CROSS-LINKING OF PHOSPHOLIPIDS TO PROTEINS IN THE ERYTHROCYTE MEMBRANE

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SUMMARY

Erythrocytes treated with the cross-linking agents difluorodinitrobenzene and suberimidate are rendered refractory to lysis. When ghosts are treated with these reagents 8.4% and 2.3% of the total lipid phosphate is cross-linked to protein by difluorodinitrobenzene and suberimidate respectively. This represents 20 and 5.8% of the amino-phospholipids. The lipids extracted from treated ghosts do not react with ninhydrin as do lipids extracted from control ghosts. Thus essentially all the amino-phospholipids of the ghosts react with these cross-linking agents and up to 20% becomes cross-linked to proteins.

Cell membranes contain phospholipids and proteins (1,2). The phospholipids are considered to exist in large part as a lipid bilayer (3,4). Some of the phospholipids are associated with proteins. Acidic phospholipids are expected to interact more with proteins than neutral phospholipids.

We have reported that some of the acidic aminophospholipids of the human erythrocyte membrane are refractory to reaction with fluorodinitrobenzene (5,6). These refractory phospholipids are considered to be tightly bound to membrane proteins. In order to determine what fraction of the aminophospholipids are closely associated to proteins we reacted erythrocyte membranes with the cross-linking agents difluorodinitrobenzene and dimethylsuberimidate and measured the lipid P which became bound to protein.

Niehaus and Wold (7) observed that dimethyladipimidate reduced the amount of protein that could be solubilized from the erythrocyte membrane. Berg et al (8) found that erythrocytes treated with difluorodinitrobenzene resisted lysis by a variety of lytic agents. These workers did not study the interaction of phospholipids with the cross-linking agents. In this paper

we provide data that difluorodinitrobenzene and dimethylsubermidate react with all the amino groups of membrane phospholipids and cross-link from 8-20% of these phospholipids to membrane proteins.

Methods

Erythrocyte ghosts were prepared by the method of Dodge et al (9). Fresh human blood and stored blood bank blood were used for these studies.

Ghosts prepared from 0.3 ml packed red cells from fresh human blood were suspended in 15 ml of Krebs buffer pH 8.5 (284 m osm)* containing 180 mg % glucose. To the ghost suspension were added 8.57 mg of 1,5-difluoro-2,4-dinitrobenzene (DFDNB) (Sigma Chem. Co.) in 0.5 ml methanol. The final concentration of DFDNB was 2.8 mM. Control ghosts were treated as above except DFDNB was omitted. After reaction at 23° C for 1 hour the ghosts were removed by centrifugation for 15 min at 16,000 rpm in a no. 30 Spinco rotor. The ghosts were extracted four times with 3 ml portions of chloroform-methanol 1:1 v/v. The extracts were combined, evaporated to dryness and the lipid dissolved in 0.2 ml of chloroform-methanol 1:1 v/v. The protein residues and the lipid extracts were digested and analyzed for total Pi (10). The lipid extracts were analyzed by TLC as described below.

Ghosts prepared from 0.9 ml of packed red cells from stored human blood bank blood were suspended in 20 ml of buffer pH 8.8 containing 10 mM NaCl and 50 mM $NaHCO_3$. To one batch of ghosts were added 70 mg of solid dimethylsuberimidate hydrochloride (Pierce Chemical Co.). The final suberimidate concentration was 13 mM. The pH of the suspension was rapidly adjusted to 8.8 by addition of 1N NaOH. Control ghosts were treated as above except the suberimidate and NaOH were omitted. The ghost suspensions were incubated at 23° C for 30 min. and centrifuged at 16,000 rpm for 15 min. The ghosts were extracted twice with 3 ml of methanol and three times with 2 ml of chloroform-methanol. The lipid extracts were combined, evaporated to dryness

* This buffer is prepared by mixing the following: 100 ml of 0.154 M NaCl, 4 ml of 0.154 M KCl, 3 ml of 0.055 M $CaCl_2$, 1 ml of 0.154 M $MgSO_4$, 21 ml of 1.3% $NaHCO_3$ and 234 mg of glucose in a final volume of 130 ml.

and the lipid dissolved in 0.4 ml of chloroform-methanol 1:1. 100 µl aliquots were used for analysis of total phosphate (10). 50 µl aliquots were chromatographed on silica gel plates (Merck & Co., Darmstadt, #SG 5763) using a solvent system of chloroform-methanol-water 65/25/4 v/v (11).

The chromatograms from the controls and the DFDNB and suberimidate treated ghosts were sprayed with 0.2% ninhydrin in acetone-lutidine 9/1 v/v. The blue-violet spots corresponding to PE and PS were scraped off the plates, extracted with methanol, the extracts concentrated to 3.0 ml and absorbance read at 565 nm in a Gilford spectrophotometer. Blank areas on the plates were scraped and extracted in order to correct for background color.

Results

Trial experiments were first performed in order to see if the erythrocyte could be protected from lysis by DFDNB and suberimidate. It was found that both of these reagents had to be used rapidly after dissolving in aqueous media. If the agents were dissolved directly in aqueous buffers or in bicarbonate solutions of 1-5% and let stand for longer than 5 minutes before use with suspensions of red cells, very little protection of the cells against lysis was achieved. Both of these cross-linking agents are rapidly hydrolyzed in alkaline aqueous medium. Since the hydrolysis of DFDNB was easy to follow spectrophotometrically, its degradation in 5% bicarbonate was studied. It was found that 24% hydrolysis occurs in 5 minutes and 78% hydrolysis occurs in 70 minutes at 23° C. For this reason the DFDNB was dissolved in methanol prior to use and the suberimidate was added as the pure solid compound. DFDNB is only sparingly soluble in the buffer system used and requires at least 30 min. to achieve complete solution at 23° C. By this time a major part becomes hydrolyzed. This can be visualized by the increase in the yellow color of the solution with time and by a concomitant increase in the absorbance at 344 nm.

Attempts to determine cross-linking of phospholipids to the membrane proteins of intact cells were complicated by the fact that these cells could not be lysed in order to remove the hemoglobin prior to lipid extraction. Lipid

extraction of the cross-linked non-lysable cells was very messy. Therefore red cell ghosts were used.

The data in Table I shows that both DFDNB and suberimidate are ef-

Table I

Cross-Linking of Phospholipids to Proteins In the Ghost Membrane*

Cross-linking Agent	Controls	Reacted
Suberimidate+		
Total extractable lipid P (μ moles)	2.71 ± 0.3 (8)	2.62 ± 0.3 (8)
P bound to protein residue (μ moles)	0.086 ± 0.015 (8)	0.15 ± 0.026 (9)a
Percent of Total Lipid P cross-linked to protein	-	2.33**
Percent of Total Amino-Phospholipid cross-linked to protein‡		5.82
Difluorodinitrobenzene++		
Total Extractable Lipid P (μ moles)	0.95 ± 0.07 (10)	0.85 ± 0.04 (10)
P Bound to Protein Residue (μ moles)	0.034 ± .004 (9)	0.102 ± .01 (10)a
Percent of Total Lipid P Cross-linked to Protein	-	8.4**
Percent of Total Amino Phospholipid Cross-linked to protein‡	-	20.2

* Experimental details given in the text

\+ Ghosts from 0.9 ml packed cells from stored blood bank blood

** This value is based on the P value after subtracting the control P.

++ Ghosts from 0.3 ml packed cells from fresh blood

The values represent the mean ± standard deviation. The number of determinations are shown in parenthesis.

‡ Based on previous analysis that the amino-phospholipids constitute 40% of the total phospholipids (12)

a Statistical analysis gave p value < .005.

fective in cross-linking some amino-phospholipids to the ghost membrane protein as evidenced by an increase in the P content of the protein residue after lipid extraction and by a concomitant decrease in the amount of organic solvent extractable lipid P. DFDNB was nearly four times more effective in cross-linking phospholipid to protein than was suberimidate. DFDNB cross-linked 8.4% of the total lipid P to proteins as compared to 2.3% with suberimidate. This represents 20% and 5.8% respectively of the total amino-phospholipids.

Both DFDNB and suberimidate react nearly completely with all the amino groups of the amino-phospholipids as evidenced by the failure of the extracted lipids from ghosts treated with cross-linking agent to react with ninhydrin (Table II). Thus all the amino groups of these phospholipids are

Table II

Ninhydrin Reaction of Amino-Phospholipids from Control and Treated Ghosts*

	Absorbance 565 nm
Control lipids	0.183
DFDNB reacted lipids	0.002
Suberimidate reacted lipids	0.002

* Experimental details are given in the test. The control amino-phospholipids were easily detected by their blue-violet color after reaction with ninhydrin. The reacted lipids gave no colored derivatives when treated with ninhydrin.

available to these reagents but at most 20% of the amino-lipids are cross-linked to protein.

In comparison to DFDNB which reacts with all the amino-phospholipids of the ghost membrane, we have found earlier (5,6) that FDNB reacts only with 75% of the membrane phosphatidylethanolamine and 26% of the ghost membrane phosphatidylserine when methanol is not employed. Since the system used with DFDNB contains 3.3% methanol whereas the system used with FDNB contained no methanol, part of this difference in extent of reaction of PE and PS may be due to an effect of methanol on the membrane. However some of this difference may be related to a structural alteration in the membrane produced by DFDNB when it cross-links protein to protein, protein to phospholipid and possibly phospholipid to phospholipid.

As observed by Berg et al (8) erythrocytes which have been reacted with DFDNB or suberimidate are very refractory to lysis by water, by sonication, by detergent (0.1 - 1.0% SDS) and by extraction with chloroform-methanol 1:1. The cells appear intact after these treatments (as observed by the light microscope). The stability of these treated cells toward lysis may be due to a cross linked network of protein and phospholipid in the membrane and/or to the intramolecular cross-linking of hemoglobin within the cell.

To our knowledge this is the first report which demonstrates the cross-linking of phospholipids to proteins in cellular membranes. These cross-linking agents can be used to see which phospholipids are juxtaposed to proteins. The data with DFDNB shows that 20% of the total membrane amino-phospholipids are closely associated with proteins. It now remains to be determined which specific proteins are associated with these phospholipids and whether both phosphatidylethanolamine and phosphatidylserine are involved.

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