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PROTON-INDUCED MEMBRANE FUSION ROLE OF PHOSPHOLIPID COMPOSITION AND PROTEIN-MEDIATED INTERMEMBRANE CONTACT

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Glycolipid-phospholipid vesicles containing phosphatidate and phosphatidylethanolamine were found to undergo proton-induced fusion upon acidification of the suspending medium from pH 7.4 to pH 6.5 or lower, as determined by an assay for lipid intermixing based on fluorescence resonance energy transfer. Lectin-mediated contact between the vesicles was required for fusion. Incorporation of phosphatidylcholine in the vesicles inhibited proton-induced fusion. Vesicles in which phosphatidate was replaced by phosphatidylserine underwent fusion only when pH was reduced below 4.5, while no significant fusion occurred (pH $\geqslant$ 3.5) when the anionic phospholipid was phosphatidylinositol. It is suggested that partial protonation of the polar headgroup of phosphatidate and phosphatidylserine, respectively, causes a sufficient reduction in the polarity and hydration of the vesicle surface to trigger fusion at sites of intermembrane contact.

Introduction

Membrane fusion constitutes a crucial step in cellular activities such as secretion by exocytosis, endocytosis and the intracellular transport of macromolecules. These various activities are most likely controlled by distinct mechanisms and it is reasonable to assume that also the fusion step involved in each case is mediated by more than one mechanism [1]. Thus, the membrane fusion step in exocytosis appears to depend on – and may be triggered by – calcium ions [2], while in other cases, it does not. For example, recent evidence indicate that during endocytosis, the internalized plasma membrane first reaches a nonlysosomal, intracellular compartment (the endosome), which has to be acidified to allow for a recycling of certain membrane components [3]. The latter suggests that acidification of the endosome may be critical to at least some of the membrane fusion events involved in the recycling of plasma membrane components following endocytosis. Studies on the infection of mammalian cells by certain enveloped viruses, such as Semliki Forest and influenza viruses, have also shown that acidification can trigger fusion between viral membrane and either a phospholipid vesicle or a cellular membrane [4,5]. In these latter cases, there is evidence that the fusion event may also directly involve peptide segments of one or more viral membrane proteins.

Studies on Ca^{2+}-induced fusion of phospholipid vesicles have led to the conclusion that membrane fusion requires both a close intermembrane

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Abbreviations: *N*-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; *N*-Rh-PE, *N*-(lissamine-Rhodamine B-sulfonyl)dioleoylphosphatidylethanolamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PELBA, phosphatidylethanol-*N*-lactobionamide; Calcein, bis[*N*,*N*′-bis(carboxymethyl)aminomethyl]fluorescein.

contact and an interference with the hydrated surfaces of the contacting membranes such that the energy barrier towards membrane intermixing is reduced below a certain limit [6–10]. Studies on the effective, hydrated size of phospholipid headgroups in bilayer vesicles by the use of steric probes have verified that headgroup hydration is reduced significantly by Ca^{2+} in the cases where vesicle fusion can be induced by this ion [11]. In the latter study, it was also found that partial protonation of the phosphate headgroup of phosphatidate at acidic pH resulted in a similar reduction of the effective size of this headgroup as did the binding of Ca^{2+}. Therefore, according to the concept above, acidification might be sufficient to trigger the fusion of membranes containing phosphatidate.

We have now tested this possibility using glycolipid-phospholipid vesicles, where intermembrane contact can be established by a lectin (*Ricinus communis* agglutinin) within a wide pH range [11]. Fusion has been followed by monitoring resonance-energy-transfer between two fluorescent phospholipid derivatives initially present in separate, but otherwise identical, vesicle populations as described by Hoekstra [8].

Materials and Methods

Phospholipids. Phosphatidylethanolamine was isolated from egg yolk [12]. Phosphatidylcholine isolated from egg yolk [13] and pure soybean phosphatidylcholine obtained from Lucas Meyer (Hamburg) were used interchangeably. Phosphatidylinositol was isolated from a crude soybean phospholipid preparation (Epicuron 510, Lucas Meyer) by chloroform extraction followed by chromatography on CM-cellulose [14]. Phosphatidate and phosphatidylserine were prepared from soybean phosphatidylcholine by treatment with cabbage phospholipase D (Boehringer, Mannheim) alone or in the presence of L-serine, respectively [14], and were then isolated by column chromatography on CM-cellulose. In some experiments, a pure phosphatidylserine purchased from Le Farm, (Cologne) was used. *N*-Rh-PE was purchased from Avanti Polar Lipids (Birmingham, AL). *N*-NBD-PE was prepared essentially as described [15].

Other chemicals. Ricinus communis agglutinin was purchased from Boehringer (Mannheim). Calcein (fluorexon) was purchased from EGA-CHEMIE (Steinheim) and was purified on Sephadex LH-20 [16]. Phosphatidylethanol-*N*-lactobionamide (PELBA) was prepared as described earlier [11].

Preparation of vesicles. Large unilamellar phospholipid vesicles were prepared by reverse-phase evaporation [17,18]. The vesicles were extruded through a 0.2 μm Uni-Pore polycarbonate membrane purchased from Bio-Rad Laboratories (Richmond, CA). All vesicle preparations contained 10 mol% PELBA and in the case of the resonance energy transfer assay, 5 mol% of either *N*-NBD-PE or *N*-Rh-PE. The remaining phospholipid content of the vesicles was varied as described in Table I.

Vesicle fusion. The resonance energy transfer assay for membrane vesicle fusion [19] was used as described [8]; the donor-acceptor pair of the resonance energy transfer being *N*-NBD-PE and *N*-Rh-PE. In the assays, vesicles containing 60 nmol of lipid were added to 2 ml of a buffer containing 0.15 M NaCl/20 mM Hepes/1 mM EDTA (pH 7.5). Half of the vesicles contained the energy donor (*N*-NBD-PE) and the other half the energy acceptor (*N*-Rh-PE). The sample was transferred to a cuvette equipped with a magnetic stirrer and was thermostated at 30°C. An Aminco-Bowman spectrophotofluorimeter was used to determine excitation and emission spectra and to measure fluorescence quenching due to resonance energy transfer. The fluorescence was recorded continuously (excitation/emission at 470/532 nm) and the values of relative fluorescence quenching were obtained as $Q_i = (F_0 - F_i)/F_0$ expressed as percentage. Here, F_i = fluorescence after i min and Q_i = relative quenching after i min.

Assessment of vesicle leakage. Calcein was encapsulated in vesicles at a self-quenching concentration (20 mM calcein/0.1 M NaCl/20 mM Hepes/1 mM EDTA, pH 7.5). Vesicles were separated from nonencapsulated calcein on a column of Sephadex G-75 equilibrated in 0.15 M NaCl/20 mM Hepes/1 mM EDTA (pH 7.5). Upon leakage from the vesicles, the calcein fluorescence became dequenched by dilution in the buffer and consequently a fluorescence increase was obtained. This

method has previously been used extensively with carboxyfluorescein [6,20]. Since calcein was found to retain a low passive permeability also at acidic pH, it was more useful for determination of proton-induced release of vesicle content. However, at pH values below 5.5, the fluorescence intensity of calcein gradually decreased. Therefore, in assays of proton-induced leakage, pH was returned to 7.4 after 5 min and the fluorescence was recorded. In each assay, 60 nmol calcein-containing vesicles were used with 10 mol% PELBA, but without the fluorophores in their lipid composition. Experimental conditions were the same as those used in the fusion assay, except that calcein fluorescence was measured at excitation and emission wavelengths of 492 and 510 nm, respectively. In order to determine the fluorescence of the total vesicle content, 50 μl of a 10% (w/v) solution of Triton X-100 was added to disrupt the vesicles. The relative leakage was calculated by using $L_i = (F_i - F_0)/(F_t - F_0)$, expressed as percentage, where L_i = relative leakage after i min, F_i = fluorescence after i min, F_t = fluorescence after disrupting the vesicles.

Use of lectin to establish intervesicle contact. Since the glycolipid PELBA was included at 10 mol% in all the vesicles, they could be brought in contact by means of the *R. communis* agglutinin, as described earlier [11,21]. To achieve this, 30 μl of a stock solution of the lectin (4 mg/ml) was added. After this addition, the vesicles were kept in the cuvette for 1 min before the fusogenic agent was added, and relative quenching or leakage was recorded.

Results

Proton-induced fusion between phospholipid vesicles was monitored by measuring resonance energy transfer between fluorophores initially incorporated in separate populations of vesicles. In addition, the release of vesicle content was determined in parallel experiments to get an estimate of the leakage accompanying membrane fusion. The fluorescence of both *N*-NBD-PE and *N*-Rh-PE was found to be essentially unaffected by the presence of lectin and by changes in pH in the range 3.5–7.4. The variation in fluorescence under these conditions did not exceed 10% of the original value (see also Fig. 1). In most experiments, changes of pH to predetermined values were accomplished by injection of a 1 M or 0.3 M solution of HCl into the final sample, under stirring. In order to improve the buffering capacity in the pH range studied, formic acid or citric acid was used instead of HCl in some experiments. Although these acids sometimes induced a slightly lower degree of resonance energy transfer than 1 M HCl, when added to the same final pH, the difference was small (see below).

Tests of proton-induced fusion and leakage were made with vesicle preparations differing in lipid composition (Table I). It was soon evident that without the use of lectin to establish intervesicle contact, no significant resonance energy transfer or leakage could be induced by acidification (pH $\geq$ 4) in any of the preparations. Adjustment of pH to more alkaline values did not induce any response in resonance energy transfer, neither in the

TABLE I

THE LIPID COMPOSITION OF VESICLE PREPARATIONS

PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidate; PI, phosphatidylinositol; PS, phosphatidylserine; fluorophore is *N*-NBD-PE or *N*-Rh-PE (see Materials and Methods). Figures within parentheses are given as mol% of total vesicle lipid mixture.

In resonance energy transfer assay	In leakage assay
PE-PA-PELBA-fluorophore (55-30-10-5)	PE-PA-PELBA (60-30-10)
PE-PC-PA-PELBA-fluorophore (35-20-30-10-5)	PE-PC-PA-PELBA (40-20-30-10)
PE-PC-PA-PELBA-fluorophore (20-35-30-10-5)	
PI-PELBA-fluorophore (85-10-5)	PI-PELBA (90-10)
PE-PI-PELBA-fluorophore (55-30-10-5)	PE-PI-PELBA (60-30-10)
PS-PELBA-fluorophore (85-10-5)	PS-PELBA (90-10)
PE-PS-PELBA-fluorophore (55-30-10-5)	PE-PS-PELBA (60-30-10)
PC-PELBA-fluorophore (85-10-5)	PC-PELBA (90-10)

absence nor the presence of lectin. The resonance energy transfer and leakage characteristics in response to acidification was, as expected, found to be determined by the acidic phospholipid species present in the vesicles. When vesicles containing 85 mol% of phosphatidylcholine were used, no significant proton-induced resonance energy transfer could be recorded, whether lectin was present or not (not shown).

Vesicles containing phosphatidate

Vesicles containing phosphatidate in mixture with phosphatidylethanolamine, with or without 20 mol% phosphatidylcholine, both showed a significant resonance energy transfer upon addition of acid even at final pH values around 6.5 (Fig. 1). The pH dependence for the two vesicle types show similarities, but the vesicles lacking phosphatidylcholine responded more extensively to small decreases of pH. The pH dependence for the vesicles containing phosphatidylcholine in the mixture suggest a two-step process with a levelling off around pH 6. The resonance energy transfer response was only slightly larger when 1 M rather than 0.3 M HCl was used for acidification, and results identical to the latter ones were obtained with citric or formic acid (not shown). Undoubtedly, the proton-induced resonance energy transfer observed with these two vesicle preparations was completely dependent on the presence of lectin (Fig. 1). When the phosphatidylcholine content of the vesicles was increased to 35 mol%, at the expense of phosphatidylethanolamine, significant proton-induced resonance energy transfer was observed only at pH values below 5 (not shown).

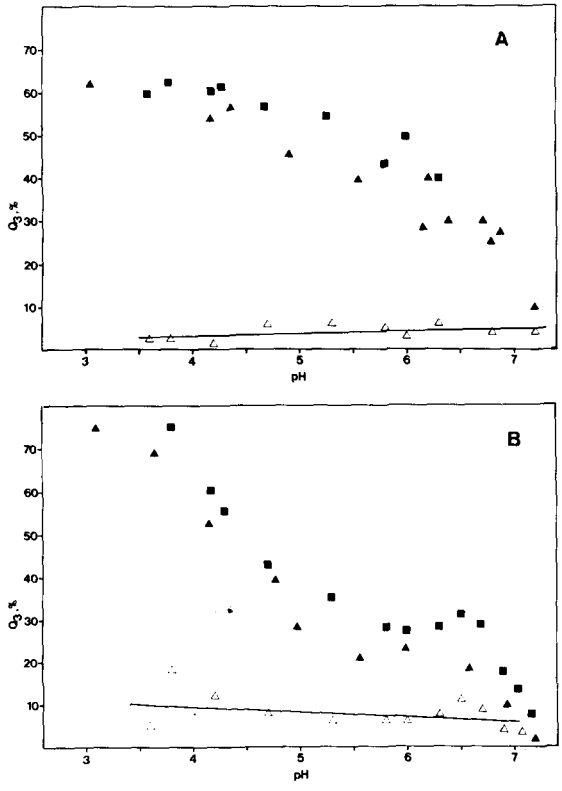

Fig. 1. Proton-induced resonance energy transfer between lipid vesicles containing phosphatidate/phosphatidylethanolamine (A) or phosphatidate/phosphatidylethanolamine and 20 mol% phosphatidylcholine (B) as a function of the final buffer pH. The total lipid composition of the vesicles is given in Table I. Q_3 = (initial fluorescence)−(fluorescence 3 min after acidification)/initial fluorescence. Adjustment of pH was made either in the absence of lectin (△) or 1 min after the addition of *R. communis* agglutinin (60 μg/ml) using 0.3 M (▲) or 1 M (■) HCl. Data from 2 or 3 different vesicle preparations are included in each figure.

As seen in Fig. 2, the proton-induced resonance energy transfer was accompanied by release of vesicle content in both types of phosphatidate-containing vesicles. In addition, the kinetics of quenching and leakage in one of the vesicle preparations at a final pH of 6.0 are presented in Fig. 3. The resonance energy transfer precedes, but is

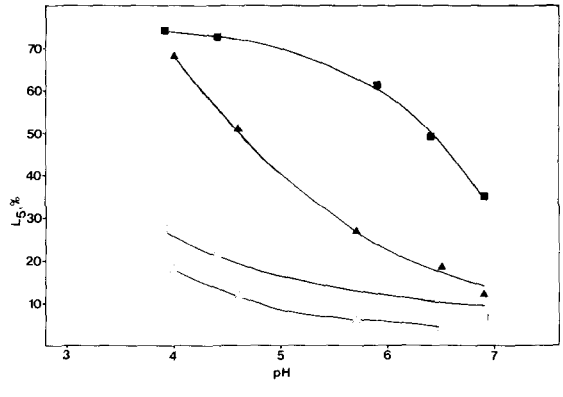

Fig. 2. Proton-induced leakage from phosphatidate/phosphatidylethanolamine (■, □) and phosphatidate/phosphatidylethanolamine/phosphatidylcholine (▲, △) vesicles in the presence (filled symbols) or absence (open symbols) of lectin. A 1 M solution of HCl was used for acidification. L_5 = (fluorescence 5 min after acidification)−(initial fluorescence)/(fluorescence after disrupting vesicles)−(initial fluorescence).

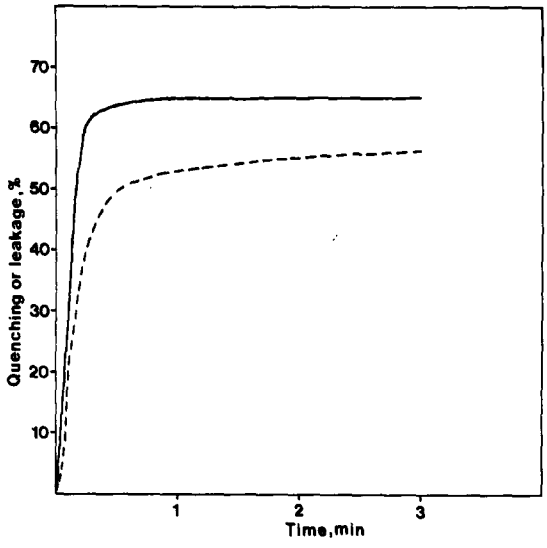

Fig. 3. Kinetics of resonance energy transfer (solid line) and leakage (broken line) for phosphatidate/phosphatidylethanolamine vesicles after adjustment of pH to 6.0 by the addition of 1 M HCl. The lectin *R. communis* agglutinin (60 μg/ml) was added 1 min before the adjustment of pH.

rapidly followed by, release of vesicle content. However, when comparing relative quenching and leakage, the fact that the quenching values do not include fusion between vesicles with the same type of fluorophore must be taken into account. Therefore, the quenching values underestimate the extent of fusion. In addition, our relative quenching values have not been corrected for the fluorescence remaining after complete intermixing and, therefore, underestimates further the extent of fusion *.

Vesicles containing phosphatidylserine or phosphatidylinositol

When the vesicles contained 85 mol% phosphatidylserine (not shown) or 30 mol% phosphatidylserine in mixture with phosphatidylethanolamine (Fig. 4A) only a very limited resonance energy transfer was seen until the final pH reached below 4.5. Below pH 4.5, however, there was a steep increase in the degree of resonance energy transfer and levels comparable to those for phosphatidate-containing vesicles were obtained

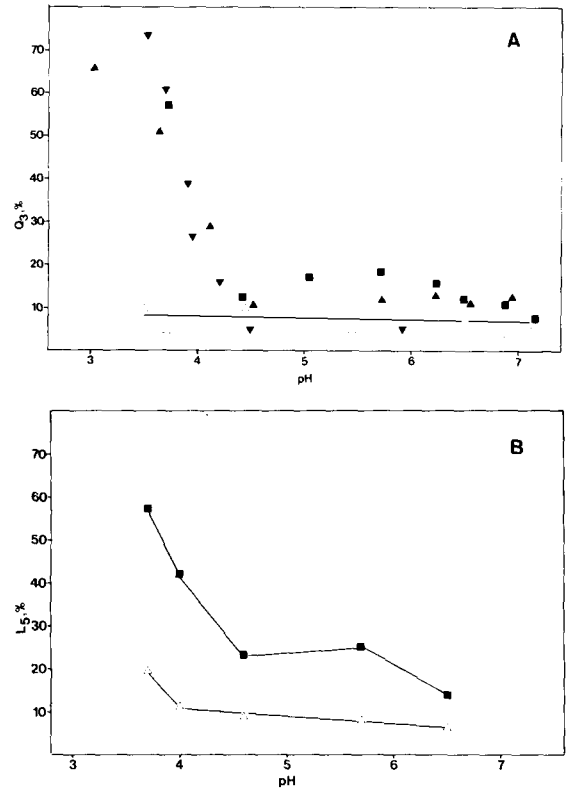

Fig. 4. (A) Proton-induced resonance energy transfer between phosphatidylserine/phosphatidylethanolamine vesicles as a function of the final buffer pH. Q_3 is defined as in Fig. 1. Adjustment of pH was made in the absence (open symbols) or presence (filled symbols) of *R. communis* agglutinin. In the latter case, either 0.3 M HCl (▲), 1 M HCl (■) or 1 M citric acid (▼) was used. Data from two separate experiments are included. (B) Proton-induced leakage from phosphatidylserine/phosphatidylethanolamine vesicles in the presence (■) and absence (△) of lectin as a function of the final buffer pH. L_5 is defined as in Fig. 2.

below pH 4.0. The proton-induced leakage of vesicle content from the phosphatidylserine-containing vesicles showed a similar dependence on the final pH as the resonance energy transfer response (Fig. 4B).

Vesicles containing phosphatidylinositol with or without phosphatidylethanolamine were resistant to proton-induced resonance energy transfer until pH reached below 5 and even in the pH range 3.5–5.0, only a very limited (less than 25%) resonance energy transfer was observed (not shown). In agreement with this, the acidification to pH 3.5–6.5 induced hardly any release of entrapped

* We find a residual, relative fluorescence of 10–13% in premixed vesicles containing both fluorophores, in agreement with results in Ref. 8.

calcein whether the vesicles were brought in contact or not.

Discussion

The results presented here show that fusion of membrane vesicles containing phosphatidate and a relatively high proportion of phosphatidylethanolamine can be induced by a very moderate increase in proton concentration, i.e., by a change in pH from 7.4 to approx. 6.5, provided that the vesicles are brought in contact by independent means.

Although fusion, in the resonance energy transfer assay used here, is equated to lipid intermixing, a solid ground for doing so has been established by the studies on Ca^{2+}-induced fusion in which assays for the intermixing of membrane lipids and entrapped vesicle contents have been compared [22,23]. Our initial experiments, using the terbium (Tb)-dipicolinate assay for vesicle fusion [24] have also indicated that the proton-induced fusion described here is indeed accompanied by intermixing of vesicle contents (not shown). However, proton-induced fusion is quite rapidly followed by leakage (see Fig. 3) and dipicolinic acid is not well retained by the vesicles at acidic pH, so the Tb-dipicolinate assay does not provide quantitive results in this case.

It is interesting to note that the phospholipid composition of the vesicles (to the extent investigated) appears to modulate proton-induced fusion in a way similar to that previously found for Ca^{2+}-induced fusion [6,7,9,10]. Thus, phosphatidate is much more fusogenic than phosphatidylserine, which is in turn more fusogenic than phosphatidylinositol. Also, in both types of fusion, phosphatidylcholine is strongly inhibitory, in contrast to phosphatidylethanolamine. These similarities support the conclusion that headgroup dehydration plays a critical role in both Ca^{2+}- and proton-induced fusion. At pH 7.4, only part of the phosphatidate carries two negative charges and one would therefore expect that its protonation increases even upon a moderate acidification. Since there are several previous indications that the effective size of the monovalent form of the phosphatidate headgroup is reduced compared to that of the divalent form [11,25,26], a proton-induced dehydration is a likely mechanism for the fusion we observed. In agreement with this, proton-induced fusion of phosphatidylserine vesicles was observed below pH 4.5, i.e., in the pH range in which protonation of the serine carboxyl group is known to occur [27]. It is also of interest to note that the pH dependence for proton-induced phase separation of phosphatidate or phosphatidylserine from mixtures with phosphatidylcholine [28] agrees very well with that for proton-induced fusion, reported here.

Morphological evidence that phosphatidate- and phosphatidylserine-containing small vesicles undergo proton-induced fusion at pH 3.0 was recently presented [29]. Our results extend this finding in several ways and also show that fusion can be induced at higher pH, i.e., in the range of pH (4.5–7.5) which is encountered in mammalian cells. In addition, under the conditions used in the present study, acidification alone was insufficient to trigger fusion and an independent means of establishing contact between the vesicles was required.

With respect to the possible physiological role of proton-induced membrane fusion, it must be pointed out that phosphatidate is normally a minor component of cellular membranes. However, increased amounts can be found after exposure to certain stimuli (see Ref. 10) and intracellular membrane vesicles generated during endocytosis have been shown to be acidified (to a pH of 4.5–5) shortly after their formation [30]. If phosphatidate is present in, or becomes generated in, such membranes, one could envision that fusion processes involved in the pinching off of vesicles during retrieval of endocytozed plasma membrane components might be triggered by the acidification of the vesicular content.

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