Phospholipid, Glycolipid, and Ion Dependencies of Concanavalin A- and Ricinus communis Agglutinin I-induced Agglutination of Lipid Vesicles

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The recent availability of a series of model glycolipids (N-alkylaldobionamides) allowed the systematic investigation of glycolipid-specific agglutination of lipid vesicles by the lectins concanavalin A (Con A) and Ricinus communis agglutinin I (RCA). The carbohydrate-binding specificities of the lectins determined which glycolipid-containing vesicles were agglutinated and which sugars specifically inhibited vesicle agglutination. Con A agglutinated negatively charged a-D-glucosyl glycolipid-containing vesicles prepared from a mixture of phosphatidyicholine (PC) and the acid phospholipids, phosphatidylserine (PS), cardiolipin (CL), or phosphatic acid (PA). The agglutination was abolished not only by methyl a-D-mannopyranoside but also by elevated ionic strength. Con A did not agglutinate a-D-glucosyl glycolipid-containing neutral vesicles composed of phosphatidyicholine (PC) or a mixture of PC and phosphatidylethanolamine (PE). Con A-induced agglutination of glycolipid-containing vesicles may involve both nonelectrostatic, lectin-carbohydrate interaction, and electrostatic interaction between Con A and vesicles.

RCA induced both neutral and negatively charged vesicles containing a-D-galactosyl glycolipids. Although the binding of sugars and of pure glycolipid micelles by RCA is not Ca²⁺-dependent, agglutination of a-D-galactosyl glycolipid-containing PC-PS vesicles had an absolute requirements for Ca²⁺ or other divalent cations and could be reversed by adding either EDTA or methyl a-D-galactopyranoside.

Lectins are carbohydrate-binding proteins of nonimmune origin which do not exhibit enzymatic activity (1). They interact with glycoproteins and glycolipids on the cell surface and can induce a variety of effects which include cell agglutination, cell adhesion to surfaces, mitogenesis, and hormone-like action. The biological effects are of considerable interest, because of potential therapeutic applications, and because of similarities with a number of hormones and toxins which bind to carbohydrate receptor sites on membranes (2–6). Lectins also induce lipid vesicle agglutination, if vesicles contain glycolipids with carbohydrate moieties, to which a particular lectin specifically binds (7–12).

Recently, a new class of model glycolipids has been synthesized, containing a variety of carbohydrate and lipophilic moieties (13). The glycolipids are synthesized by condensation of an aldonic acid (disaccharide oxidized at the C-1 position of the reducing sugar) with a long chain alkylamine to yield an N-alkylaldobionamide (Fig. 1). We have incorporated several of these glycolipids into lipid vesicles and have investigated the ability of Con A and RCA to agglutinate the vesicles. Con A, isolated from Canavalia ensiformis, is a tetrameric protein with 4 carbohydrate-binding sites. It specifically binds a-D-mannosyl or a-D-glucosyl moieties (1). RCA is one of two agglutinins that has been isolated from Ricinus communis beans (14). It is a dimer and has two binding sites which interact with n-galactosyl moieties. Con A and RCA have the potential to cross-link and agglutinate vesicles which contain appropriate surface carbohydrates. In this study, we demonstrate not only carbohydrate specificity for Con A- and RCA-induced vesicle agglutination but, in addition, lipid and ion dependencies not predicted from lectin-sugar complexation in solution.

**EXPERIMENTAL PROCEDURES**

**Vesicle Preparation**—Phospholipids and glycolipids dissolved in CHCl₃:CH₃OH (2:1) were mixed in a glass centrifuge tube, and the solvent was removed by evaporation under a stream of N₂. Buffer containing 140 mM NaCl, 50 mM Hepes (pH 7.2) was added to yield a phospholipid concentration of 1 to 2 mg/ml. The lipids were suspended by brief vortexing, followed by sonication in a bath sonicator (Laboratory Supplies Co., Inc., Hicksville, N.Y.) until the optical density of the suspension at 500 nm (A₅₀₀) was 0.06 to 0.25. Sonication was accomplished in 5 to 15 min when negative charged phospholipids were present, and in 45 min when only neutral phospholipids were present. The suspending and sonicating steps were both done under N₂.

**Lectin-induced Agglutination**—Agglutination of vesicles was followed by measuring the increase in A₅₀₀ in a Beckman single beam spectrophotometer (model DUR) of 1 ml of vesicle-containing solution. The stability of the lectin-agglutinated complex was investigated by pelleting agglutinated vesicles in an Eppendorf 5412 centrifuge (12,000 × g for 15 min). The supernatant was removed and replaced with solution containing the same glycolipid concentration as in the removed supernatant but with no lectin. Vesicles were resuspended by trituration with a Pasteur pipette, and monitored at A₅₀₀.

**Equilibrium Dialysis**—The incorporation of C-10 malt into vesicles was determined by equilibrium dialysis. Dialysis cells (3 ml) were prepared with one compartment containing vesicles composed of 1 to 2 mg of phospholipid and varying amounts of C-labeled C-10 malt without vesicles. In separate experiments, the radionabeled glycolipid was found to equilibrate across the dialysis membrane (cellulose tubing)

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1. The abbreviations used are: Con A, concanavalin A; RCA, Ricinus communis agglutinin I; C-10 malt, N-decylmaltobionamide; C-12 malt, N-dodecylmaltobionamide; C-14 malt, N-tetradecylmaltobionamide; C-14 mel, N-tetradecylmelibionamide; PC, phosphatidyicholine; PS, phosphatidylethanolamine; CL, cardiolipin; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethane-sulfonic acid.

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in less than 12 h; no significant binding to the dialysis membrane occurred during the experiment. Aliquots (100 µl) were removed from both compartments at various times, and radioactivity was determined in a liquid scintillation counter. Steady-state values were obtained by 19 h and were used to determine the amount of glycolipid incorporated in the vesicles.

**Materials**—Concanavalin A was obtained from Sigma Co., (St. Louis, Mo.). RCA, was kindly supplied by Dr. Marilyn Etzler, Univ. of California at Davis, Calif. Egg lecithin, phosphatidic acid derived from egg lecithin, bovine cardiolipin, and bovine phosphatidylserine were obtained from Avanti Biochemicals, Inc. (Birmingham, Ala.), and were routinely checked for purity by thin layer chromatography. Trace concentrations of Ca²⁺ accompanying the phospholipids was measured by atomic absorption spectroscopy and was less than 0.1 µmol/µmol of phospholipid for all phospholipids used. Stearylamine was obtained from ICN Pharmaceuticals, Inc. (Plainview, N. J.). Synthetic glycolipids were prepared as previously described by Williams et al. (13).

### RESULTS

**Effect of Lipid Composition on the Agglutination of Phospholipid Vesicles by Con A**—Con A did not agglutinate C-10 malt-containing vesicles prepared with PC or a 1:1 mixture of PC and PE (Fig. 2A). Similarly, agglutination did not occur when C-12 malt was used as the glycolipid. In long incubations (12 to 24 h), both in the presence and absence of C-10 malt, a 2-fold increase in Abs did occur, but this increase in turbidity was not inhibited in either case by methyl α-D-mannopyranoside. This latter interaction was not a carbohydrate-specific reaction, and may be related to non-carbohydrate-dependent interactions of Con A (15, 16). However, when C-10 malt-containing vesicles were prepared with a mixture of PC and the negatively charged phospholipids PS, CL, or PA, Con A induced a rapid agglutination of the vesicles (Fig. 2B). Con A also induced agglutination of C-12 malt-containing PE-PS vesicles. Agglutination of PC, PC-PE, PC-PS, PC-CL, PE-PS, or PC-PA vesicles without glycolipid did not occur. Con A did not agglutinate PC-PS (1:1) vesicles containing 10 mol-% N-decylmaltolobionamide, a glycolipid with an α-D-galactosyl moiety, rather than an α-D-glucosyl moieties. Con A-induced agglutination of negatively charged vesicles (PC-PS, PC-CL or PC-PA) with C-10 malt was inhibited almost completely by CaCl₂ (0.5 mM), was routinely present, unless otherwise indicated in experiments with Con A. CaCl₂ (0.5 mM) alone did not cause vesicle agglutination but reduced variability in Con A-induced agglutination.

**Effect of Increasing Con A Concentration and Glycolipid Concentration on Agglutination**—Increasing the Con A concentration from 0 to 800 µg/ml caused a concentration-dependent increase in agglutination of glycolipid-containing PC-CL vesicles (Fig. 3A). ΔAbs of vesicles containing C-12 malt was approximately twice as large as ΔAbs of vesicles containing C-10 malt. This is probably due to greater partitioning of the more lipophilic C-12 malt into the vesicle membranes (see dialysis experiments, below). At the highest Con A concentration used (800 µg/ml), C-12 malt-containing PC vesicles underwent an increase in Abs that was only 10% of the increase observed with C-12 malt-containing PC-CL vesicles.

Con A-specific agglutination of PC-CL vesicles was strongly dependent upon the amount of glycolipid present in the vesicles (Fig. 3B). Little or no agglutination occurred below 2.5 mol % of glycolipid; significant agglutination occurred above 5 mol % of C-10 malt or C-12 malt.

**The Effect of Increasing Proportion of Negatively Charged Phospholipid on Con A-induced Agglutination**—When the glycolipid concentration was kept constant, a larger proportion of negatively charged phospholipid (CL) resulted in an increased agglutination of vesicles by Con A (Fig. 4). Increased agglutination occurred, despite the increased electrostatic repulsion between the vesicles. Con A had no effects on CL-containing vesicles if glycolipid was absent. Increasing proportions of PS or PA also increased Con A-induced agglutination.

**Effect of Salt Concentration on Con A-induced Agglutination of Vesicles**—The possibility that ionic effects may be important in glycolipid-containing membrane-Con A interactions was investigated by determining the effects of altered ionic strength on the ability of Con A to agglutinate C-12 malt-containing PC-PS vesicles (Fig. 5). Con A agglutinated vesicles in solutions containing 0.5 mM CaCl₂ and 10 mM NaHepes (pH 7.1) but no NaCl, or containing 140 mM NaCl, 0.5 mM CaCl₂, and 50 mM NaHepes (pH 7.1). The agglutination was reversed by increasing the NaCl concentration to 280 mM or 420 mM. Con A-induced aggregation was completely inhibited in a solution containing 280 mM NaCl, 0.5 mM CaCl₂, and 50 mM NaHepes (pH 7.1). Thus, ionic effects may play a role in the interaction of Con A with negatively charged, glycolipid-containing vesicles.

**Effect of Glycolipid Concentration on Vesicle Agglutination**—One possible explanation for the lack of agglutination of PC vesicles in the presence of glycolipid is that insufficient glycolipid was incorporated into the vesicles during sonication. To examine this possibility, the binding of C-10 malt to PC and PC-PS vesicles was determined by equilibrium dialysis (Fig. 6). Approximately 50% of the C-10 malt present was incorporated into both types of vesicles in the presence of 1.8 mg/ml of phospholipid. PC vesicles, even at the highest glycolipid concentration (0.5 mM), were not agglutinated by Con A (Fig. 6). PC-CL (1:1) vesicles incorporated C-10 malt to a similar extent as PC and PC-PS vesicles (data not shown) and, as previously demonstrated, were also agglutinated by Con A. Hence, the lack of glycolipid-dependent agglutination of PC vesicles by Con A was not caused by lack of incorporation of glycolipid into the vesicles. In addition, the dialysis
**Fig. 2.** Effect of phospholipid charge on Con A-induced agglutination of glycolipid-containing vesicles. A, PC (○) and 1:1 PC:PE (△) vesicles (1.8 mg of phospholipid/ml) containing 10 mol % C-10 malt were incubated in the presence (●, △) and absence (○, ○) of 250 μg/ml of Con A. B, the same experiment as in A with 1:1 PC-PS (○), PC-CL (△, △), and PC-PA (●, △) as the phospholipid components.

**Fig. 3 (left and center).** The effects of Con A concentration (A) and vesicular glycolipid content (B) on the extent of Con A-induced agglutination of glycolipid-containing vesicles. A, 1:1 PC-CL or PC vesicles (0.9 mg of phospholipid/ml) containing C-10 malt or C-12 were incubated with various concentrations of Con A for 45 min. B, 1:1 PC-CL vesicles (0.9 mg of phospholipid/ml) containing varying amounts of C-10 malt or C-12 malt were incubated in the presence of 250 μg/ml of Con A for 1 h.

**Fig. 4 (right).** The effect of negatively charged phospholipids on the extent of Con A-induced agglutination of glycolipid-containing vesicles. PC-CL vesicles (0.9 mg of phospholipid/ml) containing 10 mol % of either C-10 malt or C-12 malt and varying proportions of PC and CL were incubated with 250 μg/ml of Con A for 45 min.

**Fig. 5.** Effect of increased ionic strength on Con A-induced agglutination of negatively charged glycolipid-containing vesicles. PC-PS vesicles (1:1) containing 10 mol % C-12 malt were prepared in solutions containing 0 (○), 140 mM (△), or 280 mM (■) NaCl. All solution contained 0.5 mM CaCl₂ and 50 mM NaHepes (pH 7.1). At 0 time, 250 μg/ml of Con A was added to vesicles. At 50 min, the NaCl concentration was increased by 280 mM in solutions with agglutinated vesicles. Vesicles in the absence of Con A had optical densities less than 0.126, which did not vary with time.

**Fig. 6.** Glycolipid incorporation into PC and PC-PS vesicles and Con A-induced agglutination. Radioactive C-10 malt incorporation into vesicles was determined by equilibrium dialysis as described under "Experimental Procedures." Con A-induced agglutination was determined on vesicles removed from the dialysis compartment.
experiments indicate that Con A-induced vesicle agglutination occurred in the presence of free glycolipid in the solution. In all experiments, C-10 malt was used at concentrations below its critical micellar concentration. The equilibrium dialysis experiments demonstrate that membrane-bound C-10 malt is in equilibrium with C-10 malt free in solution. To prepare glycolipid-containing vesicles, C-10 malt was routinely co-sonicated with phospholipid. However, C-10 malt could also be incorporated into preformed vesicles by adding the glycolipid to the aqueous phase. The incorporation was demonstrated both by equilibrium dialysis and by the ability of the vesicles to be agglutinated by Con A. When added to the aqueous solution, glycolipid was incorporated into vesicles within minutes as measured by Con A-induced agglutination.

Reversibility of Vesicle Agglutination—Vesicles containing C-10 malt were agglutinated by Con A and pelleted. When the vesicles were resuspended in Con A-free buffer, $A_{560}$ decreased less than 10% in 1 h. In contrast, methyl a-D-mannopyranoside reversed the agglutination within seconds in the presence or absence of Con A. These results suggest that the Con A-membrane glycolipid complex dissociates slowly and that methyl a-D-mannopyranoside interacts directly with the Con A-membrane glycolipid complex to accelerate dissociation of the complex.

Calcium and Phospholipid Dependence of RCA$_1$-induced Agglutination of Vesicles—RCA$_1$ specifically binds D-galactosyl moieties, but interacts with neither D-glucosyl nor D-mannosyl moieties. RCA$_1$ caused agglutination of PC (Fig. 7A) and PC-CL and PC-PA vesicles (Fig. 7C). The agglutination occurred only in the presence of D-galactose-containing glycolipid (C-14 mel). The agglutination was inhibited by methyl a-D-galactopyranoside, but not by methyl a-D-mannopyranoside. Hence, the agglutination was carbohydrate-specific.

RCA$_1$-induced agglutination of PC-PS vesicles required a divalent ion (Fig. 7B). Mn$^{2+}$, Ca$^{2+}$, and Mg$^{2+}$ in descending order of effectiveness sustained the agglutination. The agglutination of PC-PS vesicles induced by RCA$_1$ in the presence of Ca$^{2+}$ was reversed by either EDTA or methyl a-D-galactopyranoside. RCA$_1$-induced agglutination of PC vesicles did not require divalent ion. Although addition of Ca$^{2+}$ enhanced the agglutination of PC-CL and PC-PA vesicles, divalent ion was not required to obtain interaction.$^3$ Furthermore, addition of EDTA in the absence of added divalent ion had no significant effect on the agglutination of these vesicles.

The divalent ion requirement of RCA$_1$-induced agglutination of PS-containing vesicles was further investigated (Fig. 8). Increasing the fraction of PS in vesicles increased the extent of agglutination in the presence of Ca$^{2+}$.

RCA$_1$ does not contain polyvalent ions and does not require divalent ions for binding to carbohydrates in solution (1). This was confirmed in the present study with C-14 mel and various proportions of PC and PS were incubated in the presence of 350 pmol/ml of RCA$_1$ for 45 min in the presence and absence of 0.5 mM CaCl$_2$. $A_{560}$ is the steady state change of $A_{560}$ from its initial value.

Fig. 8. The effect of PS on the extent of RCA$_1$-induced agglutination of glycolipid-containing vesicles. PC-PS vesicles (0.9 mg of phospholipid/ml) containing 10 mol % C-14 mel and various proportions of PC and PS were incubated in the presence of 350 pmol/ml of RCA$_1$ for 45 min in the presence and absence of 0.5 mM CaCl$_2$. $A_{560}$ is the steady state change of $A_{560}$ from its initial value.
the presence or absence of 0.5 mM Ca\(^{2+}\), with or without 1 mM EDTA.

**DISCUSSION**

**Glycolipid and Carbohydrate Specificity of Lectin-induced Agglutination of Lipid Vesicles**—The recent synthesis and availability of a series of glycolipids with defined lipid and carbohydrate moieties (13) has allowed us to systematically investigate glycolipid-specific agglutination of lipid vesicles by the lectins Con A and RCA1, Con A, which specifically binds α-D-mannosyl or α-D-glucosyl moieties, agglutinated vesicles containing α-D-glucosyl-containing glycolipids but not α-D-galactosyl-containing glycolipids. The agglutination was inhibited specifically by methyl α-D-mannopyranoside but not by methyl α-D-galactopyranoside. Conversely, RCA1, which specifically binds β-galactosyl moieties, agglutinated vesicles with β-galactosyl-containing glycolipids. The RCA1-induced agglutination was inhibited by methyl α-D-galactopyranoside, but not by methyl α-D-mannopyranoside. Hence, the carbohydrate-binding specificities of the lectins determine which glycolipid-containing vesicles are agglutinated and which sugars specifically inhibit vesicle agglutination.

**Phospholipid and Ion Requirements**—Perhaps the most striking finding of this study was the discovery that the species of phospholipid, the charge of the phospholipid, and the presence of divalent ion can profoundly influence the ability of lectins to agglutinate glycolipid-containing vesicles. The phospholipid requirement was most evident with Con A. Con A induced agglutination of α-D-glucosyl glycolipid-containing vesicles which were negatively charged but induced little or no agglutination of α-D-glucosyl glycolipid-containing vesicles which were neutral or positively charged. The agglutination was prevented by elevated ionic strength. The results are consistent with earlier findings that electrostatic forces are involved in the interaction of Con A with negatively charged polyelectrolytes (17, 18). It is unlikely that ionic effects occur at the carbohydrate binding site, since increasing the ionic strength from 0 to 4 mM has little effect on Con A-neutral sugar interaction in solution (19). Hence, Con A-induced agglutination of glycolipid-containing, negatively charged, lipid vesicles may involve interactions of the vesicles with both a carbohydrate-specific binding site and a positively charged region on the lectin molecule.

The amount and type of glycolipid is an important determinant of the agglutinability of glycolipid-containing PC vesicles. Orr et al. (12) recently reported that Con A aggregates PC vesicles containing synthetic thiomannosides linked to cholesterol. Incorporation of as much as 12 mol % thiomannosyl glycolipid was required for agglutination, which is more than the maximum amount of glycolipid incorporated into PC vesicles (9 mol %) in the present study.

Factors other than phospholipid charge and carbohydrate specificity may play a role in the ability of Con A to agglutinate lipid vesicles. For example, the chemical identity of the acyl chains of the phospholipids and receptor mobility (20) may be important in determining the sensitivity of the vesicles to Con A-induced agglutination. These factors were not investigated in the present study.

RCA1 agglutinated both neutral and negatively charged vesicles containing α-D-galactosyl glycolipids. The agglutination of PC-PS vesicles, however, had an absolute requirement for Ca\(^{2+}\) or other divalent ions, and could be reversed by adding either EDTA or methyl α-D-galactopyranoside. Because an absolute requirement for Ca\(^{2+}\) (or other divalent ions) was not observed with other negatively charged vesicles which contained cardiolipin or phosphatidic acid instead of phosphatidylserine, reduced electrostatic repulsion between PC-PS vesicles cannot account completely for the effect of Ca\(^{2+}\) on RCA1-induced agglutination. Furthermore, RCA1-induced agglutination of glycolipid-containing PC-CL or PC-PA vesicles in the absence of Ca\(^{2+}\) was greater than that of glycolipid-containing, neutral PC vesicles. RCA1 does not contain bound polyvalent ions and Ca\(^{2+}\) has no effect on its complexation of α-galactosyl moieties in solution. Because Ca\(^{2+}\) binds strongly to PS, Ca\(^{2+}\) may alter the properties of PC-PS vesicles by enhancing glycolipid accessibility to or reactivity with RCA1.

Vesicle fusion can occur upon divalent ion-induced agglutination of phospholipid vesicles (21, 22). Agglutination, by Con A or RCA1, of phospholipid vesicles identical to those in the present study was not accompanied by vesicle-vesicle fusion. Thus, agglutination does not necessarily lead to vesicle fusion.

**Biological Significance**—The lipid and ion requirements of the interaction of Con A and RCA1 with lipid vesicles may have parallels in biological systems. The enhancement by phosphatidylserine of Con A-induced secretion from mast cells has recently been demonstrated (23), and may be related to the enhancement of Con A-induced vesicle agglutination by negatively charged lipids. Several hormones and toxins, such as thyrotropin (2), abrin (4), ricin (4), cholera toxin (3, 4), and diphtheria toxin (5) bind to membrane-bound carbohydrates at the receptor site. In analogy with lectin interactions with vesicles, it is possible that the receptor sites for these hormones and toxins may be the loci for phospholipid, and ion as well as carbohydrate interactions. Indeed, the specific binding of thyrotropin to thyroid membranes is inhibited by negatively charged phospholipids (24) and the binding of diphtheria toxin is Ca\(^{2+}\)-dependent (25).

The present study demonstrates that a variety of carbohydrate moieties can be readily incorporated into phospholipid membranes, using a new class of synthetic, model glycolipids (13). Some members of this class have a high critical micellar concentration, and upon addition to the aqueous phase, are rapidly incorporated into membranes. Thus, by judicious choice of the carbohydrate and the hydrophobic components of the glycolipid, it may now be possible to incorporate into biological membranes a wide range of carbohydrates. These synthetic glycolipids, therefore, represent new and potentially powerful probes of carbohydrate-specific phenomena that occur at the cell surface. They may be useful in studies of cell-cell aggregation, cell attachment to surfaces, cell fusion, and the intracellular effects of carbohydrate-binding hormones and lectins.

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**REFERENCES**


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Con A-induced Vesicle Agglutination