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Topology of Amino Phospholipids in Bovine Retinal Rod Outer Segment Disk Membranes†

Richard C. Crain,‡ Guido V. Marinetti,* and David F. O'Brien

ABSTRACT: The topology of phosphatidylethanolamine and phosphatidylserine in bovine retinal rod disks has been studied with covalent probes. Phosphatidylethanolamine, which comprises 40% of the total membrane phospholipid, was found to be asymmetrically arranged with 18–27% located on the inner surface, 63–72% on the outer surface, and 6–14% not readily available to labeling with chemical probes. Phosphatidylserine, which comprises 10–13% of the total membrane phospholipid, was found to have an arrangement with 25–35% located on the inner surface, 25–31% on the outer surface, and 35–50% resistant to labeling with chemical probes. These results are based on the accessibility of phosphatidylethanolamine and phosphatidylserine to labeling by trinitrobenzenesulfonate at 0 °C in the presence and absence of valinomycin and to labeling by isethionyl acetimidate at 21 °C. Trinitrobenzenesulfonate very slowly penetrates the membrane at 0 °C, whereas it rapidly penetrates at either 21 or 0 °C in the presence of valinomycin. Cross-linking of retinal rod outer segment disks with the penetrating probe difluorodinitrobenzene at 21 °C is dependent on the concentration of difluorodinitrobenzene and the reaction temperature. Maximal cross-linking occurs at 75 μM difluorodinitrobenzene. At this concentration, 72% of phosphatidylethanolamine is cross-linked to phosphatidylethanolamine, 43% of phosphatidylserine is cross-linked to phosphatidylethanolamine, and 6% of the total phosphatidylethanolamine and 37% of the phosphatidylserine are cross-linked to membrane protein. Cross-linking of amino phospholipids to protein is not influenced appreciably by light exposure or temperature. Fatty acid analyses of cross-linked and non-cross-linked amino phospholipids indicate a random array of lipids with respect to their fatty acid content in the rod disk membrane.

Phospholipid asymmetry has been noted recently in a variety of biological membranes (for a review, see Gordesky, 1976; Rothman & Lenard, 1977; Bergelson & Barsukov, 1977). A number of studies used covalent chemical probes (Bretscher, 1972; Gordesky & Marinetti, 1973; Gordesky et al., 1975; Rothman & Kennedy, 1977), phospholipases (Verkleij et al., 1973; Chap et al., 1975; Nilsson & Dallner, 1977), or phospholipid-exchange proteins (Bloj & Zilversmith, 1976; Rothman et al., 1976) to measure asymmetry in both biological and artificial membranes. Furthermore, a reaction of red cell ghosts with the cross-linking agent DFDNB[1] has indicated the existence of clusters of amino phospholipids (Marinetti, 1977).

Several properties of rhodopsin, the major protein of the ROS disk membrane, are very sensitive to environmental factors. These properties of rhodopsin include its light sensitivity, its ability to be regenerated after bleaching, and the kinetics of its light-induced bleaching (Ebrey & Honig, 1975). In the native membrane these properties are dependent on lipid–protein interactions. Rhodopsin can be delipidated, purified, and reconstituted into phospholipid bilayers, digalactosyldiglyceride bilayers, and digitonin micelles. The rhodopsin in these recombinants exists in a regenerable configuration, a characteristic of native rhodopsin (Hong & Hubbell, 1973). It has also been found that the photochemical functionality of recombinants of rhodopsin in artificial phospholipid bilayers is relatively independent of the polar head-group composition of the bilayer but dependent on the lipid fluidity, which is determined by the degree of fatty acid unsaturation (O'Brien et al., 1977).

The composition of rod outer segment membranes which are primarily rod disk membranes has been reviewed (Daemen, 1973). The membrane protein is made up of 80–90% rhodopsin. The majority of the lipid of rod outer segment membranes is composed of phospholipids (80–90%). The lipids are characterized by a high content of polyunsaturated fatty acids. Thus, the rod outer segment membrane is highly unsaturated, has a low cholesterol content, and is very fluid. It has been suggested that the phospholipids may provide a fluid hydrophobic environment for the highly organized visual pigment system.

The present investigation was undertaken to elucidate the phospholipid topology in the ROS disk membrane. Preliminary studies (Raubach et al., 1974) have suggested that there is an asymmetric arrangement of PtdEtn and PtdSer in the disk

† From the Department of Biochemistry, The University of Rochester Medical Center, Rochester, New York 14642, and the Research Laboratories, Eastman Kodak Co., Rochester, New York 14650. *Received April 25, 1978.* This work was supported in part by Grant HLB-02063 from the National Institutes of Health. A portion of this work was presented at the American Society of Biological Chemists (ASBC) Meeting, Atlanta, Ga., June, 1978.

‡ Present address: Division of Nutritional Sciences, Cornell University, Ithaca, N.Y. 14853.

[1] Abbreviations used: ROS, rod outer segment; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; TNBS, 2,4,6-trinitrobenzenesulfonate; FDNB, 1-fluoro-2,4-dinitrobenzene; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; DMA, dimethylacetal; GC, gas chromatography; DNP, dinitrophenyl; TNP, trinitrophenyl; FDNB, fluorodinitrophenyl; Etn, ethanolamine; Ser, serine; MP, methyl picolinimidate; IAI, isethionyl acetimidate; EDTA, (ethylenedinitrilo)tetraacetic acid.

0006-2960/78/0417-4186$01.00/0

membrane. Using the membrane-impermeant probe isethionyl acetimidate (IAI), these workers concluded that 70% of the amino phospholipids are located on the outer membrane surface. This work apparently was confirmed using TNBS, another nonpenetrating probe (Litman, 1974; Smith et al., 1977). From chemical-modification studies, it has been suggested that PtdSer may be closely associated with rhodopsin (deGrip et al., 1973). This was also suggested by the resistance of two molecules of PtdSer per molecule of rhodopsin to cleavage by phospholipase C (Borggreven et al., 1971). In this paper, we present data on the arrangement of PtdEtn and PtdSer in the ROS disk membrane, including asymmetry, nearest-neighbor analysis, and fatty acid analysis of cross-linked and noncross-linked PtdEtn molecules.

Materials and Methods

Frozen, dark-adapted bovine retinas were obtained from George A. Hormel Co. Methyl picolinimidate (MP), IAI, and 2,4,6-trinitrobenzenesulfonate (TNBS) were from Pierce Chemical Co. 1,5-Difluoro-2,4-dinitrobenzene (DFDNB) and 1-fluoro-2,4-dinitrobenzene (FDNB) were purchased from Eastman Organic Chemicals. Hexane, chloroform, and methanol were obtained from Fisher Scientific Co. and were distilled before use. Valinomycin was obtained from Sigma Chemical Co. Silica gel plates (silica gel 60, 0.25-mm thick) were obtained from E. Merck-Darmstadt.

Rod outer segment (ROS) disks were prepared as previously described (Smith et al., 1975). Rhodopsin concentration was determined after solubilization of membranes in detergent from the absorbance at 500 and 280 nm. Phosphate analysis was done spectrophotometrically after digestion in 70% perchloric acid (Harris & Popat, 1954).

Reaction of Chemical Probes with ROS Disks at 21 °C. ROS disks (12–15 nmol of rhodopsin) were reacted at 21 °C with 2 mM TNBS or FDNB (FDNB was added in methanol; the final concentration of methanol was less than 0.5%) in 20 mL of buffer containing 100 mM NaCl, 100 mM $NaHCO_3$, 3 mM KCl, and 1 mM EDTA at pH 8.5. This buffer was used in all subsequent experiments. The reaction was stopped after 30 min by centrifugation at 39 000*g* for 15 min. The membrane pellet was washed once, and the resulting pellet was homogenized in 0.2 mL of 0.2 M sodium buffer (pH 7.0). The lipids were extracted by suspension of the homogenized membranes in 3.8 mL of chloroform–methanol, 1:1 (Folch et al., 1957). The protein was removed by centrifugation. The chloroform–methanol extract was removed quantitatively, and 1.9 mL of chloroform and 1.14 mL of 0.74% aqueous KCl were added. Following centrifugation, the organic phase was taken to dryness under nitrogen, and the lipid was applied quantitatively to silica gel thin-layer plates. The chromatograms were developed in chloroform–methanol–water–acetic acid (65:25:4:2). The reacted PtdEtn and PtdSer were scraped from the plates and eluted with methanol. Quantitation of DNP-PtdEtn (R_f 0.7) and DNP-PtdSer (R_f 0.33) was accomplished spectrophotometrically at 345 nm (ϵ 1.71 × 10^4 cm^{-1} M^{-1}). TNP-PtdEtn (R_f 0.73) and TNP-PtdSer (R_f 0.37) were quantitated by their absorbances at 337 nm (ϵ 1.57 × 10^4 cm^{-1} M^{-1}). Unreacted PtdEtn (R_f 0.53) and PtdSer (R_f 0.23) were detected by spraying the plates with ninhydrin. The bands were removed and the lipids were eluted by heating at 50 °C for 30 min in 4 mL of 1% concentrated HCl in methanol and then with 5 mL of methanol. The combined extracts were transferred to Kjeldahl flasks, the methanol was evaporated, and the total phosphate was determined after digestion with perchloric acid. Within experimental error, the total reacted plus unreacted PtdEtn and PtdSer added up to the total phosphorus in unreacted membranes.

ROS disks (11.5 nmol of rhodopsin) were added to 20 mL of buffer containing 15 mM IAI at pH 8.5 and 21 °C. Every 40 min for up to 160 min, 1-mL aliquots of 300 mM IAI were added. The pH was readjusted to 8.5. To follow the time course of amidination of PtdEtn and PtdSer, two samples were removed every 40 min and the membranes spun down as described above. The lipids were extracted, applied to thin-layer plates, and developed in chloroform–methanol–concentrated NH_4OH (65:25:4). The plates were dried, the bands were visualized by iodine treatment, and *N*-acetimidoyl-PtdEtn and unreacted PtdSer were scraped off and quantitated as described above. The identification of the *N*-acetimidoyl derivatives of PtdEtn has been described previously (Crain & Marinetti, 1978). Two products are formed which can be resolved from unreacted PtdEtn. In this solvent system they have R_f values of 0.60 and 0.42, compared to 0.31 for unreacted PtdEtn. The identification of the *N*-acetimidoyl derivatives of PtdSer is more complicated in a membrane system because they comigrate with other membrane phospholipids. A. Shaw has found that two products are also found for the reaction of PtdSer (Serdary Research Laboratories), which have R_f values of 0.30 and 0.15, compared to 0.07 for unreacted PtdSer in the above solvent system. Quantitation of the percent PtdEtn reacted was derived from comparison of the reacted PtdEtn products to the total PtdEtn from control membranes. The percent reaction of PtdSer was derived from comparison of the amount of unreacted PtdSer in treated membranes to the total PtdSer from control membranes.

ROS disks (11 nmol of rhodopsin) were added to 30 mL of buffer containing MP (5–30 mM) at pH 8.5 and 21 °C. After 1-h reaction, the lipids were extracted and separated on TLC as described above for the IAI derivative. *N*-Picolinimidoyl-PtdEtn (R_f 0.62), unreacted PtdEtn (R_f 0.31), and unreacted PtdSer (R_f 0.08) were scraped off and quantitated by analysis of total phosphate after digestion. The total unreacted and reacted PtdEtn added up to that in unreacted membranes.

Time Course of the Reaction of Disks with TNBS at 0 and 21 °C—Valinomycin Effect. ROS disks (11–11.5 nmol of rhodopsin) were added to 20 mL of buffer at pH 8.5 containing 2 mM TNBS at 0 or 21 °C. The reaction was stopped after 2–120 min by acidification to pH 6.8 with 5 mL of buffer and centrifugation for 20 min at 39 000*g*. TNP-PtdEtn, TNP-PtdSer, and unreacted PtdEtn and PtdSer were quantitated after thin-layer chromatography in chloroform–methanol–concentrated NH_4OH (65:25:4). The experiment was repeated in the same buffer at 0 °C, except 100 mM NaCl was replaced by 50 mM NaCl and 50 mM KCl and 10 μM valinomycin was included.

Cross-Linking of ROS Disks with DFDNB at 21 °C. ROS disks (13–15 nmol of rhodopsin) were added to 20 mL of buffer containing 50–150 μM DFDNB added in methanol to bring the final concentration of methanol to 0.5%. Controls were incubated in buffer without DFDNB. The reactions were carried out at 21 °C and stopped after 2 h by centrifugation at 39 000*g* for 15 min. The pellets were resuspended in the same buffer at pH 6.8. After centrifugation, the membranes were homogenized in 0.2 mL of buffer at pH 6.8. The lipids were extracted as described previously. The protein precipitates were digested in 0.5 mL of 70% perchloric acid, and the phosphate content was determined. The lipid extract was brought to dryness under nitrogen, hydrolyzed for 2 h at 100 °C in 3 mL of 3 N HCl, and extracted twice with 4 mL of ethyl acetate. The ethyl acetate extracts were taken to dryness, applied quantitatively to thin-layer plates, and developed in

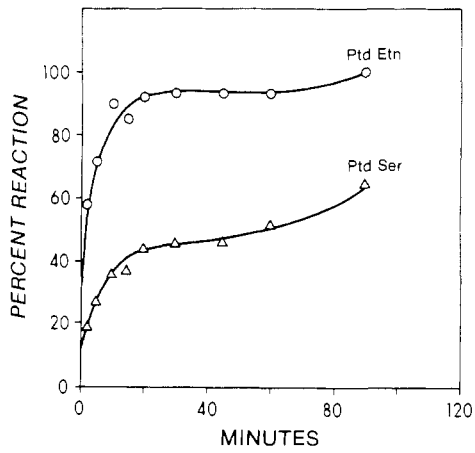

FIGURE 1: Labeling of PtdEtn and PtdSer of ROS disks by TNBS as a function of time at 21 °C. TNP-PtdEtn and TNP-PtdSer were determined spectrophotometrically as described in the text. Each point represents the average of duplicate determinations. Values from two preparations of ROS disk membranes are included.

chloroform–methanol–water (65:25:4). Etn-DNP-Etn, FDNP-Etn, Etn-DNP-Ser, FDNP-Ser, and Ser-DNP-Ser were quantitated spectrophotometrically (Marinetti & Love, 1974) after eluting from the chromatograms with methanol.

Cross-Linking of ROS Disks with DFDNB at 37 °C. ROS disks (11 nmol of rhodopsin) were added to 20 mL of buffer containing 100 μM DFDNB and 0.5% methanol at pH 8.5. Controls were incubated in the same buffer without DFDNB. After incubating for 3 h at 37 °C, the reaction was stopped and aliquots of the samples were analyzed as described above. The remainder were analyzed for fatty acid composition as described by Marinetti and Crain (1978).

Results

Reaction of PtdEtn and PtdSer with TNBS and FDNB at 21 °C. Evaluation of whether PtdEtn and PtdSer are asymmetrically arranged in the ROS disk membrane was performed initially with TNBS as the vectorial probe. Conditions were chosen to saturate available amino groups of PtdEtn and PtdSer. Our initial studies at 21 °C and pH 8.5 showed that 94 ± 1% of the PtdEtn and 43 ± 4% of the PtdSer reacted with TNBS after 30 min in the light or dark. The permeable probe FDNB reacted with 86 ± 5% of PtdEtn and 63 ± 7% of PtdSer. The asymmetry of PtdEtn and PtdSer could not be decided until we determined whether TNBS was indeed a nonpenetrating probe in the ROS disk membrane.

It has been our previous experience that temperature and the type of buffer can significantly alter the permeability characteristics of TNBS in the red cell membrane (Gordesky et al., 1975). A similar finding with respect to temperature was observed with TNBS on *B. megaterium* (Rothman & Kennedy, 1977). Therefore, the time course of reaction of ROS disks with TNBS at 21 and 0 °C was examined. At 21 °C, 94% of the PtdEtn reacts within 30 min, while only 45% of the PtdSer reacts (Figure 1). This confirms our results mentioned above. However, fewer PtdEtn and PtdSer react with TNBS at 0 °C (Figure 1).

Time Course of the Reaction of TNBS with PtdEtn and PtdSer at 0 °C. Because TNBS is present in excess of the total membrane amino groups, pseudo-first-order kinetics will result if all the PtdEtn or PtdSer molecules have the same reactivity and accessibility. Therefore, a semilogarithmic plot of the time course of reaction would yield a single linear component. However, at 0 °C more than one component is observed (Figure 2a). The reaction of both PtdEtn and PtdSer shows

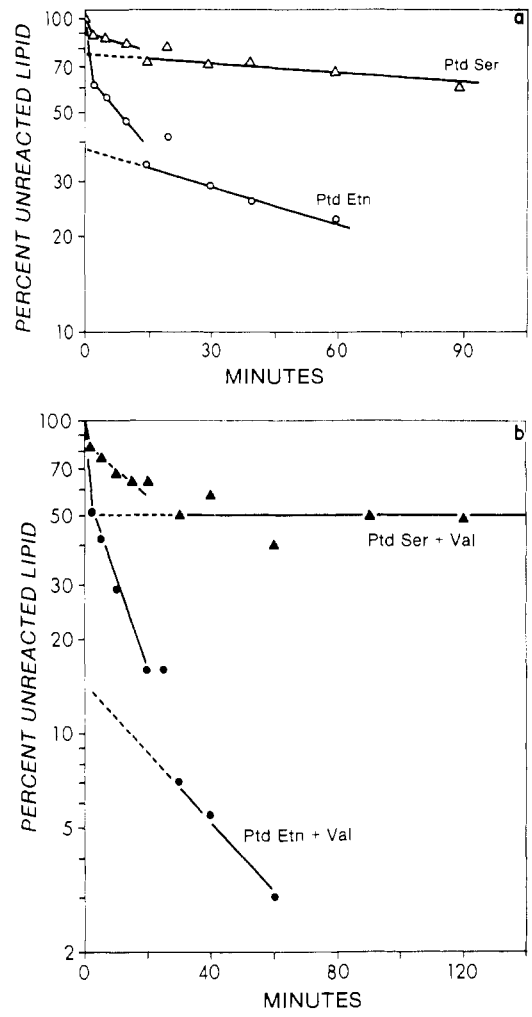

FIGURE 2: Semilog plots of the time course of labeling of PtdEtn and PtdSer of ROS disks by TNBS at 0 °C. The log of percent unreacted PtdEtn and PtdSer is plotted as a function of the time of reaction with 2 mM TNBS: (a) labeling of ROS disk membranes in the absence of valinomycin; (b) labeling of ROS disk membranes in the presence of 10 μM valinomycin. The reaction conditions are given in the text.

both a fast and a slow component. For PtdEtn, the slow component accounts for 37% of the total PtdEtn and reacts with a half-time of 43 min. The fast component, which was calculated by subtracting the slow component (Figure 2a), accounts for 63% of the total PtdEtn and reacts with a half-time of 8 min. For PtdSer, the slow component has a half-time of 300 min, while the fast component has a half-time of 14 min and accounts for 25% of the total PtdSer.

In order to determine if the slow labeling of PtdEtn or PtdSer is due to the penetration of TNBS or to membrane rearrangement and exposure of more PtdEtn and PtdSer molecules, the reaction was repeated at 0 °C in the presence of 10 μM valinomycin (Figure 2b). Valinomycin has been shown to cause penetration of TNBS into red cells (Marinetti et al., 1978), as measured by labeling of hemoglobin. In the presence of valinomycin, two components are observed for the reaction of PtdEtn and one component for the reaction of PtdSer. For PtdEtn, the fast component reacts with a half-time of 8 min and accounts for 86% of the total PtdEtn, while the slow component reacts with a half-time of 25 min and accounts for 14% of the total PtdEtn. For PtdSer, the fast component accounts for 50% of the total PtdSer and has a half-time of 11 min. The remaining 50% of the total PtdSer does not react by 120 min. We therefore conclude that the slow-labeling component of PtdEtn and PtdSer at 0 °C is primarily due to TNBS

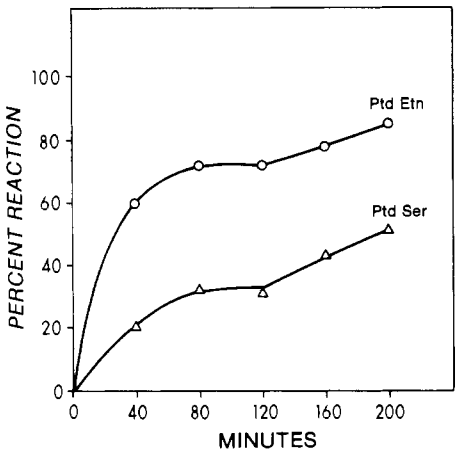

FIGURE 3: Labeling of PtdEtn and PtdSer of ROS disks by isethionyl acetimidate as a function of time at 21 °C. Percent labeling of lipid is plotted as a function of the time of reaction with isethionyl acetimidate. Experimental details are described in the text. Each point represents the average of duplicate determinations in a single preparation of ROS disk membranes.

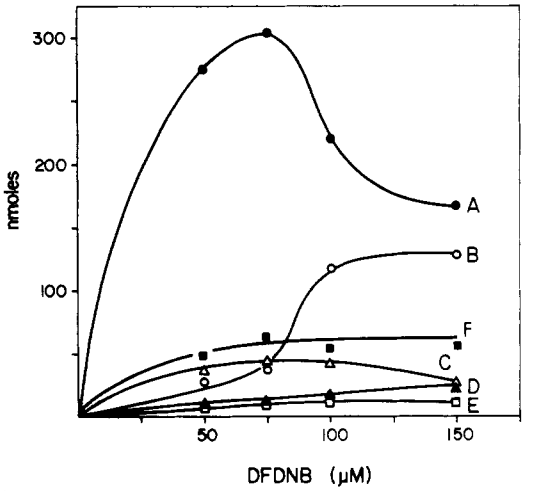

FIGURE 4: Cross-linking of PtdEtn and PtdSer of ROS disks as a function of concentration of DFDNB at 21 °C. PtdEtn and PtdSer in each reaction product are plotted vs. the concentration of DFDNB. The reaction conditions are given in the text. The reaction products are: (A) PtdEtn-DNP-PtdEtn; (B) FDNP-PtdEtn; (C) PtdEtn-DNP-PtdSer; (D) FDNP-PtdSer; (E) PtdSer-DNP-PtdSer; (F) phospholipid-DNP-protein.

penetration into the disk. The rapidly reacting PtdEtn and PtdSer molecules at 0 °C are considered to be on the outer membrane surface. The PtdEtn and PtdSer molecules which react rapidly at 0 °C in the presence of valinomycin are considered to represent the fractions of PtdEtn and PtdSer which are readily accessible on the inner- and outer-membrane surface. The possibility that valinomycin induces an alteration in the membrane, making additional PtdEtn and PtdSer available to TNBS, cannot be ruled out. There is no matrix marker that easily can be used to measure TNBS penetration. From the previous results in the red cell and from the similarity between the log plots at 21 and 0 °C in the presence of valinomycin, we think that the increased labeling is caused by TNBS penetration. These data indicate that 63% of PtdEtn and 25% of PtdSer occur on the outer-membrane surface, while 23% of PtdEtn and 25% of PtdSer occur on the inner-membrane surface. That fraction of PtdSer (50%) which does not react with TNBS at 0 °C in the presence of valinomycin is considered to be masked by tight binding to membrane protein. The PtdEtn (14%) which reacts slowly in the presence of valinomycin is believed to occur in a different configuration, being less accessible than the rapidly reacting PtdEtn.

Reaction of PtdEtn and PtdSer with MP and IAI at 21 °C. The resistance of 50% of the total PtdSer to labeling by TNBS was also observed in the studies using the permeable hydrophilic probe MP. A concentration profile showed that about 90% of PtdEtn is readily reactive at 21 °C, whereas only 50–60% of PtdSer is reactive. Thus, a certain fraction of PtdEtn and PtdSer molecules is not available to react with TNBS, MP, and FDNB. These PtdEtn and PtdSer molecules possibly are tightly associated with ROS membrane protein. This is supported by cross-linking studies with DFNDB, as shown in the next section.

The distribution of PtdEtn and PtdSer in the ROS disk membrane was confirmed using the membrane-impermeable hydrophilic probe IAI (Whiteley and Berg, 1974). Labeling of PtdEtn plateaus at 72% of the total PtdEtn between 1 and 2 h (Figure 3), while labeling of PtdSer plateaus at 31% of the total PtdSer. These reactive PtdEtn and PtdSer molecules are concluded to be localized on the exterior membrane surface. Further reaction causes a linear increase in labeling, presumably due to membrane rearrangement or leakiness. This is probably caused by IAI hydrolysis products (isethionyl acetate and ammonia).

TABLE I: Reaction of ROS Disk Membranes with DFDNB at 21 °C.[a]

react. prod	prod yield[b] (nmol)	PtdEtn equiv nmol	PtdEtn equiv %	PtdSer equiv nmol	PtdSer equiv %
FDNP-Etn	48 ± 9	48	12		
Etn-DNP-Etn	150 ± 4	300	72		
Etn-DNP-Ser	45 ± 2	45	11	45	43
FDNP-Ser	11 ± 1			11	11
Ser-DNP-Ser	5 ± 0.5			10	10
PtdEtn and PtdSer cross-linked to protein	63 ± 6[c]	23	6[d]	38	37[d]

[a] The sample contained 12 nmol of rhodopsin. The DFDNB concentration was 75 μM. The other experimental details are given in the text. [b] Values are the mean ± standard deviation of six determinations. [c] Calculated by phosphate analysis on delipidated protein and corrected for control endogenous phosphate. [d] Calculated using total lipid, total lipid reacted, and total lipid cross-linked to protein. The total amount of PtdEtn was 416 nmol and the total amount of PtdSer was 104 nmol per sample containing 12 nmol of rhodopsin.

Cross-Linking Studies of PtdEtn and PtdSer Using DFDNB. These studies were aimed at determining lipid–lipid and lipid–protein neighbors in the membrane. A concentration profile of cross-linking of the amino phospholipids in the ROS disk membrane is shown in Figure 4. The profile was found to be independent of the bleaching of rhodopsin. At 21 °C, the maximal cross-linking was obtained at 75 μM DFDNB. Below this concentration, incomplete reaction is achieved. Above this concentration, an increased fraction of monoreacted amino phospholipid is achieved. A broad plateau was found for the cross-linking of protein to amino phospholipid by DFDNB in the range of 50–150 μM.

At 21 °C under maximal cross-linking conditions, 72% of the PtdEtn was found as PtdEtn-DNP-PtdEtn, 12% was found as FDNP-PtdEtn, and 11% was found as PtdEtn-DNP-PtdSer. By comparison, 10% of the total PtdSer was found as PtdSer-DNP-PtdSer, 11% as FDNP-PtdSer, and 43% as PtdEtn-DNP-PtdSer. Table I gives data on the amount of each of the cross-linked products. From the amount of lipid phosphate cross-linked to protein, the amount of PtdEtn and PtdSer

TABLE II: Analysis of Fatty Acids and Fatty Aldehydes of PtdEtn and PtdSer of ROS Disk Membranes and of PtdEtn + PtdSer Cross-linked to Membrane Protein.[a]

fatty acid and aldehyde	PtdEtn	PtdSer	control[b]	theoret[c] DFD-NB	exptl DFDNB
16:0 DMA	0.7 ± 0.2	tr[e]		0.3	
16:0	13.0 ± 0.5	5.4 ± 0.3	53.9	8.7	7.2± 01
16:1	0.3 ± 0.01	0.5 ± 0.04		0.4	
17:1 + 18:0 DMA	1.3 ± 0.06	0.2 ± 0.01		0.7	0.7 ± 0.06
18:0	27.1 ± 0.3	23.8 ± 0.4	19.2	25.2	23.3 ± 0.25
18:1	4.0 ± 0.06	2.6 ± 0.06		3.2	2.5 ±0.01
18:2	1.3 ± 0.01	0.4 ± 0.03		0.8	0.5 ± 0.01
20:0	0.3 ± 0.01			0.1	tr
20:0 H	8.1 ± 0.2	8.0 ± 0.1		8.0	9.5 ± 0.1
20:2	0.1 ± 0.01				
20:3	0.7 ± 0.06	0.3 ± 0.02		0.5	0.5 ± 0.06
20:4	4.8 ± 0.06	0.3 ± 0.02		2.5	4.2 ± 0.15
22:2			13		
22:4	3.1 ± 0.03	6.1 ± 0.1		4.8	5.1 ± 0.15
22:5	0.7 ± 0.03	0.7 ± 0.1		0.7	
22:6	34.6 ± 0.8	34.3 ± 0.5	13.9	34.5	40.5 ± 0.5
24:X[d]	tr[e]	10.1 ± 0.1		5.8	6.0 ± 0.2

[a] Values are the mean ± standard deviation of percent area of each component analyzed in triplicate. [b] Fatty acid analysis of delipidated protein. For experimental conditions, see the text. [c] Theoretical fatty acid analysis arrived at from estimation of PtdEtn and PtdSer bound to protein. [d] Identification based on previous analysis (Anderson and Maude, 1970). [e] tr, trace component.

TABLE III: Analysis of Fatty Acids and Fatty Aldehydes of PtdEtn, FDNP-PtdEtn, and PtdEtn-DNP-PtdEtn.[a]

fatty acid	PtdEtn	FDNP PtdEtn	PtdEtn DNP-PtdEtn
16:0 DMA	0.7 ± 0.2	0.36 ± 0.01	tr[b]
16:0	13.0 ± 0.5	9.7 ± 0.2	9.9 ± 0.01
16:1	0.3 ± 0.01	0.22 ± 0.01	0.9 ± 0.09
17:1 + 18:0 DMA	1.3 ± 0.06	1.3 ± 0.01	2.6 ± 0.2
18:0	27.1 ± 0.3	28.0 ± 0.3	27.0 ± 0.3
18:1	4.0 ± 0.06	3.2 ± 0.03	6.8 ± 0.03
18:2	1.3 ± 0.01	0.7 ± 0.01	1.0 ± 0.06
20:0	0.3 ± 0.01	0.3 ± 0.06	
20:0 H	8.1 ± 0.2	8.6 ± 0.1	7.6 ± 0.3
20:2	0.1 ± 0.01		
20:3	0.7 ± 0.06	0.9 ± 0.1	
20:4	4.8 ± 0.06	4.9 ± 0.1	5.1 ± 0.3
22:4	3.1 ± 0.03	3.6 ± 0.1	3.0 ± 0.5
22:5	0.7 ± 0.03	0.6 ± 0.05	
22:6	34.6 ± 0.08	37.7 ± 0.5	36.2 ± 0.5

[a] The ROS disk membranes were reacted with 100 μM DFDNB at 37 °C. The values represent the mean ± standard deviation of one sample analyzed in triplicate and are the percent area of each component relative to the total area of all components. [b] tr, trace component.

accounted for by all other products and the total amount of PtdEtn and PtdSer from control membranes, it was determined that 6% of the total PtdEtn and 37% of the total PtdSer were cross-linked to protein. This represents approximately 2 mol of PtdEtn and 3 mol of PtdSer cross-linked to protein for each mole of rhodopsin in the sample.

Fatty Acid Analysis of Lipid Cross-linked to Lipid and Lipid Cross-linked to Protein. In order to determine if cross-linking was affected by temperature and to test whether there is a nonrandom arrangement of specific molecular species of PtdEtn in the membrane at physiological temperature, ROS disk membranes were cross-linked at 37 °C for 3 h in the presence of 100 μM DFDNB. Under these conditions, less cross-linking of lipid to lipid is produced compared to 21 °C. However, slightly greater cross-linking of PtdEtn and PtdSer to protein is observed. It was found that 11% of the total PtdEtn and 39% of the total PtdSer molecules were cross-linked to protein at 37 °C. This represents approximately 3 mol of PtdEtn and 4 mol of PtdSer cross-linked to protein for each mole of rhodopsin in the sample.

The fatty acid composition of control PtdEtn and PtdSer, FDNP-PtdEtn, PtdEtn-DNP-PtdEtn, lipid cross-linked to protein, and endogenous protein-bound lipid was evaluated. Both PtdEtn and PtdSer are characterized by a high content of polyunsaturated fatty acids. PtdSer contains a unique fatty acid which has been identified as an unsaturated 24 carbon fatty acid (Anderson & Maude, 1970). This fatty acid is designated as 24:X and runs with a retention time of a 24:4 fatty acid. We find that 35 nmol of PtdEtn and 47 nmol of PtdSer are cross-linked to protein in each sample. Therefore, 57% of the lipid cross-linked to protein (designated as lipid-DNP-protein) is accounted for by PtdSer and 43% by PtdEtn. Using these values, a theoretical fatty acid composition was calculated for the lipid-DNP-protein. Table II represents the results of fatty acid analysis of control PtdSer, control PtdEtn, control delipidated protein (representing endogenous tightly bound lipid), lipid-DNP-protein, and the calculated fatty acid composition of lipid-DNP-protein. It was found that delipidated control membrane proteins contain less than 2% of the total fatty acids cross-linked to membrane proteins. It was therefore neglected in calculating the theoretical fatty acid composition of lipid cross-linked to protein. The fatty acid composition of the amino phospholipid cross-linked to protein was found to be in very good agreement with the calculated composition (Table II), except for the 22:6 fatty acid. Likewise, the fatty acid composition of FDNP-PtdEtn and PtdEtn-DNP-PtdEtn was not different from unreacted PtdEtn (Table III). These results suggest a random array of PtdEtn in the highly fluid ROS disk membrane as contrasted to the nonrandom array of PtdEtn in the less fluid red cell membrane (Marinetti & Crain, in press).

Purity of the Membrane Preparation. Experiments using chemical labeling to determine membrane topology are critically dependent on the purity and integrity of the membrane studied. ROS disks are an excellent membrane for study due to both their purity and their osmotic integrity (Smith et al., 1975). Further evidence confirms the purity of our membrane preparation. The membranes were found to contain 28.5 ± 1.7 mol of PtdEtn and 9.7 ± 2.3 mol of PtdSer per mole of rhodopsin. These values agree well with the literature values of 27.3 ± 1.2 mol of PtdEtn and 8.4 ± 0.6 mol of PtdSer per mole of rhodopsin (deGrip et al., 1973). Moreover, the fatty acid analyses of PtdEtn and PtdSer are in good agreement with those found by Anderson and Maude (1970). For PtdEtn we found 34.6% of the total fatty acid to be 22:6, 27% to be 18:0, and 0% to be 24:X. For PtdSer, we found 34.4% of the total fatty acid to be 22:6, 23.8% to be 18:0, and 10.1% to be 24:X (Table III). Anderson and Maude found 31.7% of 22:6, 21.9% of 18:0, and 1.4% of 24:X for PtdEtn and 32% of 22:6, 19.2% of 18:0, and 10% of 24:X for PtdSer.

Discussion

In this report the topology of PtdEtn and PtdSer in the ROS disk membrane is examined. We have attempted to find if they are asymmetrically arranged, occur in clusters, and are tightly associated with membrane protein. PtdEtn was found to be asymmetrically arranged with 63–71% located on the exterior

membrane surface, 18–27% located on the inner surface, and 6–14% less accessible to chemical labeling. However, only 50% of the PtdSer reacted, of which 25% is on each membrane surface. The remaining 40–50% is not readily accessible to labeling by FDNB, TNBS, or MP and is assumed to be masked by tight interaction with protein.

The refractory nature of a large fraction of PtdSer to chemical labeling is not unique to the ROS disk membrane. In the membrane of intact red cells only 25% of PtdSer is labeled by FDNB and no PtdSer is labeled with TNBS (Gordesky & Marinetti, 1973; Gordesky et al., 1975). The resistance to chemical labeling of PtdSer in intact red cells has also been observed by Poensgen & Passow (1971). Furthermore, PtdSer in the red cell ghosts is refractory to cleavage by phospholipase C from *C. welchii* (Coleman et al., 1970). Phospholipase C from *B. cereus,* however, cleaves 90–100% of the PtdSer in ghosts (Verkleij et al., 1973). The close association of PtdSer to membrane protein and the biological significance of the association are supported by the observation that PtdSer is involved in the hormonal control of adenylate cyclase in rat liver plasma membranes (Rethy et al., 1972), is required for the ATPase activity of the sodium pump (Wheeler & Whattam, 1970), and is involved in nerve excitation (Cook et al., 1972).

Earlier work in our laboratory has indicated a nonrandom distribution of PtdEtn and PtdSer in the red cell membrane (Marinetti, 1977). More recent work has confirmed this conclusion, since we have found that the cross-linked PtdEtn molecules (PtdEtn-DNP-PtdEtn) are primarily the plasmalogen form of PtdEtn (Marinetti & Crain, 1978).

In order to explore further the topology of the ROS disk membrane, chemical labeling studies were performed using DFDNB, a hydrophobic cross-linking agent. The amount of lipid–lipid and lipid–protein cross-linking at both 37 and 21 °C was measured. The quantitation of amino phospholipid cross-linked to protein is in good agreement with the resistance of a portion of PtdEtn and PtdSer to labeling by TNBS, FDNB, or MP. Thus, 6–12% of PtdEtn is cross-linked to protein by DFDNB, while 6–14% is less available to reaction with TNBS, FDNB, or MP. Likewise, 35–39% of the PtdSer is cross-linked to protein by DFDNB, while 35–50% is not reactive with TNBS, MP, and FDNB.

Fatty acid analyses on FDNP-PtdEtn, PtdEtn-DNP-PtdEtn, and control PtdEtn indicate a random array of the free phospholipids (not associated with protein) with respect to fatty acid composition within the ROS disk membrane. These results suggest a random array of PtdEtn in the highly fluid ROS disk membrane, as contrasted to the nonrandom array of PtdEtn in the less fluid red cell membrane (Marinetti & Crain, in press). Fatty acid analysis of lipid–DNP-protein also suggests a random array, although the cross-linked protein is slightly enriched in 22:6.

Since rhodopsin accounts for 80–90% of the ROS membrane protein, it is probable that a major portion of the phospholipids cross-linked to protein is cross-linked to rhodopsin. The ROS membrane is known from rhodopsin-mobility measurements to be highly fluid (Poo & Cone, 1973; Liebman & Entine, 1974). It is likely that the major part of the phospholipid molecules in the ROS membrane diffuses rapidly in the plane of the membrane; therefore, the cross-linking data could represent the average of amino phospholipids near rhodopsin during the reaction. The additional observation that a similar quantity of amino phospholipids is unreactive to TNBS and is cross-linked to protein suggests that these same PtdEtn and PtdSer molecules are in close association with the rhodopsin. Since rhodopsin can be completely delipidated in detergents (Hong & Hubbell, 1973), this association cannot be covalent in nature but rather is due to ionic or hydrophobic interactions. The possible functional role of the amino phospholipids closely associated with rhodopsin is uncertain. It is noteworthy that PtdSer has been suggested to play a role in ion exchange in nerve membranes (Cook et al., 1972) and in the regulation of Ca^{2+} binding to membrane surfaces (Seeman et al. 1974; Cardinas & Ross, 1975). deGrip et al., (1973) have suggested that PtdSer in the membrane may react with liberated retinaldehyde after light bleaching of rhodopsin.

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Transmembrane Movement and Distribution of Cholesterol in the Membrane of Vesicular Stomatitis Virus†

Eric J. Patzer, J. Michael Shaw, Norman F. Moore,‡ Thomas E. Thompson, and Robert R. Wagner*

ABSTRACT: The transmembrane movement and distribution of cholesterol in the vesicular stomatitis virus membrane were studied by following the depletion of cholesterol from virions to interacting phospholipid vesicles and by exchange of radiolabeled cholesterol between virions and phospholipid–cholesterol vesicles. The kinetics of the cholesterol exchange or depletion reactions revealed the presence of two exponential rates: a rapid rate, dependent on the vesicle to virus ratio, and a slower rate, independent of the vesicle to virus ratio. The kinetics of cholesterol movement could be best interpreted by a model of the virion membrane considered as a two pool system in which ~30% of the cholesterol resides in the outer monolayer and ~70% in the inner monolayer. The half-time for equilibration of the two pools was calculated to be 4–6 h and was assumed to represent the time required for transmembrane movement of cholesterol across the bilayer. The initial rate of transfer of cholesterol from virus into vesicles increased when vesicle phospholipids contained more unsaturated and shorter chain fatty acids. Furthermore, the transfer of cholesterol appeared to occur by a collisional mechanism requiring membrane–membrane contact. Interaction with lipid vesicles did not significantly affect the integrity of the virion membrane as assessed by the relative inaccessibility of internal proteins to lactoperoxidase-catalyzed iodination and by the small loss of [^{3}H]amino acid labeled protein from the virus.

Studies of the distribution and degree of transmembrane movement of cholesterol in synthetic lipid vesicles and biological membranes have to date yielded conflicting results. Initial studies showed that synthetic liposomes (Poznansky and Lange, 1976, 1978), the erythrocyte membrane (Gottlieb, 1976), and the influenza virus membrane (Lenard and Rothman, 1976) contain cholesterol in two pools, presumably in the inner and outer monolayers; the inner monolayer cholesterol was assumed to be slowly exchangeable or nonexchangeable. In contrast, more recent evidence suggests that the cholesterol of liposomes is completely exchangeable as one pool (Bloj and Zilversmit, 1977) and the erythrocyte membrane contains two pools of cholesterol, both rapidly exchangeable (Lange et al., 1977). Clearly, more work is needed on these and other systems to establish the location and rates of transmembrane movement of cholesterol.

We undertook to study the distribution and movement of cholesterol in the membrane of vesicular stomatitis (VS)[1] virus. This virus can be purified to homogeneity in large quantities, and membrane constituents can be specifically radiolabeled during virus growth (Wagner, 1975). Since virions bud from the plasma membrane, the viral cholesterol distribution should reflect that of the host cell and other eucaryotic cell plasma membranes. Moreover, a great deal of evidence concerning the distribution of proteins and phospholipids in the viral membrane has already been obtained. Of the two membrane-associated proteins, the glycoprotein (G) has been shown to be asymmetrically oriented toward the external milieu (Schloemer and Wagner, 1975), whereas the matrix (M) protein appears to lie internal to the permeability barrier of the membrane (Moore et al., 1974). Similarly, the phospholipids and their fatty acyl chains have an asymmetric orientation with the majority of the choline phospholipids located in the external layer and the majority of amino phospholipids and polyunsaturated fatty acids located internally (Patzer et al., 1978). In previous studies it was found that >90% of the cholesterol in the VS virion membrane could be oxidized from the external surface by cholesterol oxidase after removal by phospholipase C of phospholipid head groups (Moore et al., 1977) or could be >90% depleted from the viral membrane by interaction with phospholipid vesicles (Moore et al., 1978).

In the present studies the movement of cholesterol between VS virus and lipid vesicles has been carefully examined to analyze the kinetics of the exchange process. These data have allowed us to estimate the percentage of membrane cholesterol in the inner and outer monolayers and the rates of transmembrane movement.

† From the Departments of Microbiology and Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22901. *Received March 31, 1978.* Supported by United States Public Health Service Grants AI-11112, GM-14628, and CA-18701, by National Science Foundation Grant PCM 77-00494, and by American Cancer Society Grant VC-88.

‡ Present address: Unit of Invertebrate Virology, Oxford University, Oxford OX1 3RB, England.

[1] Abbreviations used are: VS, vesicular stomatitis; G, glycoprotein; M, matrix protein; L, large protein; N, nucleocapsid protein; BHK, baby hamster kidney; PS, phosphatidylserine; PC, phosphatidylcholine; NaDodSO$_4$, sodium dodecyl sulfate; TLC, thin-layer chromatography; PBS, phosphate-buffered saline; DMPC, DPPC, and DOPC, dimyristoyl-, dipalmitoyl-, and dioleoylphosphatidylcholine; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

0006-2960/78/0417-4192$01.00/0