids were used, fusion was the exclusive form of interaction, which is in agreement with the observations of Düzgünes et al. [18]. They reported an increase in liposome stability (reduced leakiness) when mixtures of phospholipids were used instead of pure PS.

In summary we report here that true membrane fusion does occur when vesicles of pure PS are mixed with calcium, and further that this is an extremely rapid event which is detectable within 10 ms of initial mixing. The amount of fusion in such brief intervals was limited by the low concentration of liposomes used in these experiments (higher concentrations could not be used for technical reasons). Considering the low frequency of vesicle-vesicle collisions in such low concentration suspensions, and the mean vesicle-vesicle distance, it is probable that membrane fusion events are rapid enough to permit exocytosis to account for neurotransmitter release, a matter which has been questioned [19]. The comparison between this in vitro model and actual neurotransmitter release is made more favorable in this regard by the close contacts between synaptic vesicles and the release sites (active zones) in nerve terminals.

Thus liposome-liposome fusion, which has been extensively used as a model for exocytosis, matches the behavior of actual exocytosis in some respects (notably the time course and the morphology of vesicle-vesicle fusion, which closely mimics the appearance of 'complex exocytosis' in non-neural exocytotically-secreting cells), but fails to match that behavior in some other aspects (especially the ionic dependence: cellular exocytosis requires Ca^{2+} specifically and in concentrations of 10^{-4} M or lower; the model fuses with either Ca^{2+} or Mg^{2+} and requires concentrations of either ion above 1 mM and is not maximal until the concentrations approach or exceed 10 mM). Calcium specificity and sensitivity to low Ca^{2+} concentrations (10^{-6} M), which are characteristic of biological fusion processes such as exocytosis, could well be provided by special proteins. This was originally proposed and experimentally documented for fusion of isolated secretory vesicles [1,20,21], and now has been extended to artificial model systems [22–24].

References

1 Dahl, G., Ekerdt, R. and Gratzl, M. (1979) Symp. Soc. Exp. Biol. 33, 349–368
2 Papahadjopoulos, D., Poste, G. and Vail, W.J. (1979) Methods Membrane Biol. 10, 1–119
3 Papahadjopoulos, D., Poste, G., Schaeffer, B.E. and Vail, W.J. (1974) Biochim. Biophys. Acta 352, 10–28
4 Papahadjopoulos, D., Vail, W.J., Jacobson, K. and Poste, G. (1975) Biochim. Biophys. Acta 394, 483–491
5 Papahadjopoulos, D., Vail, W.J., Newton, C., Nir, S., Jacobson, K., Poste, G. and Lazo, R. (1977) Biochim. Biophys. Acta 465, 579–598
6 Portis, A., Newton, C., Pangborn, W. and Papahadjopoulos, D. (1979) Biochemistry 18, 780–790
7 Wilschut, J. and Papahadjopoulos, D. (1979) Nature 281, 690–692
8 Wilschut, J., Düzgünes, N., Fraley, R. and Papahadjopoulos, D. (1980) Biochemistry 19, 6011–6021
9 Wilschut, J., Düzgünes, N. and Papahadjopoulos, D. (1981) Biochemistry 20, 3126–3133
10 Ginsberg, L. (1978) Nature 275, 758–760
11 Ingolian, T.D. and Koshland, D.L. (1978) J. Biol. Chem. 253, 3821–3829
12 Holz, R.W. and Stratford, C.A. (1979) J. Membrane Biol. 46, 331–358
13 Kendall, D.A. and MacDonald, R.C. (1982) Biophys. J. 37, 25a
14 Szoka, F.C. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4195–4198
15 Ekerdt, R., Dahl, G. and Gratzl, M. (1981) Biochim. Biophys. Acta 646, 10–22
16 Düzgünes, N., Bearer, E. and Papahadjopoulos, D. (1982) Biophys. J. 37, 25a
17 Lucy, J.A. (1970) Nature 227, 815–817
18 Düzgünes, N., Wilschut, J., Fraley, R. and Papahadjopoulos, D. (1981) Biochim. Biophys. Acta 642, 182–195
19 Marchbanks, R.M. (1979) Symp. Soc. Exp. Biol. 33, 251–256
20 Gratzl, M. and Dahl, G. (1976) J. Cell Biol. 70, 241a
21 Dahl, G., Gratzl, M. and Ekerdt, R. (1977) Proc. Int. Union Physiol. Sci. 12, 131
22 Cohen, F.S., Zimmerberg, J. and Finkelstein, A. (1980) J. Gen Physiol. 75, 251–270
23 Zimmerberg, J., Cohen, F.S. and Finkelstein, A. (1980) Science 210, 906–908
24 Hong, K., Düzgünes, N. and Papahadjopoulos, D. (1981) J. Biol. Chem. 256, 3641–3644