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Phosphatidylethanol counteracts calcium-induced membrane fusion but promotes proton-induced fusion

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The susceptibility of phosphatidylethanol-containing lipid vesicles towards Ca^{2+}- and proton-induced fusion has been investigated, using a system of interacting vesicles. The results show that phosphatidylethanol-rich vesicles are quite resistant to Ca^{2+}-induced fusion while being highly sensitive to proton-induced fusion. Inclusion of phosphatidylethanol was also found to promote and inhibit, respectively, the proton-induced and Ca^{2+}-induced fusion of bilayer vesicles containing also phosphatidylethanolamine and either phosphatidylserine or phosphatidic acid. Thus, phosphatidylethanol affected Ca^{2+}- and proton-induced fusion in opposite directions, in contrast to the naturally occurring anionic phospholipids phosphatidic acid, phosphatidylserine and phosphatidylinositol, which affect the sensitivity to Ca^{2+}- and H^+-induced fusion in the same direction. However, the fusion competence of phosphatidylethanol vesicles in response to both Ca^{2+} and H^+ was inversely related to the apparent thickness of the polar headgroup layer, determined by using lectin-glycolipid interaction as a steric probe, as previously found for vesicles containing naturally occurring anionic phospholipids.

Introduction

Phosphatidylethanol constitutes an interesting model for some of the naturally occurring anionic phospholipids by virtue of its simple phospho-alkyl headgroup [1]. In addition, recent evidence suggests that phosphatidylethanol might form in various organs of the rat after ethanol treatment [2,3]. The properties of bilayer vesicles containing phosphatidylethanol have now been investigated with respect to susceptibility towards Ca^{2+}- and proton-induced membrane fusion. Established procedures for determination of vesicle lipid intermixing [4], vesicle leakage [5], and the intermixing of vesicle contents [6] have been employed. In addition, lectin-glycolipid interaction has been used as a steric probe [7] to determine changes in the effective size of the phospholipid headgroup upon changes in calcium ion or proton concentration. The results are compared with those previously reported for the naturally occurring anionic phospholipids phosphatidic acid, phosphatidylserine and phosphatidylinositol [8–12].

Abbreviations: *N*-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; *N*-Rho-PE, *N*-(lissamine-Rhodamine B-sulfonyl)dioleoylphosphatidylethanolamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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Materials and Methods

Chemicals

Phosphatidylethanol and phosphatidic acid

were prepared from soybean phosphatidylcholine by treatment with cabbage phospholipase D (Boehringer, Mannheim, F.R.G.) in the presence or absence of ethanol [13], and were then isolated by column chromatography on Silica gel 60 or CM-cellulose, respectively. Pure phosphatidylethanolamine and phosphatidylserine were purchased from Le Farm (Cologne, F.R.G.). The latter was converted into its sodium salt by two-phase partitioning of a chloroform solution, first against methanol/0.1 M Na_2EDTA (1 : 1 by volume) and then against methanol/0.15 M NaCl (1 : 1 by vol.). *N*-(lissamine-Rhodamine B-sulfonyl)dioleoylphosphatidylethanolamine (*N*-Rho-PE) was purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.), and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (*N*-NBD-PE) was prepared essentially as described [14]. Octadecenyllactobionamide and phosphatidylethanol-*N*-lactobionamide were prepared as earlier described [7]. *Ricinus communis* agglutinin (mol.wt. 120000) and *Arachis hypogaea* (peanut) agglutinin were purchased from Boehringer (Mannheim, F.R.G.).

Preparation of lipid vesicles

Large unilamellar phospholipid vesicles were prepared by reverse-phase evaporation followed by extrusion through a 0.2 μm Uni-pore (Nucleopore Corp.) polycarbonate membrane [15,16]. Small unilamellar vesicles were prepared by sonication of 3 μmol of the appropriate lipids in 1 ml of 0.15 NaCl/20 mM Hepes (pH 7.5), as previously described [17].

Determination of vesicle lipid intermixing

An assay based on fluorescence resonance energy transfer [4] was used to determine vesicle lipid intermixing as described [11], the fluorescent donor-acceptor pair being *N*-NBD-PE and *N*-Rho-PE (each at 5 mol%). Under these conditions the former fluorophore experiences a certain degree of self-quenching. However, it was noted that rates of resonance energy transfer under conditions of fusion were the same when the vesicles instead contained 1 mol% of the NBD-fluorophores, and thus were largely free from self-quenching. In the assay, 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). When Ca^{2+}-induced fusion was monitored, the buffer contained no EDTA. Half of the vesicles contained the energy donor (*N*-NBD-PE) and the other half the energy acceptor (*N*-Rho-PE). The sample was transferred to a cuvette equipped with an adjustable magnetic stirrer and was thermostated at 30 °C. An Aminco-Bowman spectrophotofluorimeter was used to measure fluorescence quenching due to resonance energy transfer. The fluorescence (excitation/emission at 470/532 nm) was recorded continuously, and values of relative fluorescence quenching were calculated as $Q_i = (F_0 - F_i)/F_0$ expressed as a percentage. Here, F_i = fluorescence after i min and Q_i = relative quenching after i min. Since phosphatidylethanol-*N*-lactobionamide was included at 10 mol% in vesicle preparations, vesicles could be brought in contact by preincubation with *Ricinus communis* agglutinin or peanut agglutinin (60 μg/ml) before the addition of calcium ions or protons [7,18].

Assessment of intermixing of vesicle contents and vesicle leakage

An assay based on the formation of highly fluorescent Tb-dipicolinic acid complexes upon vesicle fusion was used to determine the extent of intermixing of vesicle contents [6]. The experimental conditions were the same as previously described [9]. In some experiments the retention of the Tb-dipicolinic acid signal that developed in response to lectin followed by H^+ was assessed by readjustment of pH (using 0.1 M NaOH) and disruption of lectin-dependent intervesicle bridges (using methyl-β-D-galactopyranoside).

Calcein was encapsulated in vesicles at a self-quenching concentration (29 mM). The vesicles were freed of nonencapsulated calcein on a column of Sephadex G-75 (Pharmacia). Upon leakage from the vesicles, its fluorescence became dequenched by dilution in the buffer. The experimental conditions used in this assay have been described recently [12].

Gel chromatography

In order to assess the extent of increase in vesicle size during fusion, gel chromatography was performed using a 1 × 60 cm column of Sephacryl

S-1000 (Pharmacia) as in Ref. 19. A suspension of small unilamellar vesicles (0.5 ml, 0.6 μmol lipid/ml), labelled with a trace amount of [^{3}H]phosphatidylcholine, was applied to the column, and fractions of 1 ml were collected with a flow rate of 0.2 ml/min. Acidification was achieved using 0.3 M HCl and reversed using 0.1 M NaOH. When the vesicles were preincubated with one of the lectins, using 50 μl of a 4 mg/ml lectin solution, 50 μl of a 0.5 M solution of methyl-β-D-galactopyranoside were added to break intervesicle bonds due to lectin before chromatography. The vesicle recovery on the column varied from 60 to 80%, compared to control preparations.

Lectin-glycolipid interaction as a steric probe

Lectin-glycolipid interaction, assessed by an agglutination assay [7], was used to probe changes in the head-group layer of phosphatidylethanol vesicles upon changes in proton or calcium concentration. Small unilamellar vesicles (0.2 μmol lipid) containing 80 mol% phosphatidylethanol and octadecenyl-lactobionamide or, in control phosphatidylethanol-*N*lactobionamide (20 mol%) were incubated with varying amounts of *Ricinus communis* agglutinin for 45 min. Agglutinated vesicles were then separated by centrifugation as described earlier [7].

Results

Vesicles containing only phosphatidylethanol or mixtures with phosphatidylethanolamine

A pronounced fluorescence resonance energy transfer was induced by even a small decrease of pH, when the phospholipid vesicles contained 85, 50 or 25 mol% phosphatidylethanol in mixture with phosphatidylethanolamine and derivatives (for lipid compositions, see Table I). This proton-induced response requires lectin-mediated intervesicle contact, and was induced equally well when either *Ricinus communis* agglutinin or peanut agglutinin was used. The lipid intermixing decreased with an increasing ratio of phosphatidylethanolamine to phosphatidylethanol in the vesicles (Fig. 1). The proton-induced leakage of encapsulated calcein was rather extensive (not shown), in agreement with previous observations [12]. Experiments

TABLE I

LIPID COMPOSITION OF VESICLE PREPARATIONS USED IN THE ASSAY FOR LIPID INTERMIXING

PE, phosphatidylethanolamine; PS, phosphatidylserine; PELBA, phosphatidylethanol-*N*-lactobionamide; PA, phosphatidic acid; PEt, phosphatidylethanol. Figures within parentheses represent mol% of the total vesicle lipid mixture.

PEt/PELBA/fluorophore	(85:10:5)
PEt/PE/PELBA/fluorophore	(50:35:10:5)
PEt/PE/PELBA/fluorophore	(25:60:10:5)
PEt/PE/PA/PELBA/fluorophore	(50:5:30:10:5)
PEt/PE/PA/PELBA/fluorophore	(25:30:30:10:5)
PEt/PE/PS/PELBA/fluorophore	(50:5:30:10:5)
PEt/PE/PS/PELBA/fluorophore	(25:30:30:10:5)

using the Tb-dipicolinic acid assay [6] showed that, under the same conditions, a significant degree of vesicle content intermixing occurred (Fig. 2A, B). The extent of content intermixing was about equal for vesicles containing 85 and 50 mol% of phosphatidylethanol (not shown). A large part of the fluorescence signal was retained upon readjustment of pH and administration of methyl-β-D-galactopyranoside to disrupt lectin-dependent intervesicle bonds (Fig. 2A, broken line). The in-

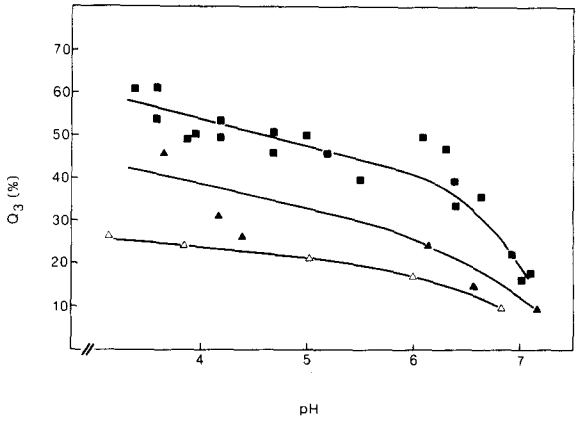


Fig. 1. Proton-induced lipid intermixing among lipid vesicle containing phosphatidylethanol (■), or mixtures of phosphatidylethanolamine and phosphatidylethanol in which phosphatidylethanol constituted 50 (▲), or 25 (△) mol% of the total vesicle lipids (see Table I). All assays contained at 60 μg/ml a lectin which was *Ricinus communis* agglutinin. Q_3 denotes ((initial fluorescence) − (fluorescence 3 min after acidification))/(initial fluorescence).

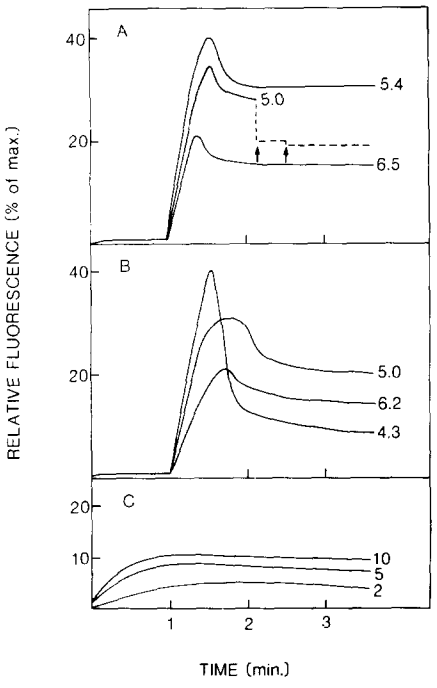


Fig. 2. Kinetics of proton-induced (A, B) and calcium-induced (C) intermixing of vesicle contents, using vesicles containing 50 mol% phosphatidylethanol, 40 mol% phosphatidylethanolamine and 10 mol% phosphatidylethanol-*N*-lactobionamide. In A and B, the figures denote the final pH after acidification at 1 min; in A, *Ricinus communis* agglutinin was used to agglutinate the vesicles and in B, peanut agglutinin was used. In C the figures denote the final Ca^{2+} concentrations (mM) after Ca^{2+} addition at time 0. The dotted line in A denotes pH readjustment (left-hand arrow) followed by addition of methyl-β-D-galactopyranoside (right-hand arrow).

termixing of vesicle contents was induced equally well by H^+ in the presence of either *Ricinus communis* agglutinin or peanut agglutinin (Fig. 2). Despite the clear assessment of vesicular lipid and contents intermixing, any significant increase in vesicle size could not be detected upon gel chromatography on Sephacryl S-1000, after reversal of the fusing and aggregating conditions (see Materials and Methods). We do not know the reason for this apparent discrepancy, but the recovery of vesicles from the column was incomplete and a selective loss of larger vesicles can not be excluded. Another possibility is that some reorganisation of vesicles into approximately the initial size may take place subsequent to content intermixing, as appears to be the case in peptide-mediated fusion between vesicles containing phosphatidic acid and phosphatidylethanolamine [20].

In contrast to the large response to low pH, only a low degree (approx. 10%) of Ca^{2+}-induced lipid intermixing (Fig. 3) or vesicle leakage (not shown) could be detected using up to 10 mM Ca^{2+}. In the presence of *Ricinus communis* agglutinin a certain increase in resonance energy transfer occurred (up to 25%) but only when the vesicles contained the lowest proportion (25 mol%) of phosphatidylethanol.

Vesicles containing phosphatidylethanol in mixtures with phosphatidylethanolamine and phosphatidic acid or phosphatidylserine

Vesicles containing 30 mol% phosphatidic acid in mixture with phosphatidylethanolamine and the glycolipid phosphatidylethanol-*N*-lactobionamide are highly susceptible to Ca^{2+}-induced fusion, even more so when the vesicles are brought in contact by the lectin *Ricinus communis* agglutinin [18]. When part of the phosphatidylethanolamine in such vesicles was exchanged for phosphatidylethanol, The Ca^{2+}-induced fusion, manifested as lipid intermixing, decreased (Fig. 4A). In vesicles containing 25 mol% phosphatidylethanol, the decrease was restricted to the lower range of Ca^{2+}-concentrations, but in vesicles containing 50% phosphatidylethanol the decrease was seen at all Ca^{2+}-concentrations tested; it was especially

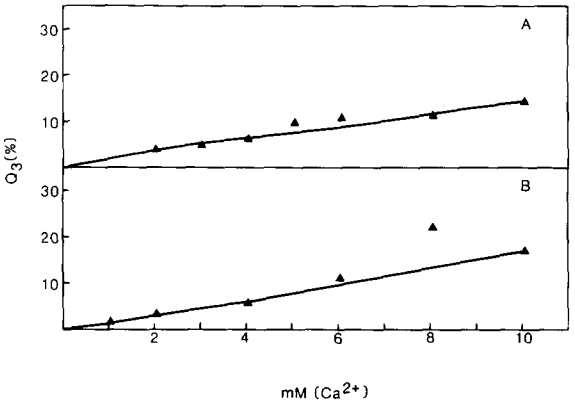


Fig. 3. Ca^{2+}-induced lipid intermixing among vesicles containing low phosphatidylethanol (A) or phosphatidylethanolamine and phosphatidylethanol (75:25) (B). No lectin was present.

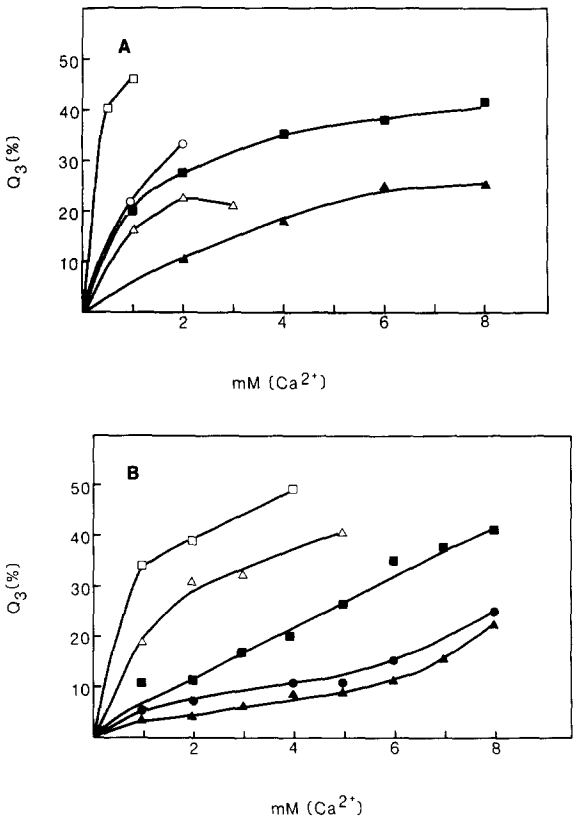


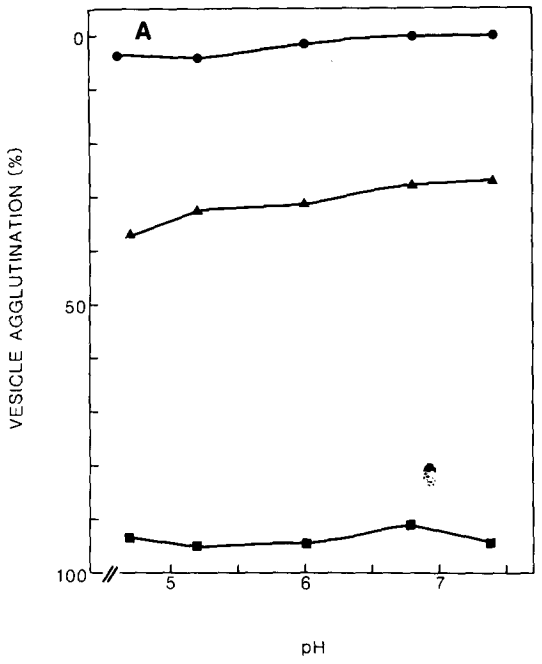
Fig. 4. Ca^{2+} induced lipid intermixing in the absence (closed symbols) or presence (open symbols) of *Ricinus communis* agglutinin (60 μg/ml), among vesicles containing phosphatidylethanolamine and 30 mol% of phosphatidic acid (A) or phosphatidylserine (B). In these mixtures 0% (■, □), 25% (●, ○), or 50% (▲, △) of the phosphatidylethanolamine had been exchanged for phosphatidylethanol.

marked in the presence of *Ricinus communis* agglutinin. Proton-induced fusion was extensive in these mixed vesicles, as would be expected from the susceptibility of both phosphatidylethanolamine-phosphatidic acid vesicles [12] and phosphatidylethanol vesicles (Fig. 1) towards proton-induced fusion.

Vesicles containing phosphatidylserine, phosphatidylethanolamine and derivatives (Table I) underwent moderate Ca^{2+}-induced fusion, but with an inclusion of only 25 mol% phosphatidylethanol in exchange for phosphatidylethanolamine it was inhibited (Fig. 4B); no additional changes occurred when the phosphatidylethanol proportion was increased to 50 mol%. It is noteworthy that the lectin-dependent enhancement of Ca^{2+}-induced fusion was more pronounced in phosphatidylethanolamine-phosphatidylserine vesicles than in vesicles containing only phosphatidylserine (and glycolipid). Proton-induced fusion, which is minimal at final pH values above 4.5 in the case of phosphatidylserine-phosphatidylethanolamine vesicles [12], increased gradually when the vesicles contained increasing amounts of phosphatidylethanol (not shown). The results were essentially the same whether small or large unilamellar vesicles were used. Also, results obtained by the use of the Tb-dipicolinic acid assay for vesicle content intermixing were consistent with those from the resonance energy transfer assay of lipid intermixing with regard to the limited response to Ca^{2+} (not shown).

Lectin – vesicle interaction as a steric probe

As previously reported [7] lectin–glycolipid interaction can, under certain conditions, be used as a steric probe for changes in the effective size of surrounding phospholipid head groups, provided that the lectin-binding group is located within the headgroup layer. Octadecenyl-lactobionamide, containing a four-member spacer arm, is a glycolipid which fulfills this requirement, while phosphatidylethanol-*N*-lactobionamide (with a ten-member arm), extends beyond the phospholipid head group layer. The lack of steric effects when phosphatidylethanol-*N*-lactobionamide, is used is illustrated in Fig. 5A, where small unilamellar vesicles containing phosphatidylethanol (80%) and this glycolipid are shown to be readily agglutinated, irrespective of pH. When phosphatidylethanol-*N*-lactobionamide was replaced by octadecenyl-lactobionamide (fig. 5B) agglutination at neutral pH was weaker, but it was enhanced to the level seen with phosphatidylethanol-*N*-lactobionamide upon lowering of pH. This indicates that the steric inhibition of lectin binding to octadecenyl-lactobionamide at neutral pH gradually disappears upon acidification and no longer occurs at, or below, pH 5. On the other hand, the lectin-induced agglutination of octadecenyl-lactobionamide-containing vesicles was only weakly enhanced by Ca^{2+} (Fig. 6). This is consistent with the low degree of lipid intermixing and fusion induced by this ion (see above).



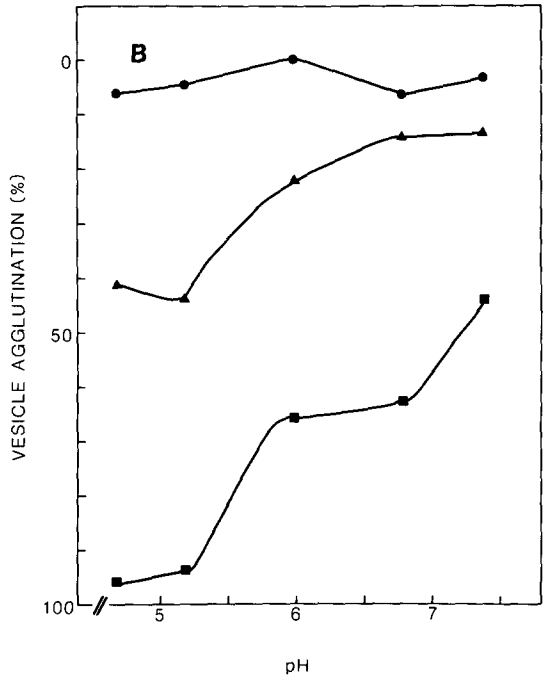


Fig. 5. Modulation by pH of lectin binding to, and agglutination of, vesicles containing phosphatidylethanol and 20 mol% phosphatidylethanol-*N*-lactobionamide (A) or octadecenyl-lactobionamide (B). Agglutination assay mixtures contained either 0 (●), 40 (▲), or 120 (■) μg/ml of the lectin *Ricinus communis* agglutinin.

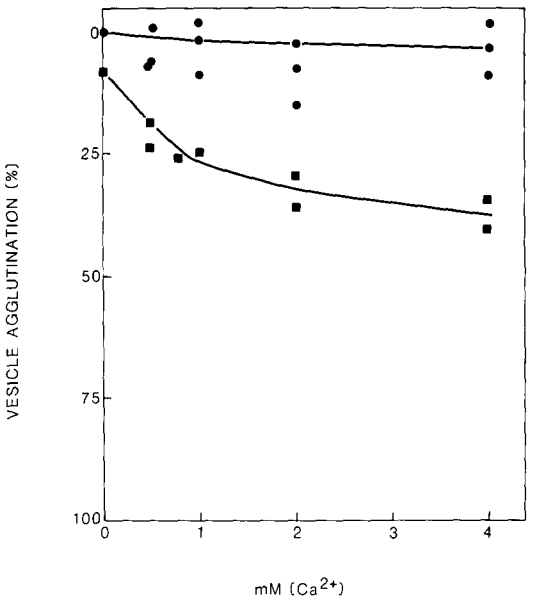


Fig. 6. Ca^{2+} modulation of the lectin-induced agglutination of vesicles containing phosphatidylethanol and 20 mol% octadecenyl-lactobionamide, using either 0 (●), or 40 (■) μg/ml of *Ricinus communis* agglutinin.

Discussion

The results presented here indicate that phosphatidylethanol vesicles are quite resistant to Ca^{2+}-induced fusion and that this lipid acts as an inhibitor of Ca^{2+}-induced fusion when other negatively charged phospholipids are present in the membrane. On the other hand, it strongly promotes a lectin-dependent, proton-induced fusion and predominates over phosphatidylserine, which in itself does not trigger pH-dependent fusion until pH 4.5 [12]. The results obtained by using lectin-glycolipid interaction as a steric probe indicate that the previous concept, that a decrease in the hydration shell of lipid vesicles is necessary for fusion [21,22], applies also in the case of Ca^{2+}- or proton-induced fusion of phosphatidylethanol-containing vesicles.

The proton-induced membrane fusion between phosphatidylethanol-containing vesicles is accompanied by vesicle content intermixing, but any significant increase in vesicle size has so far not been documented. A lack of size increase in the fusion product could be related to the finding that

the size of phosphatidic acid vesicles decreases when pH is increased [23,24] if we assume that phosphatidylethanol vesicles have similar characteristics, since pH is readjusted to 7.4 before gel chromatography in our experiments. Furthermore, vesicles containing phosphatidic acid and phosphatidylethanolamine showed no or minimal increase in size after polyhistidine-mediated, proton-induced fusion, as assessed by electron microscopy [20]. This is also the case for phosphatidylethanol-containing vesicles.

Vesicles containing phosphatidic acid, phosphatidylserine, phosphatidylglycerol or phosphatidylinositol differ greatly in susceptibility towards Ca^{2+}-induced fusion [8,9,25]. While phosphatidylinositol vesicles are completely resistant, those containing phosphatidylglycerol start to fuse at approximately 10 mM Ca^{2+}, a somewhat higher concentration than that needed for phosphatidylethanol vesicles. This could reflect the difference in size and polarity between the headgroups.

A previous study strongly suggested that phospholipid headgroup protonation was the trigger for proton-induced fusion among vesicles containing phosphatidic acid or phosphatidylserine [12]. However, this explanation may not be applicable in the case of phosphatidylethanol, since this phospholipid does not undergo protonation in the pH range investigated [1,26]. One possibility that can not be excluded is that the lectin protein plays a role in the fusion step by direct interaction with the vesicle bilayer, although one would not expect then that fusion would be supported equally well by two different lectins. Another possible explanation would be a proton-induced conformational change in the phosphoethanol headgroup, leading to a reduction in its polarity. Such a conformation change could require both acidification and a close intermembrane contact [21].

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References

1 Browning, J.L. (1981) Biochemistry 20, 7123–7133
2 Alling, C., Gustavsson, L., Månsson, J.-E., Benthin, G. and Änggård, E. (1984) Biochim. Biophys. Acta 793, 119–122
3 Benthin, G., Änggård, E., Gustavsson, L. and Alling, C. (1985) Biochim. Biophys. Acta 835, 385–389
4 Struck, D.K., Hoekstra, D. and Pagano, R.E. (1981) Biochemistry 20, 4093–4099
5 Straubinger, L.M., Hong, K., Friend, D.S. and Papahadjopoulos, D. (1983) Cell 32, 1069–1079
6 Wilschut, J. and Papahadjopoulos, D. (1979) Nature 281, 690–692
7 Sundler, R. (1984) Biochim. Biophys. Acta 771, 59–67
8 Wilschut, J., Duzgunes, N. and Papahadjopoulos, D. (1981) Biochemistry 20, 3126–3133
9 Sundler, R. and Papahadjopoulos, D. (1981) Biochim. Biophys Acta 649, 743–750
10 Nir, S., Bentz, J., Wilschut, J. and Duzgunes, N. (1983) Prog. Surface Sci. 13, 1–124
11 Hoekstra, D. (1982) Biochemistry 21, 2833–2840
12 Bondeson, J., Wijkander, J. and Sundler, R. (1984) Biochim. Biophys. Acta 777, 21–27
13 Comfurius, P. and Zwaal, F.A. (1977) Biochim. Biophys. Acta 488, 36–42
14 Monti, J.A., Christian, S.T. and Shaw, W.A. (1978) J. Lipid Res. 19, 222–228
15 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194–4198
16 Duzgunes, N., Wilschut, J., Hong, K., Fraley, R., Perry, C., Friend, D.S., James, T.L. and Papahadjopoulos, D. (1983) Biochim. Biophys. Acta 732, 289–299
17 Sundler, R., Alberts, A.W. and Vagelos, P.R. (1978) J. Biol. Chem. 253, 4175–4179
18 Sundler, R. and Wijkander, J. (1983) Biochim. Biophys. Acta 730, 391–394
19 Nozaki, Y., Lasic, D.D., Tanford, C. and Reynolds, J.A. (1982) Science 217, 366–367
20 Wang, C.-Y. and Huang, L. (1984) Biochemistry 23, 4409–4416
21 Rand, R.P. (1981) Annu. Rev. Biophys. Bioeng. 10, 277–314.
22 Sundler, R., Duzgunes, N. and Papahadjopoulos, D. (1981) Biochim. Biophys. Acta 649, 751–758
23 Hauser, H., Gains, N. and Müller, M. (1983) Biochemistry 22, 4775–4781
24 Gains, N. and Hauser, H. (1983) Biochim. Biophys. Acta 731, 31–39
25 Rosenberg, J., Duzgunes, N. and Kayalar, C. (1983) Biochim. Biophys. Acta 735, 173–180
26 Träuble, H. and Eibl, H. (1975) in Functional Linkage in Biomolecular Systems, (Schmitt, F.O., Schneider, D.M. and Crother, D.M., eds.), pp. 59–101, Raven Press, New York