Influence of Glycophorin Incorporation on Ca^{2+}-Induced Fusion of Phosphatidylserine Vesicles†

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ABSTRACT: The effect of incorporation of glycophorin, the major integral sialoglycoprotein of the erythrocyte membrane, into bovine brain phosphatidylserine (PS) vesicles on the Ca^{2+}-induced fusion of these vesicles has been investigated. Fusion was monitored by the terbium–dipicolinic acid fluorescence assay for the mixing of aqueous contents of the vesicles and by a resonance energy transfer assay that follows the intermixing of membrane lipids. The Ca^{2+}-induced fusion of PS vesicles is completely prevented by incorporation of glycophorin (molar ratio of PS/glycophorin = 400–500:1) for Ca^{2+} concentrations up to 50 mM. The ability to fuse is partially restored after treating the glycophorin-containing vesicles with neuraminidase, which removes the negatively charged sialic acid residues of glycophorin. Fusion is further facilitated by trypsin treatment, removing the entire extravesicular glycosylated head group of glycophorin. However, Ca^{2+}-induced fusion of enzyme-treated glycophorin–PS vesicles proceeds at a slower rate and to a smaller extent than fusion of protein-free PS vesicles. The influence of the aggregation state of the glycophorin molecules on fusion has been investigated in experiments using wheat germ agglutinin (WGA). Addition of WGA to the glycophorin–PS vesicles does not induce fusion. However, upon subsequent addition of Ca^{2+}, distinct fusion occurs concomitantly with release of vesicle contents. The inhibition of Ca^{2+}-induced fusion of PS vesicles by incorporation of glycophorin is explained by a combination of steric hindrance and electrostatic repulsion between the vesicles by the glycosylated head group of glycophorin and a direct bilayer stabilization by the intramembranous hydrophobic part of the glycophorin molecule.

Membrane fusion is a fundamental event in many subcellular and cellular functions. It plays for instance a key role in processes such as endocytosis, exocytosis, and fertilization (Poste & Allison, 1973; Silverstein et al., 1977). In addition, important applications of membrane fusion are found in the area of cell hybrid production (Knutton, 1979) and the potential use of phospholipid vesicles in transferring drugs and other substances into cells (Papahadjopoulos, 1978).

About the mechanism(s) of these fusion events and their control, not much is known, although the role of Ca^{2+} as inducer of fusion is well established in certain cases, e.g., the exocytotic release of hormones and neurotransmitters (Douglas, 1974; Normann, 1976). The transient character of fusion and the chemical and structural complexity of biological membranes have seriously complicated mechanistic studies. Therefore, studies on membrane fusion have resorted to simple model membrane systems such as divalent cation induced fusion of phospholipid vesicles containing acidic phospholipids [for review, see Nir et al. (1983)].

The influence of several factors, e.g., head-group composition of mixed phospholipid vesicles (Düzgünes et al., 1981a,b; Sundler & Papahadjopoulos, 1981), bilayer curvature (Nir et al., 1982), divalent cation specificity (Wilschut et al., 1981; Bentz et al., 1983), the incorporation of glycolipids in the phospholipid bilayer (Sundler & Wijkander, 1983; Düzgünes et al., 1984a; Hoekstra et al., 1985), lipid-phase separations and membrane hydration (Hoekstra, 1982b), and ionotropic and thermotropic phase transitions (Düzgünes et al., 1984b), on fusion of in particular phosphatidylserine- (PS-)[1] containing vesicles has been examined.

From these studies the following picture of vesicle fusion has evolved. In Ca^{2+}-induced fusion the first requirement is aggregation of the vesicles. Upon aggregation, fusion is initiated by close apposition of the participating bilayers, which is achieved by dehydration of the polar phospholipid head groups, possibly by the formation of a trans calcium–acidic phospholipid complex (Portis et al., 1979). Then somehow, a local destabilization of the bilayer occurs, which exposes the hydrophobic interior of the bilayers at the points of contact, and fusion follows.

Since biological membranes consist not only of phospholipids but also of proteins and since membrane proteins might also play an important role in fusion of biomembranes (Lucy, 1984), e.g., in its regulation, for example in the case of virus–membrane fusion (Helenius et al., 1980; Simons et al., 1982; Wilschut & Hoekstra, 1984), we decided to investigate the influence of an incorporated membrane-spanning protein on phospholipid vesicle fusion.

Glycophorin, the major integral sialoglycoprotein of the human erythrocyte, was chosen to serve this purpose, because it can be isolated and purified in large quantities and has

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[1] Abbreviations: DPA, dipicolinic acid; EDTA, ethylenediaminetetraacetic acid; GlcNAc, *N*-acetyl-D-glucosamine; N-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; N-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; PAS, periodic acid–Schiff's reagent; PS, phosphatidylserine; RET, resonance energy transfer; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; WGA, wheat germ agglutinin.

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biochemically been well characterized [for review, see Marchesi et al. (1976)]. An additional advantage of glycophorin is that reconstituted glycophorin–phospholipid vesicles have been thoroughly characterized in many respects. Studies have been performed on the structure of these vesicles (MacDonald & MacDonald, 1975; van Zoelen et al., 1978a), on their physicochemical properties (van Zoelen et al., 1982; Taraschi & Mendelsohn, 1980; Mendelsohn et al., 1981; Rüppel et al., 1982), on their barrier properties (van der Steen et al., 1982, 1983; van Hoogevest et al., 1983a,b, 1984), and on the influence of glycophorin on lipid polymorphism (Taraschi et al., 1982a,b, 1983).

Furthermore, the capacity of wheat germ agglutinin (WGA), a lectin containing multiple binding sites for sialic acid and *N*-acetylglucosamine (Bhavanandan & Katlic, 1979), both present in glycophorin (Irimura et al., 1981), to agglutinate glycophorin-containing vesicles (Taraschi et al., 1982b; Grant & Peters, 1984), provides us with a model system for protein-mediated intermembrane recognition and contact, which is very likely to play an important role in fusion of biological membranes and its control.

To get insight into the influence of glycophorin on vesicle fusion, we have investigated in this study the effect of incorporation of glycophorin on Ca^{2+}-induced fusion of PS vesicles. Fusion was followed with the terbium–dipicolinic acid (Tb–DPA) assay (Wilschut et al., 1980), which monitors the mixing of aqueous contents and can also be used to measure release of vesicle contents (Bentz et al., 1983), and with the resonance energy transfer (RET) assay, which detects the intermixing of membrane lipids, as a result of vesicle fusion (Struck et al., 1981; Hoekstra, 1982b).

Experimental Procedures

Materials

Phosphatidylserine (PS) was isolated from bovine brain, by general procedures (Papahadjopoulos & Miller, 1967; Sanders, 1967) with some modifications. In order to avoid the harsh chemical conditions imposed on PS in these procedures, in particular the elution of the product from a diethylaminoethyl-(DEAE-) cellulose column with glacial acetic acid (which in addition is difficult to remove), this step was replaced by column chromatography and high-performance liquid chromatography (HPLC) under milder chemical conditions.

Briefly, bovine brain was extracted with chloroform/methanol (1:1 v/v) (Sanders, 1967) after which the total lipid extract was precipitated with acetone. The acetone precipitate was dissolved in diethyl ether and stored for 2 days at 4 °C. Subsequently, the precipitated substance was removed (Folch, 1942). A crude PS fraction was obtained by chromatography of the lipid extract on a column (80 × 7 cm) packed with polygosil 60 (40–63-μm mesh, Macherey-Nagel, Düren, FRG), using chloroform/methanol/ammonia/water (50:50:2:1 v/v/v/v) as eluent.

Finally, two successive high-performance liquid chromatography steps were found to be essential to remove small amounts of residual phosphatidylethanolamine and phosphatidylcholine. The first was performed on a column (500 × 50 mm) packed with polygosil 60 (5–20-μm mesh, Macherey-Nagel, Düren, FRG) with chloroform/methanol/ammonia/water (50:50:2:1 v/v/v/v) as the solvent system; the second was on a column (250 × 22 mm) packed with Lichrosorb Si-60 (5-μm mesh, Merck, Darmstadt, FRG) with chloroform/methanol/ammonia/water (68:28:2:2 v/v/v/v) as eluent.

The PS was converted into the sodium form according to Papahadjopoulos & Miller (1967). This conversion was checked to be 100 ± 1%, by flame-emission spectroscopy as described by Smaal et al. (1984). All manipulations were carried out under a nitrogen atmosphere. The procedure yielded about 2 g of NaPS/kg of bovine brain (wet weight). The final product did not contain any detectable impurities as judged from high-performance thin-layer chromatography with chloroform/methanol/acetic acid/water (75:45:3:1 v/v/v/v) as the solvent system (Allan & Cockcroft, 1982).

Glycophorin was isolated and purified from human erythrocyte ghosts as described previously (Taraschi et al., 1982a). The purified protein showed two bands (PAS I and PAS II) on sodium dodecyl sulfate–polyacrylamide gels stained with Coomassie blue and with periodate–Schiff reagent (Fairbanks et al., 1971). The glycophorin contained 2.1 μmol of sialic acid/mg of protein and less than 0.8 mol of phosphorus/mol of glycophorin.

N-NBD-PE and N-Rh-PE were purchased from Avanti Polar Lipids Inc. (Birmingham, AL). $TbCl_3 \cdot 6H_2O$ was obtained from Aldrich Chemical Co. (Milwaukee, WI). Dipicolinic acid (DPA), Tes, and *N*-acetyl-D-glucosamine were purchased from Sigma (St. Louis, MO). L-Histidine and trypsin [bovine, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK) treated to remove chymotrypsin activity] were obtained from Merck (Darmstadt, FRG). Wheat germ agglutinin (WGA) was purchased from Boehringer Mannheim GmbH (FRG). Neuraminidase from *Vibrio cholerae* (protease free) was obtained from Koch-Light Ltd. (Haverhill, U.K.). All other chemicals were of analytical grade.

Methods

Preparation of Vesicles. Glycophorin–PS vesicles were made by reconstitution of glycophorin into unilamellar vesicles according to the method of MacDonald & MacDonald (1975), as applied to PS (van Zoelen et al., 1978a). A mixture of 1.3 mg of glycophorin and 10 μmol of bovine brain PS (lipid/glycophorin molar ratio 400:1) was dissolved in chloroform/methanol/1 mM Tris, pH 7.4 (150:75:1 v/v/v), and dried by rotatory evaporation during at least 2 h under vacuum. In experiments using the RET assay, the appropriate amounts of N-NBD-PE and N-Rh-PE were added to the PS, prior to the mixing with glycophorin. The mixed glycophorin–PS film was resuspended in 1 mL of the appropriate buffer. The resulting vesicles were centrifuged for 10 min at 10000*g* (at 4 °C) to remove large structures, containing no or little protein (van Zoelen et al., 1978a). In order to collect the glycophorin-containing vesicles, the supernatant was centrifuged at 37000*g* for 30 min (4 °C).

These glycophorin–PS vesicles were found to have a 400–500:1 phospholipid:protein molar ratio as determined by analysis of sialic acid [according to Warren (1959)] and phosphorus [according to Rouser et al. (1970)], in agreement with van Hoogevest et al. (1984).

Large unilamellar protein free PS vesicles were prepared by reverse-phase evaporation (Szoka & Papahadjopoulos, 1978) with some modifications (Wilschut et al., 1980; Düzgünes et al., 1983). The vesicles were sized by extrusion through polycarbonate Unipore membranes (Bio-Rad) with a pore size of 0.2 μm.

Tb–DPA Fusion Assay. The Tb–DPA fusion assay was performed as described in detail by Wilschut et al. (1980). Vesicles were made in either (i) 2.5 mM $TbCl_3$ and 50 mM sodium citrate, (ii) 50 mM DPA (sodium salt) and 20 mM NaCl, or (iii) 1.25 mM $TbCl_3$, 25 mM sodium citrate, 25 mM DPA, and 10 mM NaCl. All solutions were buffered with 2 mM Tes and 2 mM L-histidine, pH 7.4, and contained 0.2 mM NaN_3.

Unencapsulated material was removed by gel filtration on Sephadex G-75 (Pharmacia) with 1 mM EDTA, 100 mM NaCl, 2 mM Tes, and 2 mM L-histidine, pH 7.4, as elution buffer.

For purposes of calibration, portions of the vesicle preparations were passed through another Sephadex G-75 column to remove EDTA from the external medium, with the above elution buffer without EDTA. In the case of protein-free PS vesicles, these gel filtration steps were carried out at room temperature. Since glycophorin-containing vesicles prepared by the McDonald procedure exhibit an increased permeability toward solutes with a molecular weight less than 900 as compared to protein-free vesicles (van der Steen et al., 1983; van Hoogevest et al., 1983a, 1984), the gel filtration steps of these vesicles were carried out at 4 °C in order to reduce leakage of the vesicle contents.

Fusion of the vesicles was measured by monitoring the formation of the fluorescent Tb–$(DPA)_3^{3-}$ complex upon mixing of the aqueous contents of Tb- and DPA-containing vesicles.

Fusion measurements were carried out in a quartz cuvette containing 2 mL of a 1:1 mixture of Tb vesicles (type i) and DPA vesicles (type ii) at a final lipid concentration of 50 μM in 0.1 mM EDTA, 100 mM NaCl, 2 mM Tes, and 2 mM L-histidine, pH 7.4. The EDTA is present to prevent Tb–DPA complex formation outside the vesicles (Wilschut et al., 1980).

Fluorescence was measured in a Perkin-Elmer MPF-3 fluorescence spectrophotometer. The temperature was maintained at 25 °C, and the solution in the cuvette was continuously mixed with a magnetic stirrer. Concentrated solutions of WGA and $CaCl_2$ were injected with Hamilton syringes directly into the cuvette via a small hole in the cuvette compartment, thus allowing fluorescence intensities to be continuously followed.

The Tb–DPA complex was excitated at 276 nm, and emission was measured at 545 nm, with a cut-off filter (permitting light at wavelengths greater than 520 nm) between sample and monochromator, in order to reduce the contribution of light scattering to the fluorescence signal. The remaining light scattering contribution was measured by performing the experiments with vesicles prepared in 100 mM NaCl, 2 mM Tes, and 2 mM L-histidine, pH 7.4, and constituted at most 10% of the 100% Tb fluorescence value in the case of protein-free vesicles and 30% of this value in the case of glycophorin–PS vesicles. The fluorescence data were corrected for these numbers.

Fluorescence intensities are always expressed as percentages of the fluorescence intensity obtained when all Tb present in the vesicles has complexed with DPA, the 100% Tb fluorescence value. In order to determine this 100% Tb fluorescence value, the trapped volume of the vesicles, and the passive permeability properties of the vesicles toward the encapsulated solutes, vesicles were used that had been separated from external EDTA. Upon addition of an excess of DPA (final concentration 20 μM) to a cuvette containing Tb vesicles (50 nmol of lipid), leakage of Tb from these vesicles, which had occurred after the gel filtration steps, could be determined. The amount of leakage was expressed as percentage of the amount of Tb initially present in the vesicles. This latter value was obtained by the addition of sodium cholate (1% w/v) to the cuvette, causing all Tb to complex with DPA. From this value and the known lipid concentration, the trapped volume could be calculated. Subtraction of the amount of leakage from the amount of Tb initially present in the vesicles yielded the amount of Tb present in the vesicles at the time of cholate addition, the 100% Tb fluorescence value.

The trapped volume of DPA-containing vesicles and leakage of DPA from these vesicles were determined in the same way, except that in this case an excess of $TbCl_3$ (5 μM) was added. In the case of vesicles containing the fluorescent complex, the trapped volume could be calculated from the initial fluorescence. Leakage could be determined by addition of EDTA (0.1 mM). The release of internal aqueous contents during fusion was measured by monitoring the dissociation of preencapsulated Tb–DPA complex (type iii vesicles) (Bentz et al., 1983), under the same conditions as in the fusion experiments.

RET Assay for Lipid Mixing. The RET assay employed by us has been described in detail by Struck et al. (1981) and Hoekstra (1982b). Two populations of vesicles were made, one containing 0.5% N-NBD-PE and 1% N-Rh-PE and the other devoid of fluorescent lipids, in a solution comprising 100 mM NaCl, 2 mM Tes, and 2 mM L-histidine, pH 7.4.

Ca^{2+}-induced fusion of these vesicles results in dilution of the fluorescent phospholipids, thus decreasing the efficiency of resonance energy transfer between donor and acceptor. The extent of fusion was measured by following the emission signal of NBD (530 nm) upon excitation at 450 nm.

Measurements were carried out in 2 mL of a 1:1 mixture of labeled and unlabeled vesicles at a final lipid concentration of 50 μM, in 0.1 mM EDTA, 100 mM NaCl, 2 mM Tes, and 2 mM L-histidine, pH 7.4. Instrumental conditions were the same as those in the Tb–DPA assay except that in the RET assay no cut-off filter was used.

The fluorescence signal was corrected for the contribution of light scattering by subtraction of the signal caused by unlabeled vesicles from the total signal. The light scattering never exceeded 3% of the total signal in the case of protein-free vesicles and 10% of the total signal in the case of glycophorin–PS vesicles.

The initial NBD emission intensity in the fusion experiments, when no lipid mixing has yet occurred, is taken as 0% fusion. The final NBD emission intensity, corresponding to complete lipid mixing, which is considered to represent 100% fusion, was obtained from independently prepared vesicles containing 0.25% N-NBD-PE and 0.5% N-Rh-PE ("100% vesicles"). The amount of resonance energy transfer is approximately proportional to the extent of probe intermixing. The extent of fusion can then be determined by comparing the relative fluorescence level in the fusion experiment with the fluorescence level of the "100% vesicles" and is expressed as percentage fluorescence increase (Struck et al., 1981; Hoekstra, 1982b; Hoekstra & Martin, 1982).

Upon addition of Ca^{2+} to labeled vesicles, a decrease of the NBD fluorescence level occurred, probably due to self-quenching of N-NBD-PE caused by Ca^{2+}-induced phase separation in the bilayer of the vesicles (Hoekstra, 1982a,b; Hoekstra & Martin, 1982). Since this effect coincided with the occurrence of Ca^{2+}-induced fusion (measured as an increase of NBD fluorescence), a correction was necessary in order to obtain the fluorescence signal caused by fusion only. For this purpose, all fusion conditions were also applied to "100% vesicles" in parallel experiments. These experiments enabled us to follow the change in fluorescence exhibited by these vesicles upon addition of WGA and/or $CaCl_2$ and to correct the fluorescence signal obtained in fusion experiments for this change. Under the experimental conditions used, the decrease of the fluorescence level exhibited by the "100% vesicles" amounted to at most 10% of the initial fluorescence level (at 25 mM Ca^{2+}, 3 min after Ca^{2+} injection). The

fluorescence increase percentages obtained in fusion experiments with protein-free vesicles were corrected by multiplication by the appropriate correction factor, derived from the parallel experiment with the "100% vesicles".

In the case of Ca^{2+} addition to PS or glycophorin–PS vesicles, the decrease in fluorescence of the "100% vesicles" could be reversed by the addition of a 4-fold molar excess of EDTA over Ca^{2+}, in agreement with Hoekstra (1982b). However, in the case of "100% glycophorin–PS vesicles" that had been incubated with WGA prior to Ca^{2+} injection, a rise of the fluorescence signal relative to its starting level was observed upon addition of a 4-fold molar excess of EDTA over the Ca^{2+} present. This rise was Ca^{2+} concentration dependent, and under the experimental conditions used its maximal value was 7% of the initial fluorescence level (3 min after Ca^{2+} injection, final concentration 25 mM; vesicles preincubated for 5 min with a 10-fold molar excess of WGA). At present there is no clear explanation for this small change in fluorescence. In view of this observation, the extent of fusion of glycophorin–PS vesicles after incubation with WGA was determined after addition of EDTA to both fusion mixture and "100% vesicles". For reasons of consistency, the same procedure was followed for glycophorin–PS vesicles when no WGA was involved. EDTA was administered as an aliquot of a 0.5 M solution, 10 mM Tris, pH 7.4, to a 4-fold molar excess over the Ca^{2+} present, at certain points of time after Ca^{2+} addition.

The use of the corrections reported above introduced a certain inaccuracy in the quantification of the data, which was estimated to be in total at most 20% of the percentages of fluorescence increase found. In all experiments where WGA is involved, the amount of WGA is expressed on a molar basis, relative to the amount of glycophorin present in the vesicles, assuming the molar ratio PS:glycophorin to be 400:1.

Pretreatments of the Glycophorin–PS Vesicles with Enzymes. Glycophorin–PS vesicles at a total lipid concentration of approximately 1 mM were incubated with either 10% (w/w) trypsin with respect to glycophorin or 0.04 unit of neuraminidase/mg of glycophorin, during 1 h at 37 °C in a buffer containing 100 mM NaCl, 2 mM Tes, and 2 mM L-histidine, pH 7.4. Subsequently, these vesicles were centrifuged at 37000*g* for 30 min (at 4 °C), and the enzyme-containing supernatant was discarded. By means of sialic acid determination (Warren, 1959), it was shown that trypsin removed 77 ± 5% and neuraminidase 71.5 ± 5% of the sialic acid present, in agreement with van Zoelen et al. (1978a).

Results

The influence of glycophorin incorporation on the Ca^{2+}-induced fusion of PS vesicles was first investigated by the Tb–DPA assay. In Figure 1, the fusion characteristics of the protein-free large unilamellar PS vesicles are depicted. In agreement with Wilschut et al. (1980), it was found that the threshold Ca^{2+} concentration for fusion was 2.5 mM. Higher concentrations of Ca^{2+} showed a higher initial fusion rate followed by a gradual decrease of fluorescence, due to the collapse of the vesicles and the formation of cochleated structures (Papahadjopoulos et al., 1975).

In contrast, addition of Ca^{2+}, even up to concentrations of 50 mM, to glycophorin-containing vesicles (molar ratio of PS:glycophorin = 400–500:1) did not cause any detectable development of fluorescence although the turbidity of the solution slightly increased. For the absence of the formation of the fluorescent complex, four explanations are possible:

(1) The fusion might be prevented by binding of the Ca^{2+} ions to sialic acid residues on the protein. However, this is most unlikely since Ca^{2+} is present in a large molar excess (up to 13 000-fold) over the sialic acid. In addition, it has been shown that both before and after trypsin treatment of glycophorin–cardiolipin vesicles the Ca^{2+} binding is similar (Taraschi et al., 1983).

(2) Due to the increased bilayer permeability by the incorporation of glycophorin (van Hoogevest et al., 1984), the vesicles do not contain sufficient Tb and/or DPA to allow a proper detection of the Tb–DPA complex. However, considering Table I, which compares trapped volumes and passive permeabilities toward Tb, DPA, and the Tb–DPA complex of protein free vesicles with those obtained for glycophorin-containing vesicles, it is clear that, despite the fast rate of leakage of in particular DPA, sufficient amounts of both Tb and DPA (which is initially present in 25-fold molar excess over Tb) are still contained within the vesicles at the time of the assay.

(3) The glycophorin–PS vesicles do fuse, but the fusion cannot be monitored due to an immediate release of contents and/or the entry of EDTA and Ca^{2+} (Bentz et al., 1983). In order to evaluate this possibility, release of vesicle contents was followed by measuring the dissociation of fluorescent Tb–DPA complex, preencapsulated in the vesicles, upon ad-

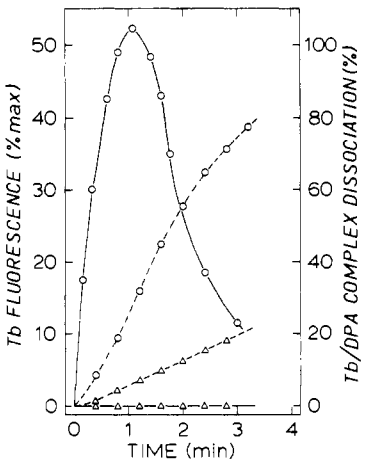

FIGURE 1: Ca^{2+}-induced fusion (—) and release of contents (--) of PS vesicles (○) and glycophorin–PS vesicles (△), as measured by the Tb–DPA assay. $CaCl_2$ was injected as an aliquot of a 1.0 M solution at $t = 0$ to a final concentration (not corrected for the 0.1 mM EDTA, present in the medium) of 25 mM. Fusion was measured as the mixing of aqueous contents of separate vesicle populations (each with a lipid concentration of 25 μM) containing either Tb or DPA and is presented as the percentage of encapsulated Tb complexed to DPA. The release of vesicle contents was measured with vesicles (with a lipid concentration of 50 μM) containing the Tb–DPA complex and shows the percentage of the fluorescent complex that has dissociated due to leakage from, and the entry of medium into, the vesicles. For experimental details, see Experimental Procedures.

Table I: Trapped Volumes and Passive Permeability Properties of PS Vesicles and Glycophorin–PS Vesicles Containing Tb, DPA, or the Tb–DPA Complex[a]

	obsd trapped vol (μL/μmol)		leakage (%/h)	
	PS	glycophorin–PS	PS	glycophorin–PS
Tb^{3+}	3.6	2.8	0.1	6.5
DPA^{2-}	3.6	1.0	0.3	15
$Tb-(DPA)_3^{3-}$	3.6	1.0	0.4	15

[a] Trapped-volume and permeability properties (i.e., leakage of vesicle contents) were determined as described under Experimental Procedures upon storing the vesicles, after the gel filtration steps, at 0 °C for 24 and 4 h in the case of PS and glycophorin–PS, respectively. The estimated relative errors in the values found for the trapped volume and the leakage amount to 10% and 20%, respectively.

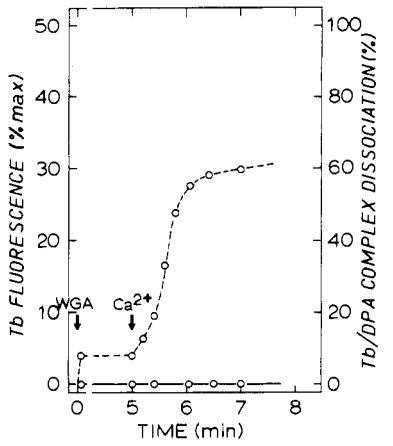

FIGURE 2: Effect of WGA on Ca^{2+}-induced fusion (—) and release of contents (--) of glycophorin–PS vesicles, as measured by the Tb–DPA assay. WGA was added at $t = 0$ from a 4.5 mg/mL solution to a final concentration of 112.5 μg/mL, which corresponds to a 25-fold molar excess of WGA over the glycophorin present. $CaCl_2$ was introduced as an aliquot of a 1.0 M solution to a final concentration of 25 mM. The lipid concentration was 50 μM.

dition of Ca^{2+}. The dashed lines in Figure 1 show that the glycophorin–PS vesicles exhibited a small release of contents upon addition of Ca^{2+}, while protein-free vesicles after an initially almost nonleaky fusion lost their contents to a much larger extent, in accordance with Wilschut et al. (1980, 1983).

(4) Taking into account the above findings, the most probable explanation for the lack of fluorescence increase is that glycophorin–PS vesicles do not fuse upon addition of Ca^{2+}.

Since WGA is able to agglutinate glycophorin–lipid vesicles and aggregate glycophorin in the plane of the membrane (Taraschi et al., 1982b; Grant & Peters, 1984), it is of interest to investigate a possible WGA effect on the glycophorin-induced blockade of Ca^{2+}-induced fusion of PS vesicles. After addition of a 25-fold molar excess of WGA over glycophorin, a very slight increase of turbidity occurred, accompanied by an immediate small release of vesicle contents (as detected by Tb–DPA leak, Figure 2). These effects, which are probably caused by, respectively, agglutination of the vesicles and aggregation of glycophorin, were also found with glycophorin–dioleoylphosphatidylcholine and glycophorin–erythrocyte lipid vesicles (van der Steen et al., 1983). An increase of fluorescence was not observed either during the incubation with WGA (5 min, 25 °C) or upon subsequent Ca^{2+} injection (up to 25 mM) (Figure 2). In contrast to the situation where the vesicles were not preincubated with WGA, a fast release of vesicle contents did occur when Ca^{2+} was added after incubation with WGA. This release of contents even proceeded at a faster rate than in the case of Ca^{2+} addition to protein-free vesicles (compare Figures 1 and 2).

In view of the fast release of vesicle contents upon Ca^{2+} addition to the WGA-pretreated glycophorin–PS vesicles, the absence of an increase in Tb fluorescence in the fusion assay cannot be taken as evidence against vesicle fusion. In addition, it is theoretically possible that outer monolayer fusion occurs without mixing of aqueous compartments. In order to get insight in this possibility and to avoid problems concerning the permeability of the glycophorin–PS vesicles, the RET assay for lipid mixing was applied, further investigating the possible fusion of glycophorin–PS vesicles.

In Figure 3 the time course of vesicle lipid mixing in the presence of 5 mM Ca^{2+} is shown. The protein-free large unilamellar PS vesicles displayed efficient lipid mixing upon Ca^{2+}-induced fusion. Addition of Ca^{2+} to glycophorin–PS vesicles did not cause any increase of fluorescence. This is even

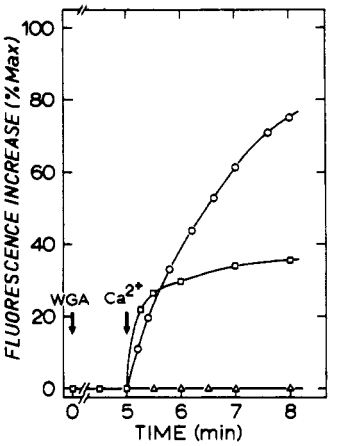

FIGURE 3: Ca^{2+}-induced fusion of PS vesicles (○), glycophorin–PS vesicles (△), and glycophorin–PS vesicles after incubation with WGA (□), as measured by the RET assay. $CaCl_2$ was introduced as an aliquot of a 1.0 M solution to a final concentration of 5 mM. The total lipid concentration was 50 μM. The incubation of glycophorin–PS vesicles with WGA (45 μg/mL, corresponds to a WGA:glycophorin molar ratio of 10) was carried out for 5 min prior to the injection of Ca^{2+}. For experimental details and the determination of the percentages of fluorescence increase, see Experimental Procedures.

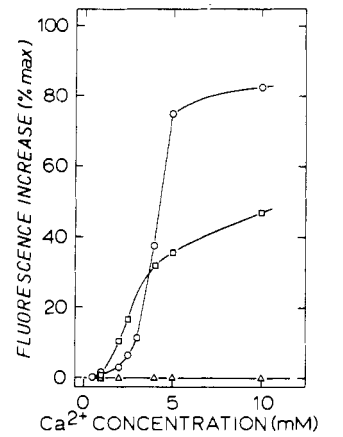

FIGURE 4: Dependency of vesicle fusion on the Ca^{2+} concentration for PS vesicles (○), glycophorin–PS vesicles (△), and glycophorin–PS vesicles after incubation with WGA (□), as measured by the RET assay. $CaCl_2$ was injected as aliquots of either a 0.1 M solution to final concentrations smaller than 5 mM or a 1.0 M solution to final concentrations of 5 mM and higher. The total lipid concentration was 50 μM. The incubation of glycophorin–PS vesicles with WGA (45 μg/mL, corresponds to a WGA:glycophorin molar ratio of 10) was carried out for 5 min prior to the addition of Ca^{2+}. The percentages of fluorescence increase were determined 3 min after $CaCl_2$ injection as described under Experimental Procedures.

true at higher concentrations of Ca^{2+} (Figure 4), confirming the findings obtained with the Tb–DPA assay, in that glycophorin incorporated in the bilayer of PS vesicles completely prevents the Ca^{2+}-induced fusion.

Incubation of the vesicles with WGA did not cause any lipid mixing. However, subsequent addition of Ca^{2+} resulted in an increase of fluorescence that initially proceeded at a faster rate than it did in the corresponding experiment of Ca^{2+}-induced fusion of protein-free PS vesicles, although the final level was lower (Figure 3). These observations are confirmed in Figure 4, which shows that at lower concentrations of Ca^{2+} glycophorin–PS vesicles, preincubated with WGA, exhibited a higher extent of lipid mixing that protein-free vesicles, of which fusion was induced by the same amount of Ca^{2+}.

To investigate whether the efficient Ca^{2+}-induced fusion of the glycophorin–PS vesicles preincubated with WGA is due to the receptor properties of glycophorin for WGA, experi-

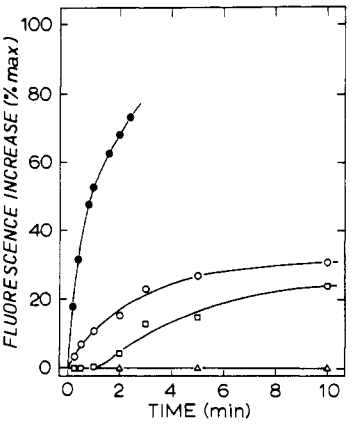

FIGURE 5: Ca^{2+}-induced fusion of PS vesicles (●) and glycophorin–PS vesicle after incubation without enzyme (△), with trypsin (○), or with neuraminidase (□), as measured by the RET assay. $CaCl_2$ was introduced as aliquots of a 1.0 M solution to a final concentration of 25 mM. The total lipid concentration was 50 μM. The glycophorin–PS vesicles were incubated with trypsin (10% w/w, glycophorin) or neuraminidase (0.04 unit/mg glycophorin) during 1 h at 37 °C.

ments were performed with the monosaccharide *N*-acetyl-D-glucosamine (GlcNAc), which specifically binds to WGA and thus inhibits its agglutinating properties (Bhavanandan & Katlic, 1979; Monsigny et al., 1980; Maget-Dana et al., 1981). Since WGA has a much higher affinity for glycophorin than for GlcNAc (Bhavanandan & Katlic, 1979), a large molar excess of GlcNAc over glycophorin is required to prevent the interaction of WGA with glycophorin. When in our experiments 8 mM GlcNAc was added to glycophorin–PS vesicles prior to incubation with WGA (molar ratio GlcNAc/WGA ~6500:1), no significant fusion (less than 1% increase in fluorescence) was observed upon addition of 5 mM Ca^{2+}. The fusion of protein-free PS vesicles was not affected by the presence of 8 mM GlcNAc.

Incubation of the protein-free PS vesicles with WGA (45 μg/mL) during 5 min prior to the addition of Ca^{2+} slightly decreased the extent of fusion to some 85% of its value when no WGA was involved. This effect is probably due to the fact that WGA is a basic protein with an isoelectric point of 8.5 (Rice & Etzler, 1975; Monsigny et al., 1979) and thus could be aspecifically bound to the negatively charged PS vesicles, thereby slightly inhibiting Ca^{2+}-induced fusion. From these experiments it can be concluded that specific binding of WGA to the carbohydrate moieties of glycophorin is responsible for enabling Ca^{2+} to induce fusion of glycophorin–PS vesicles.

In order to investigate which part of the glycophorin molecule is responsible for the inhibitory effect on Ca^{2+}-induced fusion of PS vesicles, the glycophorin–PS vesicles were treated with various enzymes. Removal of the negatively charged sialic acid residues by neuraminidase treatment made the vesicles susceptible to fusion by Ca^{2+} (Figure 5), although the rate of fusion was still slow as compared to protein-free vesicles. In addition, the threshold concentration of Ca^{2+} for fusion of these vesicles was about 6 mM, as compared to 2.5 mM for the protein-free vesicles (compare Figures 4 and 6). Trypsin treatment, which removes the entire bulky extravesicular glycosylated head group of glycophorin (Tomita & Marchesi, 1975), facilitated Ca^{2+}-induced fusion of glycophorin–PS vesicles to a larger extent (Figures 5 and 6).

The contents mixing assay revealed that under these conditions maximally 5% of the Tb–DPA complex had formed 90 s after the addition of Ca^{2+}. In view of the leakiness of the glycophorin-containing vesicles, which is unchanged after trypsin treatment (van Hoogevest et al., 1983), these numbers

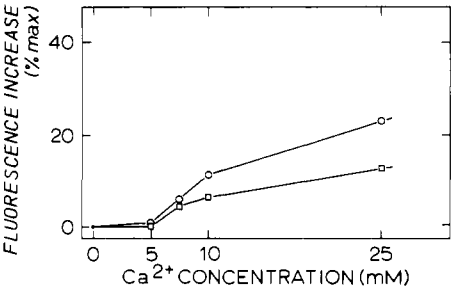

FIGURE 6: Ca^{2+}-concentration dependency of fusion of trypsin (○) and neuraminidase-treated glycophorin–PS vesicles (□). $CaCl_2$ was injected as aliquots of a 1.0 M solution; the total lipid concentration was 50 μM. The percentages of fluorescence increase were determined 3 min after Ca^{2+} injection.

cannot be interpreted in terms of fusion efficiency.

Fusion of protein-free PS vesicles under the same conditions proceeded at a faster rate and to a larger extent (Figure 5) from which it can be concluded that the remainder of the protein, also after trypsin treatment, still imposes a barrier toward the Ca^{2+}-induced fusion. Control experiments revealed that incubation of protein-free PS vesicles with neuraminidase did not significantly affect the Ca^{2+}-induced fusion, whereas trypsin treatment of protein-free PS vesicles decreased the extent of Ca^{2+}-induced fusion to some 80% with respect to untreated vesicles, probably due to some binding of the basic trypsin to the vesicles.

Discussion

In this study it has been demonstrated, with two independent fusion assays, that incorporation of glycophorin into large unilamellar PS vesicles to a molar ratio PS:glycophorin of 400–500:1 prevents the Ca^{2+}-induced fusion of these vesicles, up to Ca^{2+} concentrations of 50 mM (Figures 1 and 4). This finding is in line with the suggestion that glycophorin is involved in the protection of erythrocytes against fusion (Tanner, 1978).

Insight into the nature of the fusion inhibitory effect can be derived from the enzyme treatments of the glycophorin–PS vesicles. Removal by neuraminidase of the extravesicular negatively charged sialic acid residues takes away part of the barrier toward Ca^{2+}-induced fusion. Fusion is further facilitated upon removal of the entire bulky glycosylated head group of glycophorin from the outside of the vesicles, by treatment with trypsin. Compared to protein-free PS vesicles, however, these trypsin-treated vesicles still show a limited extent of fusion.

Since the fusion event begins with two consecutive steps, first, agglutination of the vesicles and, second, bilayer destabilization at the contact site of the two approaching bilayers, the inhibitory effect of glycophorin can be explained as follows. Considering the enzyme treatment experiments (Figures 5 and 6), it can be stated that due to steric hindrance, caused by the bulky extravesicular glycosylated head group, the bilayers are not able to come into close apposition. In addition, the charged sialic acid residues present on the head group will cause repulsion between two opposite glycophorin-containing bilayers. Furthermore, the remainder of the glycophorin molecule after trypsin treatment still limits fusion as compared to protein-free vesicles, either due to the steric hindrance from the 9–10 amino acid residues that remain outwardly extended from the lipid–protein vesicles after treatment with trypsin (Segrest et al., 1974) or to a direct bilayer stabilization, resulting from hydrophobic interactions between the intramembranous part of glycophorin and the hydrophobic phospholipid chains. The existence of such hydrophobic interactions has been established

by Raman spectroscopy (Mendelsohn & Taraschi, 1980; Taraschi et al., 1981) and differential scanning calorimetry (van Zoelen et al., 1978b).

Further insight into the mechanism of the inhibition of vesicle fusion by glycophorin incorporation has been obtained from experiments with WGA. Addition of WGA to glycophorin–PS vesicles does not cause fusion. However, subsequent addition of Ca^{2+} induces distinct fusion (Figure 3), accompanied by a dramatic release of vesicle contents (Figure 2). In discussing these results, it is useful to recall that WGA can affect the glycophorin–PS vesicles in two ways. First, the glycophorin molecules can be aggregated in the plane of the bilayer by the interaction with the multivalent lectin. Second, WGA can agglutinate the vesicles by bridging glycophorin molecules in separate vesicles (Taraschi et al., 1982b; Grant & Peters, 1984). It is of interest to know by which of these two mechanisms WGA promotes the Ca^{2+}-induced fusion of glycophorin–PS vesicles. The lower threshold Ca^{2+}-concentration value for the fusion of glycophorin–PS vesicles after incubation with WGA (Figure 4) and the initially fast rate of fusion of these vesicles (Figure 3), relative to fusion of protein-free PS vesicles, suggest a fusion mechanism in which WGA first agglutinates the glycophorin–PS vesicles, after which less Ca^{2+} would be necessary to induce fusion. If WGA would enable Ca^{2+}-induced fusion to proceed by clearing the area of vesicle contact from the fusion inhibiting glycophorin molecules, a similar Ca^{2+} requirement should be expected as in fusion of protein-free vesicles.

It is of interest to compare our results with those obtained in recent studies on the effect of glycolipids on the Ca^{2+}-induced fusion of acidic phospholipid-containing vesicles (Sundler & Wijkander, 1983; Düzgünes et al., 1984a). Incorporation of 5–10 mol % of several glycolipids into these vesicles drastically reduces the rate and extent of Ca^{2+}-induced fusion and in certain cases even completely prevents it, as we report for the glycophorin-containing vesicles. Incubation of the glycolipid-containing vesicles with a lectin, specific for the carbohydrate group of the glycolipid, prior to the addition of Ca^{2+}, greatly enhances fusion.

For phosphatidylethanolamine–phosphatidate vesicles containing a synthetic glycolipid, it was found that the Ca^{2+} requirement for fusion in the presence of the appropriate lectin is even much lower than in the case of Ca^{2+}-induced fusion of glycolipid-free vesicles [compare Sundler & Wijkander (1983) and Sundler et al. (1981)]. This finding also is very similar to that obtained in the WGA-facilitated Ca^{2+}-induced fusion of glycophorin-containing PS vesicles, suggesting a common mechanism in which the establishment of lectin-mediated cross-linking of receptors on more than one vesicle enhances Ca^{2+}-induced fusion.

There is a striking similarity between the inhibitory effect of glycophorin on the Ca^{2+}-induced fusion of PS vesicles and the influence of glycophorin on lipid polymorphism (Taraschi et al., 1982a,b, 1983). Under conditions where dioleoylphosphatidylethanolamine (DOPE), or cardiolipin in the presence of Ca^{2+}, would prefer the hexagonal (H_{II}) phase, incorporation of glycophorin promotes a lipid bilayer organization. After trypsin treatment, the bilayer stabilization is lost, and the hexagonal (H_{II}) phase is formed, the extent of which depends on the lipid:glycophorin ratio. Furthermore, WGA addition to the glycophorin–cardiolipin–Ca^{2+} and the glycophorin–DOPE systems triggers a bilayer to hexagonal (H_{II}) phase transition. The latter process is thought to be caused by both the "clearing effect" and the "agglutinating effect" of WGA on the glycophorin-containing vesicles (Taraschi et al., 1982b). Similar rearrangements of the glycophorin molecules occur upon incubation with WGA on glycophorin–PS vesicles, leading to an increased tendency to fuse by the action of Ca^{2+}.

This reinforces the conclusion that the fusion inhibitory effect of glycophorin is due to a combination of steric hindrance of close apposition and a direct bilayer stabilization by the protein molecule.

Our finding that a lectin is capable of modulating the process of Ca^{2+}-induced fusion of glycophorin-containing PS vesicles indicates that proteins, functioning as receptors and effectors, might play an important role in mediating and regulating fusion of biological membranes. This particularly might be the case in receptor-mediated endocytosis (Goldstein et al., 1979) and exocytotic events (Hong et al., 1981, 1982). Furthermore, this result might be relevant in explaining the involvement of lectins in the fusion of myoblasts (Gartner & Podleski, 1975; MacBride & Przybylski, 1980).

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Registry No. Ca, 7440-70-2; *N*-acetylglucosamine, 7512-17-6.

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