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STUDIES ON THE EFFECTIVE SIZE OF PHOSPHOLIPID HEADGROUPS IN BILAYER VESICLES USING LECTIN-GLYCOLIPID INTERACTION AS A STERIC PROBE

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An experimental approach is described which provides information about the relative, effective size of phospholipid headgroups in bilayer vesicles. It is based on determination of the binding of lectins (*Ricinus communis* agglutinin or concanavalin A) to synthetic glycolipids inserted in such vesicles, using a vesicle agglutination assay. It is shown that the ability of a glycolipid containing a shorter (4-member) spacer arm to bind the appropriate lectin is highly sensitive to the headgroup structure of the surrounding phospholipid in mixed glycolipid-phospholipid vesicles. Furthermore, when the phospholipid was phosphatidate a change in protonation or in monovalent counter-ion species (Li^+, NH_4^+, $N(CH_3)_4^+$ or Na^+) significantly influenced lectin binding. The interference with lectin binding described above was reduced when the glycolipid spacer arm was extended from a 4- to a 6-member length. Furthermore, the sensitivity to phospholipid headgroup structure or to changes in the ionic environment was completely eliminated when the glycolipid contained a longer (10- or 12-member) spacer arm between the hydrophobic part and the lectin-binding group. It is concluded that the modulation of lectin binding in the former case is due to steric inhibition determined by the effective (hydrated) size of the various phospholipid headgroups.

Introduction

There are well-known differences in physicochemical properties among naturally occurring phospholipids which differ in polar headgroup structure; e.g. in their gel-liquid crystalline phase transition temperature and regarding their ability to form lamellar or non-lamellar structures in water [1,2]. Differences in the polarity and hydration of the lipid headgroups may play a significant role in these respects. Furthermore, the ability of anionic phospholipid vesicles to undergo Ca^{2+}-induced fusion has been found to vary dramatically with headgroup structure in a manner that does not correlated with the charge properties of the vesicles [3,4]. It was instead suggested that the degree of dehydration of the constituent phospholipid headgroups upon binding of Ca^{2+} may determine whether fusion occurs [4].

The present work was undertaken to gain independent information on the hydrated size of phospholipid headgroups in bilayer vesicles and on the modulation of this parameter by the ionic environment. To this end an experimental technique has been developed in which lectin binding to glycolipids inserted in phospholipid vesicles is used as a steric probe. It is based on the previous observation that lectin binding to glycolipids in a phospholipid bilayer occurs only if the carbohydrate

Abbreviations:

OMBA,	octadecenyl-maltobionamide
OLBA,	octadecenyl-lactobionamide
OMELBA,	octadecenyl-melibiobionamide
PEMBA,	phosphatidylethanol-*N*-maltobionamide
PELBA,	phosphatidylethanol-*N*-lactobionamide
PE-MELBA,	phosphatidylethanol-*N*-melibiobionamide
Hepes,	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

groups extend beyond the phospholipid head group layer of the vesicles [5–7]. In a recent report [8] we provided initial evidence that by placement of the lectin-binding group sufficiently close to the headgroup layer, lectin binding becomes sterically regulated by the surrounding phospholipid headgroups. This approach has now been further developed and validated using another lectin (*Ricinus communis* agglutinin) and several different glycolipids.

Material and Methods

Concanavalin A was obtained from Serva Biochemicals (Heidelberg) and *Ricinus communis* agglutinin (mol. wt. 120 000) from Boehringer (Mannheim).

Phospholipids

Pure soybean phosphatidylcholine was obtained from Lucas Meyer (Hamburg). Where indicated, phosphatidylcholine from egg yolk [9] or synthetic dimyristoylphosphatidylcholine (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), purchased from Medmark (Munich), was used. Phosphatidylethanolamine was isolated from egg yolk [10] and dimyristoylphosphatidylethanolamine (1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine) was from Medmark (Munich). Phosphatidylinositol was isolated from a crude soybean phospholipid preparation (Epikuron 510, Lucas Meyer) by chloroform extraction followed by chromatography on CM-cellulose [11]. Extensive elution of the column (8–10 bed vol.) with chloroform-methanol (70 : 30, by vol.) before elution of phosphatidylinositol with chloroform/methanol (60 : 40, by vol.) was found necessary for complete separation of the latter from phosphatidate. Phosphatidate, phosphatidylglycerol and phosphatidylserine were prepared from soybean phosphatidylcholine by treatment with cabbage phospholipase D (Boehringer, Mannheim) alone or in the presence of glycerol or of L-serine, respectively [11], and were then isolated by column chromatography on CM-cellulose.

Phosphatidyl[*Me*-^{3}H]choline was prepared from phosphatidyl-*N*,*N*-dimethylethanolamine [12] and [*Me*-^{3}H]methyliodide according to Stoffel et al. [13] and was isolated by column chromatography on silic acid.

All phospholipids used gave a single spot upon two-dimensional thin-layer chromatography; solvent I: chloroform/methanol/28% ammonia (65 : 35 : 8, by vol.); solvent II: *n*-butanol/acetic acid/water (60 : 20 : 20, by vol.). They were stored in sealed ampoules in chloroform solution at −30°C under argon.

Synthetic glycolipids

Octadecenyl-maltobionamide (OMBA), octadecenyl-lactobionamid (OLBA) and the corresponding octadecyl compounds as well as octadecenyl-melibiobionamide (OMELBA) were synthesized according to Williams et al. [14]. The octadecyl compound were isolated by preparative thin-layer chromatography on silica gel G; solvent: chloroform/methanol/4 M ammonia (65 : 35 : 5, by vol.) while the octadecenyl glycolipids were isolated by column chromatography on silicic acid using chloroform/methanol mixtures saturated with 28% aqueous ammonia as solvent. For quantitative determination of these glycolipids the long chain amine was liberated by acid hydrolysis in 2 M HCl at 100°C for 2 h. It was then determined as the trinitrophenyl-derivative, after reaction with 2,4,6-trinitrobenzene sulfonate (TNBS) and two-phase partitioning. The isolated compounds were free from alkylamine as shown by the absence of TNBS-reactive material in unhydrolyzed samples and by thin-layer chromatography.

Phosphatidylethanol-*N*-maltobionamide (PEMBA), -lactobionamide (PELBA) and -melibiobionamide (PE-MELBA) were prepared by a modification of the procedure referred to above [14]. To a solution of 100 mg of phosphatidylethanolamine in 10 ml of chloroform/methanol (1 : 1, v.v) was added 20 µl of triethylamine and then, slowly under continuous stirring at room temperature, a 5–10-fold molar excess of lactonized malto- (or lacto- or melibio-) bionic acid in methanol. After 4 h the reaction was terminated by two-phase partitioning through the addition of chloroform and 0.2 M KH_2PO_4. The lower phase was washed with methanol/water (1 : 1, v/v) taken to dryness and dissolved in chloroform. Thin-layer chromatography showed in each case extensive (over 80% by phosphorous analysis) conversion of phosphatidylethanolamine to a single, slower migrating, ninhydrin-negative compound. The

product of the reaction with maltobionic acid migrated slightly ahead of that derived from lactobionic acid. A similar difference was noted between OMBA and OLBA. The glycophospholipids were isolated by column chromatography on silicic acid (using chloroform-methanol mixtures as eluent) and were shown: (a) to contain phosphorous, (b) to have the same fatty acid composition as the original phosphatidylethanolamine, (c) to release ethanolamine upon acid hydrolysis (6 M HCl, 100°C for 24 h) and (d) to bind concanavalin A (PEMBA) or *Ricinus communis* agglutinin (PELBA and PE-MELBA) with the expected carbohydrate specificity when incorporated in phospholipid vesicles (see Results). A sample of PELBA was also shown to contain equimolar amounts of phosphorous and galactose (within 5%).

Lipid vesicles

Appropriate lipids (6 or 10 μmol), including [^{3}H]phosphatidylcholine (5–10 nmol) as a radiolabeled tracer, were mixed in chloroform and taken to dryness. Sonicated vesicles were prepared in 0.1 M NaCl/20 mM Hepes (pH 7.2) as previously described [15]. Large unilamellar vesicles were prepared by reversed-phase evaporation followed by extrusion through Unipore filters of 0.2 and 0.1 μm pore size (Bio-Rad Laboratories) [16].

Lectin-induced agglutination

In the standard assay lipid vesicles (0.2 μmol lipid), containing 20 mol% glycolipid, were incubated with the appropriate lectin and additions as indicated in 1 ml of 0.1 M NaCl/20 mM Hepes (pH 7.2) at 20°C. After 45 min of incubation the mixture was centrifuged at 4500 rpm for 15 min in a fixed-angle rotor. The radioactivity of the whole incubation mixture and of the supernatant after centrifugation was determined by liquid scintillation counting.

In some experiments agglutination was also followed by continuous registration of the turbidity at 500 nm, using a Cary 210 spectrophotometer (Varian Instruments).

Results and Discussion

Characteristics of the agglutination assay

Previous studies on the agglutination of lipid vesicles by lectins have usually focused on the initial rate of agglutination, which has been determined by turbidimetry [5–7,17]. However, in the present study vesicle agglutination was to be used as a measure of the equilibrium binding of lectin. Under these conditions turbidity measurements on vesicles differing in lipid composition did not provide quantitative results. Therefore, a simple and reproducible quantitative assay, involving sedimentation of agglutinated vesicles by low-speed centrifugation, was developed.

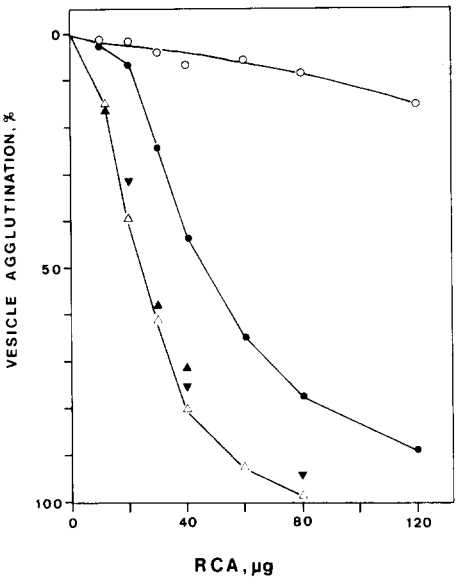

Fig. 1. Effect of surface density of glycolipid (PELBA) on the agglutination of PELBA/phosphatidylcholine vesicles by *Ricinus communis* agglutinin (RCA). The vesicles contained either 5 (○), 10 (●), 15 (▼), 20 (△) or 30 (▲) mol% PELBA.

Sonicated vesicles containing PELBA in mixture with phosphatidylcholine were used to establish basic experimental conditions. Vesicles containing between 15 and 30 mol% of the glycolipid were equally well agglutinated by *Ricinus communis* agglutinin, with 25 ± 2 (mean ± range) μg of lectin required for half-maximum agglutination (Fig. 1). This means that lectin binding to at most 1.5% of the glycolipid exposed on vesicles containing 20 mol% glycolipid (corresponding to four lectin molecules per vesicle) was sufficient for half-maximal agglutination. Despite that, agglutination was markedly inhibited when the glycolipid content was reduced below 10–15 mol% (Fig. 1). In order to minimize influences from

variation in surface density of glycolipid, which could arise because of the different surface area occupied by the phospholipids used, 20 mol% glycolipid was incorporated in the vesicles in all subsequent experiments. The agglutination induced by *Ricinus communis* agglutinin was in all cases carbohydrate specific since it was completely prevented, or reversed, by methyl-β-D-galactoside but unaffected by methyl-α-D-glucoside and did not occur when PEMBA (which contains a terminal α-D-glucose residue) was substituted for PELBA.

The time-course of lectin-induced agglutination of vesicles has in several cases been followed both by turbidity measurements and by using the sedimentation assay described above. Agglutination induced by *Ricinus communis* agglutinin occurred rapidly and approached equilibrium in 5–15 min depending on the lipid composition of the vesicles. No further agglutination occurred when the time of incubation was extended from 45 min to 3 h. Concanavalin A-induced agglutination occurred somewhat slower, but apparent equilibrium was always reached within 45 min. In most of the experiments described, galactolipids and *Ricinus communis* agglutinin were used rather than glucolipids and concanavalin A. The latter lectin requires bound divalent cations and may exist as dimer or tetramer depending on pH [18] and these properties could, potentially, have influenced the results in some of the experiments.

TABLE I

AGGLUTINATION OF GLYCOLIPID (PELBA)/PHOSPHOLIPID VESICLES BY *RICINUS COMMUNIS* AGGLUTININ. LACK OF SENSITIVITY TO PHOSPHOLIPID HEADGROUP STRUCTURE

Vesicles containing 20% PELBA and phospholipid as indicated were incubated with varying amounts of *Ricinus communis* agglutinin (RCA) in buffer containing 0.1 mM EDTA. Agglutination was determined by centrifugation (see Methods). Values are mean and range for three separate preparations.

Phospholipid	RCA, C_{50} [a] (μg/ml).
Phosphatidylcholine	25 ± 2
Phosphatidylethanolamine	26 ± 3
Phosphatidylinositol	23 ± 2
Phosphatidylserine	28 ± 4
Phosphatidate	24 ± 3

[a] Lectin concentration for agglutination of 50% of the vesicles.

TABLE II

EFFECT OF pH, IONIC STRENGTH AND Ca^{2+} ON THE AGGLUTINATION OF PELBA/PHOSPHOLIPID (20:80) VESICLES INDUCED BY *RICINUS COMMUNIS* AGGLUTININ

Values are means from two separate experiments. RCA, *Ricinus communis* agglutinin. C_{50} is defined as in Table I.

Phospholipid	pH	NaCl (M)	LiCl (M)	Ca^{2+} (mM)	RCA, C_{50} (μg/ml)
Phosphatidylcholine	7.2	0.1	–	1.0	27
	7.2	0.6	–	–	28
Phosphatidylinositol	7.2	0.1	–	1.0	23
	5.4	0.1	–	–	36
	8.5	0.1	–	–	24
	7.2	0.2	–	–	22
	7.2	0.4	–	–	20
	7.2	–	0.1	–	23
	7.2	–	0.3	–	22

Agglutination of vesicles containing glycolipids with a 10-member spacer arm

As already shown (Fig. 1) phosphatidylcholine vesicles containing 20 mol% PELBA were well agglutinated by *Ricinus communis* agglutinin. When phosphatidylcholine in such vesicles was replaced by phosphatidylethanolamine or by any of several different anionic phospholipids agglutination was induced in a virtually identical manner (Table I). This agglutination was only minimally affected by changes in pH or ionic strength or by subaggregating concentrations of Ca^{2+} (Table II).

The influence of the various phospholipids and the presence of divalent cations on concanavalin A-induced agglutination of vesicles containing PEMBA (20 mol%) instead of PELBA was also investigated for comparison. Also here, agglutination was largely unaffected by the structure of the vesicle phospholipid and by the presence of Ca^{2+} or Mg^{2+} (not shown). These results indicate that the glycolipids PEMBA and PELBA had a spacer arm sufficiently long to make lectin binding insensitive to changes in the phospholipid headgroup region.

Glycolipids with a 4-member spacer arm in zwitterionic phospholipid vesicles

The above results contrast sharply with the sensitivity to changes in both phospholipid structure and the ionic environment found when glycolipids containing a shorter (4-member) spacer group were used. When OLBA was incorporated in phosphatidylcholine vesicles the vesicles could not be agglutinated by *Ricinus communis* agglutinin in agreement with earlier findings, using OMBA and concanavalin A [8,17]. This suggests that the binding of lectin was prevented by the phosphocholine head group, since agglutination was observed when part of the phosphatidylcholine was replaced by phosphatidylethanolamine (Fig. 2). As indicated in Fig. 2, both the binding of concanavalin A to OMBA and the binding of *Ricinus communis* agglutinin to OLBA was affected in a very similar way by changes in the proportions of phosphatidylcholine and phosphatidylethanolamine in the vesicles. Also, substitution of phosphatidylcholine from egg yolk or dimyristoylphosphatidylcholine (agglutination assayed at 30°C) for the soybean phosphatidylcholine did not change these results.

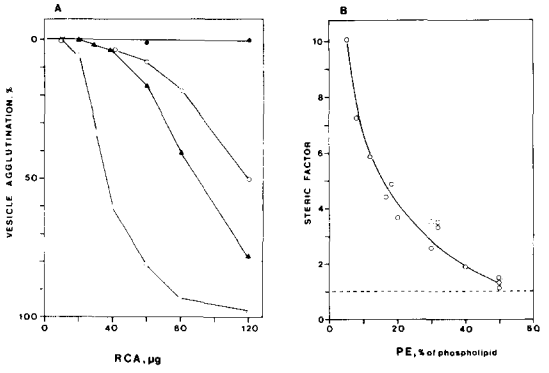

Fig. 2. Lectin-induced agglutination of zwitterionic lipid vesicles containing varying proportions of phosphatidylcholine and phosphatidylethanolamine (PE). (A) The vesicles contained 20 mol% glycolipid (OLBA) and either phosphatidylcholine alone (●) or phosphatidylcholine/phosphatidylethanolamine (65:15, ○), (55:25, ▲) or (40:40, △) RCA, *Ricinus communis* agglutinin. (B) Data from (A) and from Ref. 8 are included. The 'steric factor' is defined as the concentration of lectin required for half maximal agglutination of vesicles containing OLBA (or OMBA) divided by that required for vesicles containing the longer glycolipids PELBA (or PEMBA). Each point represents one vesicle preparation. The vesicles contained 20 mol% of either OLBA (○), or OMBA (△), as the glycolipid.

The phosphocholine headgroup is chemically more bulky than the phosphoethanolamine headgroup, but differences in the degree of hydration of the two phospholipids is considered more important in determining their different physical properties in excess water [2]. Rand and co-workers [19] have determined the repulsive force that develops between approaching phospholipid bilayers and attributed the steep increase occurring at bilayer separations of 15–30 Å to the water of hydration. The hydration 'shell', with a diffuse outer border, was found to be 10–20 Å thick for a phosphatidylcholine bilayer and 6–8 Å for phosphatidylethanolamine [20]. In the present system, the more hydrated phosphocholine headgroups would be expected to interfere more strongly with lectin binding and this was also found to be the case.

It is likely that the lectins had to penetrate into the outer, less tightly bound, part of the hydration 'shell' in order to bind to OLBA or OMBA since the carbohydrate group in these glycolipids would be localized only a few Ångström out from the head group surface. Alternatively, the glycolipid may have been somewhat displaced vertically, i.e. pulled out relative to the vesicle surface, upon lectin binding. In any case, the energy of interaction should be the same when the *Ricinus communis* agglutinin binds to OLBA in different phospholipid environments or to PELBA, since the lectin-binding group is the same in all cases.

Glycolipids with a 4-member spacer arm in vesicles of anionic phospholipids

We recently reported that concanavalin A-induced agglutination of vesicles containing OMBA and three different anionic phospholipids varied with head group structure and was modified by Ca^{2+} in a differential way [8]. Results quite analogous to those were obtained when *Ricinus communis* agglutinin and OLBA were used instead of concanavalin A and OMBA (not shown); the only exception being that a weak electrostatic contribution to the binding of *Ricinus communis* agglutinin to OLBA-phosphatidate vesicles (in the absence of Ca^{2+}) was noted. This was eliminated by increasing the concentration of NaCl to at least 0.2 M. It was also found that the enhancing effect of Mg^{2+} on *Ricinus communis* agglutinin-induced ag-

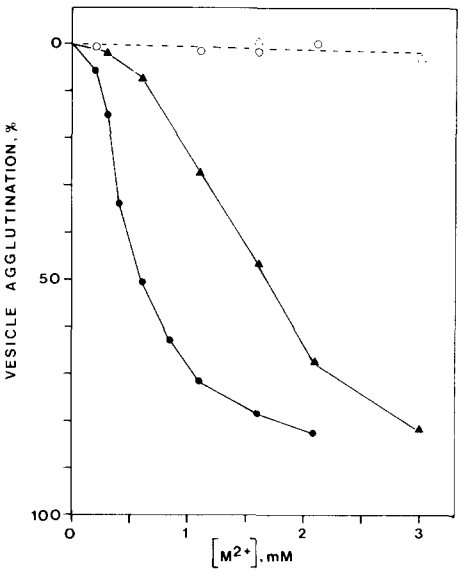

Fig. 3. The effects of Ca^{2+} and Mg^{2+} on lectin-induced agglutination of OLBA/phosphatidylinositol (20:80) vesicles. Ca^{2+}, ○, ●; Mg^{2+}, △, ▲. Open symbols: controls without lectin; filled symbols: *Ricinus communis* agglutinin added (40 μg/ml).

glutination was weaker than that of Ca^{2+} (Fig. 3). This further supports the proposal that divalent cations enhance lectin binding and thereby vesicle agglutination by causing dehydration (and size reduction) of the anionic headgroups [8], since Mg^{2+} is less effective in this respect than Ca^{2+}.

Using OLBA-phosphatidate vesicles the influence of pH and of some monovalent salts on the agglutination induced by *Ricinus communis* agglutinin have also been investigated. Phosphatidate is known to undergo protolysis with apparent pK_a at, or slightly below, neutral pH [21]. Below the pK_a (i.e. in the monovalent form) phosphatidate has a higher gel-liquid crystalline transition temperature than at, or above, the pK [22]. One reason for this could be that the singly charged headgroup is less hydrated (and therefore less bulky) than the doubly charged phosphate group. As shown in Fig. 4, the glycolipid-phosphatidate vesicles were more easily agglutinated at lower than at higher pH. This is consistent with a reduction in the (hydrated) size of the phosphatidate headgroup upon protonation since the interaction between glycolipid and *Ricinus communis* agglutinin per se remains essentially constant in this pH range (Table II).

It is also clear from Fig. 4 that replacement of NaCl (0.3 M) by LiCl significantly enhanced agglutination. Since the enhancement occurred irrespective of pH in the whole range investigated it appears unlikely that it was due to a displacement of the protolysis of phosphatidate towards more alkaline pH. A more likely explanation is that phosphatidate (Li^+) is less strongly hydrated than phosphatidate (Na^+). The fact that an increase in the concentration of LiCl to 0.5 M did not reduce the enhancement of agglutination further supports this conclusion. The lithium salt of phosphatidylserine has also been shown to pack significantly more tightly than the sodium salt [23], a finding consistent with a lower degree of hydration of the former salt form.

Substitution of ammonium or tetramethylammonium (TMA) ions for sodium ions has also been studied. In ammonium chloride, agglutination was enhanced to about the same degree as in LiCl while (TMA)Cl had no effect compared to NaCl (Fig. 5).

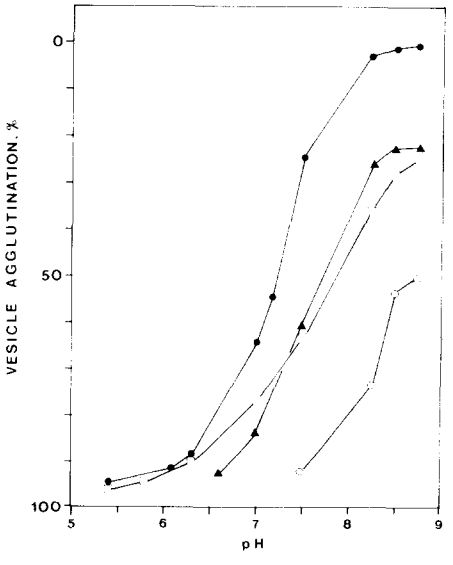

Fig. 4. Effect of pH on lectin-induced agglutination of OLBA/phosphatidate (20:80) vesicles. The buffer contained either 0.3 M NaCl (●, ▲) or 0.3 M LiCl (□, ○). *Ricinus communis* agglutinin was present at 25 (□), 40 (○, ●), or 80 (▲) μg per ml, respectively. Controls without lectin or with lectin and methyl-β-D-galactoside showed less then 5% agglutination.

Glycolipids with a 6-member spacer arm

If steric effects on lectin binding underlie the modulation of agglutination observed with vesicles containing OLBA and OMBA one would expect these effects to gradually weaken with increasing length of the glycolipid spacer arm. As already shown they are not detectable when a lipid containing a 10-member spacer arm (i.e., PELBA) is used. Because of the structural difference between OLBA and PELBA, it was considered important to investigate also a closer structural analog of OLBA differing in spacer arm length. OMELBA, and also PE-MELBA, were therefore used in some experiments. The latter, containing a 12-member spacer group, was used to assess the lectin requirement for agglutination since these glycolipids contain an α-D-galactose as lectin-binding group instead of the β-epimer. In agreement with the selectivity reported for *Ricinus communis* agglutinin [24] almost twice as much of the lectin (46 ± 4 μg) was required for half-maximal agglutination of PE-MELBA-containing vesicles compared to those containing PELBA, irrespective of the phospholipid composition of the vesicles.

The agglutination of phosphatidate and phosphatidylinositol vesicles containing OMELBA or OLBA, respectively, were then compared. As shown in Table III, phosphatidate vesicles containing OMELBA or PE-MELBA were agglutinated equally well, while OLBA-containing vesicles required more *Ricinus communis* agglutinin for half maximal agglutination than those containing PELBA. Thus, extension of the spacer arm length from 4 to 6 atoms virtually eliminated the weak interference with lectin binding by phosphatidate (Na^+). Our results with phosphatidylinositol vesicles also demonstrated a weakening of the (steric) inhibition. Thus, in OMELBA-containing vesicles a significantly lower concentration

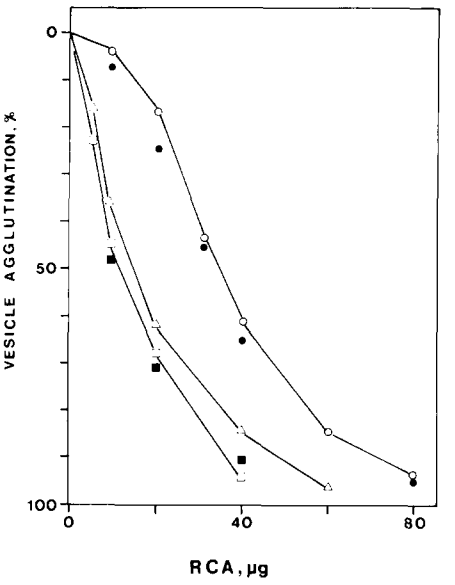

Fig. 5. Effect of monovalent counter-ion species on lectin-induced agglutination of phosphatidate vesicles containing OLBA (20 mol%). The buffer (pH 7.2) contained 0.3 M chloride salt of Na^+ (○), $(TMA)^+$ (●), NH_4^+ (△) or Li^+ (□), or 0.5 M LiCl (■). RCA, *Ricinus communis* agglutinin.

TABLE III

COMPARISON OF GLYCOLIPIDS CONTAINING A 4-MEMBER (OLBA) OR A 6-MEMBER SPACER ARM (OMELBA) IN PHOSPHATIDATE AND PHOSPHATIDYLINOSITOL VESICLES

All vesicle preparations contained 20 mol% glycolipid. In experiments with phosphatidate-containing vesicles, the monovalent salt concentration was 0.3 M, RCA, *Ricinus communis* agglutinin.

Phospholipid	Glycolipid	Monovalent salt	Ca^{2+} (mM)	RCA, C_{50} (μg/ml)	Steric factor [a]
Phosphatidate	OLBA	NaCl	–	36	1.5
	OLBA	LiCl	–	26	1.1
	OMELBA	NaCl	–	42	0.9
Phosphatidyl-inositol	OLBA	NaCl	0.6	44	1.8
	OLBA	NaCl	1.2	24	1.0
	OMELBA	NaCl	0.4	44	1.0

[a] Defined as C_{50} (OLBA)/C_{50} (PELBA) and C_{50} (OMELBA)/C_{50} (PE-MELBA), respectively.

of Ca^{2+} was required for half-maximal lectin-induced agglutination than when OLBA was used (Table III).

Effect of the size of glycolipid-phospholipid vesicles

So far, only experiments using sonicated vesicles have been described. Such vesicles have been well characterized and they were, due to their small size and insensitivity to osmotic gradients, well suited for these experiments. However, both variation in vesicle size and in the partitioning of individual lipid components between the inner and outer half of the vesicle bilayer could occur with changes in lipid composition. Certain key observations made with sonicated vesicles were therefore investigated using larger vesicles (approx. 0.1 μm diameter). These include: (A) the modulation of lectin-induced agglutination by the phosphatidylcholine/phosphatidylethanolamine ratio in zwitterionic vesicles containing OLBA; (B) the drastic enhancement of agglutination of anionic, OLBA-containing, vesicles by Ca^{2+} and (C) the absence of the above effects when PELBA, containing a longer spacer arm, was used instead of OLBA. Experiments using PELBA-containing large vesicles confirmed the last point and also showed that in this system a much smaller amount of *Ricinus communis* agglutinin in absolute terms, (4 ± 1 μg) was required for half-maximal agglutination as compared to the system of sonicated vesicles. This corresponds to a slightly higher amount of lectin per vesicle, if all vesicles in the former system have a diameter of 0.1 μm. However, also some smaller vesicles are generated by the reverse-phase procedure and the number of lectin molecules per vesicle, required for agglutination, therefore may remain essentially unchanged. Also in these, larger, vesicles lectin binding and vesicle agglutination was considerably enhanced by substitution of phosphatidylethanolamine for phosphatidylcholine. The 'steric factor' was reduced from far over 10 to 2.5 to 1.2 when 25 and 50% of the phosphatidylcholine was replaced by phosphatidylethanolamine, in agreement with the results for sonicated vesicles (cf. Fig. 2B). The enhancement of agglutination of anionic vesicles by divalent cations was also very similar in large as compared to small, sonicated vesicles (not shown).

Concluding remarks

The present study provides strong evidence that lectin-glycolipid interaction may be used as a steric probe, to help define the hydrated size of phospholipid headgroups in bilayer vesicles. Until recently [19,20] only limited information on the degree of hydration of phospholipids other than phosphatidylcholine has been available, although the potential importance of headgroup hydration, in stabilizing bilayer structures [1,19,25] and controlling membrane fusion [4,26,27] has become increasingly recognized. Our results with anionic phospholipids (Ref. 8 and the present study) clearly show that the lectin-glycolipid probe is sensitive not only to differences in chemical 'bulkiness' of the phospholipid headgroups but also to changes in their degree of hydration, induced by changes in the ionic environment. Further development of the present technique, to include direct measurements of the amount of lectin bound, should allow also an assessment in absolute terms of the vertical extension of phospholipid head groups.

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