CROSS-LINKING OF PHOSPHOLIPID NEIGHBORS IN THE ERYTHROCYTE MEMBRANE

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Summary

Erythrocyte ghosts were reacted with difluorodinitrobenzene. The lipids were extracted and hydrolyzed with 3N HCl to yield water soluble dinitrophenyl derivatives of serine and ethanolamine. These derivatives were separated by thin layer chromatography and compared to synthetic standards. The results show that only 5% of the total extractable amino-phospholipids becomes cross-linked. Cross-linking of PE to PS occurs to a greater extent than cross-linking of PE to PE or PS to PS. These results suggest that these amino-phospholipids occur primarily as lipoprotein sub-units on the inner surface of the membrane and that a small fraction occurs as a phospholipid bilayer.

Neighbor analysis of individual phospholipids of biological membranes remains essentially an unexplored problem in membrane biology. We have shown that up to 20% of the amino-phospholipids in erythrocyte ghosts become cross-linked to proteins when difluorodinitrobenzene (DFDNB) is used as the cross-linking agent (1). In this paper we report the cross-linking of neighboring amino-phospholipids (phosphatidylserine and phosphatidylethanolamine) of erythrocyte ghosts by difluorodinitrobenzene (DFDNB). The chemical synthesis of the five possible products resulting from the reaction of ghosts amino-phospholipids with difluorodinitrobenzene (DFDNB) was first carried out (2).

Although the cross-linking of phospholipids to proteins or of phospholipids to phospholipids only recently has begun to be examined in cell membranes, the cross-linking of proteins (either intramolecular or intermolecular) has been studied extensively (3). The cross-linking of proteins in the isolated erythrocyte membrane has been studied by Steck (4).

The neighbor analysis of phospholipids with phospholipids and with

DFDNB-1,5-difluoro-2,4-dinitrobenzene, PE-phosphatidylethanolamine, PS-phosphatidylserine.

proteins may give more insight into the topographical distribution of these components in the cell membrane and reveal the extent of non-random arrangement of lipids and proteins in the membrane and the extent to which lipids occur as a bilayer. The asymmetric arrangement of neuraminic acid (5), acetylcholine esterase (6,7), cholesterol (8) and phospholipids (9-11) has been reported for the erythrocyte membrane.

Materials and Methods

Erythrocyte ghosts were prepared by the method of Dodge et al. (12) using 5 mM Tris buffer pH 7.5 containing 1 mM EDTA. Red Cross bank blood was used in these studies. The erythrocytes were washed three times in isotonic saline before making ghosts. Washed ghosts from 1.0 ml packed cells were suspended in 20 ml of 120 mM $NaHCO_3$-40 mM NaCl buffer pH 8.6 and treated with 50 µl of DFDNB (4.08 mg Sigma Chem. Co.) in methanol. After 30 min reaction at 23°C the ghosts were treated with another 50 µl aliquot of DFDNB and reacted for another 30 min. The ghosts were removed by centrifugation at 15,000 rpm for 15 min in a Spinco No. 30 rotor and washed twice with 15 ml of $NaHCO_3$-NaCl buffer pH 8.6 containing 0.5% bovine serum albumin (Sigma; Fraction V). The ghosts were extracted twice with 8 ml of chloroform-methanol 1:1 for 15 min at 23°C. The chloroform-methanol extracts were evaporated to dryness under nitrogen at 40°C. The yellow lipid residue was dissolved in 3 ml of 3N HCl and hydrolyzed for 2 hrs at 100°C. The suspensions were filtered through sintered glass funnels and the eluates collected in 16 x 100 mm test tubes. The eluates were then made basic with excess $NaHCO_3$ and extracted three times with 3 ml of ethyl acetate (extract I). The phases were separated by centrifugation at 2000 rpm for 5 min. Extract I contains FDNP-ethanolamine, and DNP-bis-ethanolamine. The water phase was then acidified with concentrated HCl and extracted three times with 3 ml each of ethyl acetate (extract II). The phases were separated by centrifugation. Extract II contains FDNP-serine, DNP-bis-serine, serine-DNP-ethanolamine and HO-DNP ethanolamine. The ethyl acetate extracts were evaporated to dryness under nitrogen at 40°C and the residues dissolved in 0.4 ml chloroform-

methanol 1:1 and transferred quantitatively to 20 x 20 cm silica gel glass plates (Merck & Co., Darmstadt, SG-5763). Derivatives from extract I were run in chloroform-methanol-water 65:25:4 v/v. Derivatives from extract II were run first in chloroform-methanol-water 65:25:4 v/v, then in chloroform-methanol-water 50:40:10 v/v for only a distance (about 3 cm) just enough to move the DNP-bis-serine from the origin. The yellow bands were scraped off and eluted three times with methanol in 16 x 100 mm test tubes. After each extraction the tubes were centrifuged at 2000 rpm for 5 min to separate the silica gel from the methanol extracts. The extracts from the bis-derivatives and from HO-DNP-ethanolamine were adjusted to 2.0 ml. The extracts from the mono-derivatives were adjusted to 10 ml. These final extracts were centrifuged at 2000 rpm for 5 min and the absorbance of the clear supernatants was determined on a Gilford spectrophotometer at the λ max of each derivative (2).

Results and Discussion

The cross-linking of neighboring amino-phospholipids in the erythrocyte membrane of DFDNB might involve the serine and ethanolamine groups of phosphatidylethanolamine (PE), phosphatidylserine (PS), their plasmalogen forms (Pl-PE, PL-PS) and their lyso-forms (lyso-PE, lyso-PS, lyso-Pl-PE and lyso PL-PS). This gives rise to over 30 possible cross-linked products depending on the concentration and proximity of these various amino-phospholipids in the membrane. However, if one can minimize the formation of the lysophospholipids and since only PE has a considerable amount of plasmalogen form in the human red cell, one might obtain nine dinitrophenyl derivatives of the intact phospholipids but only 5 dinitrophenyl derivatives of their HCl hydrolysis products. The problem is not this simple since both the lyso-derivatives of PE, PS and Pl-PE occur in appreciable amounts in ghosts and hence they cannot be ignored. The HCl hydrolysis of the dinitrophenylated phospholipid thus offers the simplest system for analysis. It is complicated by some hydrolysis by HCl of the dinitrophenyl derivatives. Serine-DNP-ethanolamine was the most labile. It was hydrolyzed to the extent

TABLE I

Analysis of the HCl Hydrolysis Products of Dinitrophenylated Erythrocyte Ghost Phospholipids

	Compound[a]	Rf value[b]	Absorbance[c]	nmoles[d]	% of Total
(1)	FDNP-Et	0.87	0.94 ± 0.02	672.8	66.8
(2)	HO-DNP-Et[e]	0.79	0.10 ± 0.04	8.2	0.81
(3)	Et-DNP-Et	0.71	0.20 ± 0.03	16.2	1.61
(4)	FDNP-Ser	0.33	0.39 ± 0.01	277.1	27.5
(5)	Et-DNP-Ser	0.28	0.29 ± 0.02	24.1	2.39
(6)	Ser-DNP-Ser	0.07	0.11 ± 0.01	9.2	0.91

[a] Et = ethanolamine, Ser = serine, HO = hydroxy.

[b] Rf values by thin layer chromatography on silica gel plates using chloroform-methanol-water 65:25:4 v/v as solvent. The DNP-serine derivatives 4,5 and 6 were further characterized by conversion to their methyl ester derivatives (2). In this system DFDNB has an Rf of 0.92, FDNP-OH has an Rf of 0.54 and HO-DNP-serine has an Rf of 0.30.

[c] Represents the actual absorbance at the λ max of each compound (2). Values represent the mean ± S.D. of 6 different experiments. Samples (1) and (4) were dissolved in 10 ml of methanol; samples (2), (3), (5), (6) were dissolved in 2 ml of methanol.

[d] Calculated on the basis of the extinction coefficients of each compound (2).

[e] HO-DNP-Et arises from the HCl hydrolysis of Ser-DNP-Et, FDNP-Et and Et-DNP-Et. The major part arises from Ser-DNP-Et.

of 25% in two hours yielding primarily HO-DNP-ethanolamine + serine. By analysis for serine and ethanolamine in the HCl hydrolysate of the intact dinitrophenylated ghost lipids one can ascertain how much hydrolysis occurred in each of the products. This was possible since the reaction of DFDNB with the ghosts phospholipid goes essentially to completion and thus reacted PE and PS

are not present to yield serine and ethanolamine. The analysis of the HCl hydrolysate of the dinitrophenylated lipids showed serine to be the major amino-containing product. The TLC analysis of the hydrolysis products also showed a band having the chromatographic mobility of HO-DNP-ethanolamine. This latter product and serine were formed by the hydrolysis of serine-DNP-ethanolamine.

The analysis of the HCl hydrolysis products of the dinitrophenylated ghost lipids is given in Table I. The raw data showed six dinitrophenyl derivatives were separated by TLC. Five of these (components 1,3,4,5 and 6) have Rf values and spectra identical to the synthetic standards (2). Component 2 has been identified as HO-DNP-ethanolamine by the fact that it is obtained by acid or alkaline hydrolysis of serine-DNP-ethanolamine.

The data in Table I permits the estimation of the degree and type of cross-linking which occurs between the neighboring amino-phospholipids in the erythrocyte membrane. This analysis shows that only 5% of these amino-phospholipids becomes cross-linked to themselves and that 95% is not cross-linked. The intermolecular cross-linking of the amino-phospholipids occurs between PE-PE, PE-PS and PS-PS. The cross-linking between PE with PS is higher than that between PE and PE or between PS and PS.

It must be pointed out that the analysis shown in Table I does not include 20% of the total amino phospholipids which become cross-linked to protein (1). Thus the total amount of cross-linking of the amino-phospholipids to themselves and to proteins involves approximately 25% of all these membrane lipids. Since these lipids are localized primarily on the inner surface of the membrane (9-11) one might expect them to be sufficiently close together so that cross-linking should be enhanced. Moreover, if these phospholipids exist as a bilayer and if the bilayer is a major form in which PE and PS occur in the membrane, one might expect the degree of cross-linking to be greater than 5%. This relatively small degree of phospholipid-phospholipid cross-linking coupled with our earlier finding that phospholipid-protein cross-linking was 20% indicates that PE and PS which are localized

on the inner surface of the erythrocyte membrane are spatially separated by proteins. This leads us to conclude that the phospholipid bilayer does not constitute a major arrangement of PE and PS in the erythrocyte membrane. Lipoprotein sub-units containing PE and PS would be consistent with our data. The outer surface of the erythrocyte membrane is rich in lecithin, sphingomyelin and cholesterol and may very well exist to a large extent as a lipid bilayer (8,10).

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Addendum

We have used the cross-linking agent 4,4'-difluoro 3,3'-dinitrodiphenylsulfone under the same conditions as the difluorodinitrobenzene. With the difluorodinitrodiphenylsulfone the extent of cross-linking of phosphatidylethanolamine to phosphatidylethanolamine and of phosphatidylethanolamine to phosphatidylserine is much greater than the cross-linking which occurs with difluorodinitrobenzene. The cross-linking of phosphatidylserine to phosphatidylserine also occurs but to a lesser extent. The mono-derivatives and bis-derivatives of the difluorodinitrophenylsulfone with serine and ethanolamine have been synthesized.

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