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A SPIN LABEL STUDY OF THE URINARY BLADDER LUMINAL MEMBRANE

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Spin label experiments have been carried out on the urinary bladder luminal membrane of the bovine transitional epithelium employing the 5-, 7-, 12-, and 16-doxyl substituted stearic acid methyl esters, and compared for reference to similarly labeled bovine erythrocytes. The bladder membranes are significantly different from the bovine red blood cell membranes and show a lower order and polarity near the membrane surface. This fact and the general similarity of results for the bladder and isolated plaque membranes suggests that the highly organized proteins of the bladder membrane may act as a coat on the lipid bilayer and, while intrinsic in nature, do not significantly perturb the hydrophobic core of the lipid bilayer.

Introduction

Closed packed oligomeric aggregates cover 70–80% of the surface of the luminal membrane of the mammalian transitional epithelium [1–4]. The subunits (hexagons) that protrude 5 nm over the surface of the luminal leaflet are associated in the plane of the membrane in roughly circular paracrystalline domains or plaques of 0.5–2 μm in diameter separated by interplaque membrane deprived of subunits (hinge membranes). Details of the structure of these paracrystalline plaques are known at about 3.5 nm resolution and more precise information is being collected by low dose electron microscopy and three dimensional reconstruction [5,6].

In contrast little is known about the organization of the plaque membrane in the axis perpendicular to the plane of the membrane. There is not agreement about the molecular weight of the subunit protein [7,10,8], the numbers of protomers comprised by the individual hexagon (6 or 12), the interaction of the oligomeric aggregates with the lipid bilayer or cytoplasmic proteins [3] or the degree of penetration of the protein in the hydrophobic core of the bilayer [9].

In this paper we approach some of these problems by ESR spectroscopy. We have evaluated order and flexibility gradient parameters of the bilayer hydrophobic environment using fatty acid spin labels incorporated in isolated luminal membranes, detergent isolated plaques and liposomes of extracted lipids in an effort to gain information on the organization of the lipid bilayer and lipid-protein interactions in this system. We attempt to determine to what extent the long range protein order observed in these membranes influences the lipid order and dynamics and, to the extent it does, to define the most probable protein-lipid interaction. We are interested in the degree of penetration of the protein subunits in the lipid bilayer and if this organization is shared by all membranes with a regular array of subunits. Parallel studies were carried out on bovine erythrocytes, their ghosts, and liposomes of red blood cells extracted lipids to establish a line of comparison between membranes with a regular array of subunits and membranes with a random distribution

of proteins. We have concluded that the lipid bilayer of the luminal membrane of the urinary bladder is more fluid and less ordered than the erythrocyte membrane near the surface of the membrane, and that the protein subunits apparently do not extend deeply into the bilayer.

Material and Methods

1. Isolation of bladder luminal membranes

Cow urinary bladders obtained from a local slaughterhouse were used as a source of transitional epithelium luminal membranes. The organs were kept at 4°C until ready to be processed and all the subsequent steps of membrane isolation were carried out at this temperature. The epithelium was gently scraped out with a spatula and collected in 100 ml of 8.5% w/w sucrose/0.02% sodium azide/1 mM EDTA/0.1 mM DMPF (phenylmethyl sulfonyl fluoride) in 10 mM phosphate buffer, pH 7.8. The cell suspension was homogenized by 20 strokes in a glass homogenizer using a glass pestle. The homogenate was brought to 500 ml with the same solution, filtered through four layers of cheese cloth and centrifuged at 5000 rpm ($3000 \times g$) for 10 min in a GSA rotor using the RC-2B Sorvall centrifuge. The pellet was discarded and the pooled supernatant centrifuged at 15000 rpm ($19000 \times g$) for 60 min in a 30 Beckman rotor. The pellet was resuspended in 43% sucrose in phosphate buffer and the following discontinuous sucrose gradient was prepared in a 40 ml nitrocellulose tube: 5 ml of 50% w/w sucrose were deposited in the bottom of the tube as a cushion and overlaid with 5 ml aliquot of 43% sucrose resuspended pellet, 10 ml of 37% sucrose in phosphate buffer, 10 ml of 34% w/w sucrose and 8 ml of 10 mM phosphate buffer on top. The gradient was centrifuged at 25000 rpm ($81000 \times g$) in a Rotor SW 28 in the L-5 Beckman centrifuge for 4 h. Bands were located at all the interfaces at the end of the run. The material located in the 37/34% w/w interface contained large sheets of hexagonal membranes, a small amount of mitochondrial fragments, and smooth membranes. Further purification of this fraction was carried out on a continuous gradient of 40 to 20% sucrose in 10 mM phosphate buffer. The luminal membranes equilibrated in a zone around 34% w/w sucrose ($d = 1.14 \cdot ml^{-1}$) after centrifugation at 35000 rpm ($147 \times g$) in a rotor SW 50-1 for 12 h. Aliquots of the membrane suspension were frozen in liquid nitrogen and stored at −50°C until processed for ESR spectroscopy.

2. Preparation of isolated plaques

Isolated luminal membranes were resuspended in 10 mM phosphate buffer, pH 8.0, containing 0.5% of sodium deoxycholate and incubated at room temperature for 15 min with mild agitation. The suspension was then centrifuged at 15000 rpm for 20 min in a Rotor SW 41 in an L-5 Beckman centrifuge. The pellet was resuspended in 2 ml of 42% sucrose, separated on a discontinuous gradient consisting of a 1 ml aliquot of sample on the bottom of 5 ml nitrocellulose tubes and 1.75 ml each of 36% and 34% w/w sucrose and 0.5 ml of the same buffer and centrifuged for 4 h at 45000 rpm in a Rotor SW 50-1. A distinct band at the 34% w/w sucrose interface contains the isolated hexagonal plaques. This material was dialyzed against 10 mM phosphate buffer containing 0.02% sodium azide and 1 mM EDTA for 16 h at 4°C with two changes of buffers.

3. Preparation of bovine erythrocytes and ghosts

Erythrocytes and red blood cell ghosts were isolated from fresh-drawn heparinized cow blood according to the procedure of Steck and Kant [15]. The final pellet was resuspended in saline phosphate buffer and stored frozen at −40°C.

4. Lipid extraction and spin labelling

Lipids were extracted from isolated bladder membranes with chloroform/methanol (2 : 1, v/v) saturated with Argon and containing 0.0005% of butylated hydroxytoluene. The chloroform phase was dried in a rotary evaporator and the lipid resuspended in chloroform at a concentration of 1 mg/ml. Aliquots of lipids were mixed in a test tube with appropriate volume of spin label stock solutions in ethanol and the mixture dried with a jet of nitrogen or argon. The spin label to lipid molar ratio was maintained at 0.02. The dried lipid film was stored overnight under vacuum in the cold room to assure complete evaporation of the organic solvent.

Multilamellar liposomes were prepared by in-

cubation of the dried lipid-spin-label film with 0.5 ml of saline phosphate buffer at room temperature with gentle agitation.

5. Labelling of intact red blood cells

10 μl of a $3.2 \cdot 10^{-4}$ M stock solution of spin label in ethanol were dried in a glass tube with a jet of N_2. 0.5 ml of erythrocyte suspension was added and incubated for 30 min at room temperature under low speed vortex. The labelled red blood cells were washed twice in saline phosphate buffer and resuspended in 0.25 ml of this buffer prior to taking the spectra.

6. Labelling of isolated hexagonal membranes and red blood cells ghosts

0.5 ml of suspensions of bladder membranes or red blood cells ghosts containing 1 mg of membrane protein were labelled using the procedure described for intact red blood cells.

7. ESR Spectroscopy

5-, 7-, 12- and 16-doxyl substituted analogues of stearic acid methyl esters were purchased from Molecular Probes Inc., Plano, Texas, and utilized without further purification. $3.2 \cdot 10^{-4}$ M stock solutions of the spin labels were prepared in ethanol and stored at 4°C. The materials, labelled as described above, were placed in quartz flat cells or capillary tubes for electron spin resonance spectroscopy. The spectra were recorded on a standard V4502 Varian ESR spectrometer operating at 9 GHz at room temperature with a sweep range of 100 Gauss and a scan time of 5 or 10 min.

Calibration of the recorder was carried out by using as a primary standard potassium peroxylamine disulfonate in saturated K_2CO_3 according to Faber and Frankel [11]. The order parameter S was calculated using the distance between the outer ($T_{\|}$) and inner ($T_{\perp}$) extrema of the ESR spectra in the formalism of Gaffney [12]. Reference crystal parameters for a doxyl-aliphatic chain according to Jost et al. [14] were employed. The isotropic nitrogen hyperfine coupling constant is denoted as A_N.

Results and Discussion

The average values and standard deviations for the A_N and S parameters are given in Table I for the six types of systems investigated. All data for a given label and system taken on a given day were considered as one data point when calculating averages; thus, the parameter n, the number of data points, denotes the number of experiments carried out on the indicated system and label. In each experiment at least two and often six or more spectra were acquired for each labeled system. Results from spectra that were extremely weak (making the measurements uncertain) or which showed distortions (due likely to high label concentration exchange effects) [13] were dropped from the analysis. In cases where the standard deviation between separate runs (separate days) was less than the standard deviation from spectral measurement within a given run, the larger value was employed. This happened only infrequently and the standard deviation employed in these cases is quite in line with those observed for similar experiments. Means and standard deviations are given in Table I, and represented graphically in Fig. 1.

As shown in Fig. 2, the spectra observed for each label are quite similar in general appearance

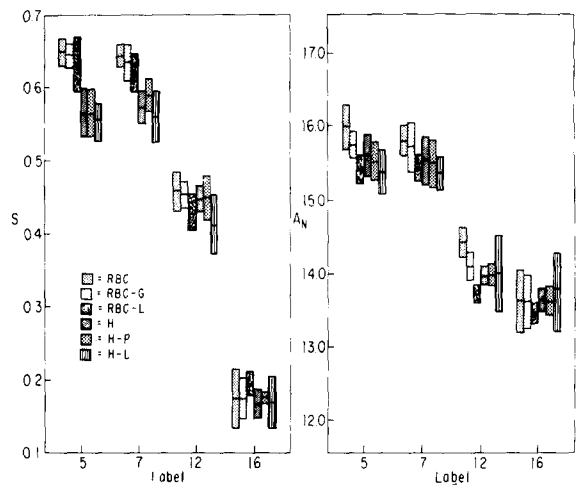

Fig. 1. Plots of the order parameters S and polarity factors A_N for the 5-, 7-, 12-, and 16-spin labels. The bars plotted have their centers at the observed averages and a size which is two standard deviations on either side of the average. The six bars for each label are in the order from left to right of red blood cell (RBC), red blood cell ghost (RBC-G), red blood cell liposomes (RBC-L), hexagonal membranes (H), hexagonal plaques (H-P), and hexagonal membrane liposomes (H-L).

TABLE I

Averages (A_N, S) and standard deviations (σ_{a_N}, σ_S) for n independent determinations of the polarity (isotropic hyperfine constant, A_N) and rigidity (order parameter, S) factors for the labels and systems studied. Abbreviations for the systems are indicated in parentheses.

	n	A_N	σ_{a_N}	S	σ_S
1. Red blood cell (RBC)					
label = 5	7	16.01	0.150	0.648	0.0093
7	5	15.82	0.099	0.643	0.0040
12	12	14.44	0.099	0.458	0.0129
16	6	13.64	0.213	0.176	0.0194
2. Red blood cell ghosts (RBC-G)					
label = 5	10	15.76	0.085	0.644	0.0081
7	7	15.73	0.158	0.635	0.0134
12	11	14.12	0.093	0.454	0.0085
16	11	13.63	0.177	0.176	0.0141
3. Red blood cell liposomes (RBC-L)					
label = 5	6	15.44	0.103	0.631	0.0188
7	7	15.45	0.083	0.619	0.0136
12	8	13.72	0.056	0.429	0.0123
16	9	13.48	0.073	0.172	0.0076
4. Hexagonal membranes (H)					
label = 5	15	15.63	0.136	0.565	0.0163
7	11	15.55	0.160	0.573	0.0106
12	10	13.99	0.056	0.449	0.0086
16	10	13.65	0.082	0.168	0.0101
5. Hexagonal plaques (H-P)					
label = 5	6	15.54	0.125	0.565	0.0156
7	47	15.51	0.159	0.589	0.0110
12	6	14.00	0.066	0.450	0.0147
16	5	13.63	0.103	0.177	0.0040
6. Hexagonal membrane liposomes (H-L)					
label = 5	7	15.40	0.154	0.557	0.0098
7	4	15.38	0.113	0.560	0.0173
12	6	14.02	0.259	0.414	0.0195
16	6	13.82	0.239	0.171	0.0176

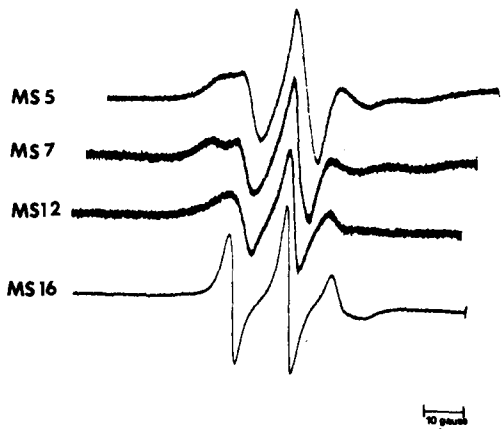

Fig. 2. ESR spectra of spin labels 5-, 7-, 12-, and 16-doxyl-stearic acid methyl ester incorporated urinary bladder membranes.

to those observed for labeled red blood cells [16] with the differences coming in the actual quantitative measurements of A_N and S. Since the various standard deviations are similar and since there is no a priori reason to think otherwise, the t-values were calculated assuming samples drawn from normal populations whose standard deviations are equal.

The results of the t-tests are shown in Table II, a significance table for the six systems investigated. The spin labels are denoted as 5, 7, 12, and 16 according to the location of the doxyl group and appear in the table at the intersection of the

TABLE II

Significance table for the six systems and four spin labels studied. Absence of an entry between pairs of systems indicates no statistical difference in a two-tailed *t*-test at the 95% level of confidence; values in parentheses indicate a probable significance (95–99% level) while unbracketed values indicate high significance (99% level). Abbreviations are as given in Table I. Only upper right entries are given.

	A_n					
	RBC	RBC-G	RBC-L	H	H-P	H-L
RBC	–	5, 12	5, 7, 12	5, 7, 12	5, 7, 12	5, 7, 12
RBC-G		–	5, 7, 12, (16)	5, (7), 12	(5), (12)	5, 7
RBC-L			–	12, 16	5, 12, 16	12, 16
H				–		5
H-P					–	
	S					
	RBC	RBC-G	RBC-L	H	H-P	H-L
RBC	–		7, 12	5, 7	5, 7	5, 7, 12
RBC-G		–	(7), 12	5, 7	5, 7	5, 7, 12
RBC-L			–	5, 7, 12	5, 7, (12)	5, 7
H				–	(7)	12
H-P					–	(7), 12

two systems being compared only if there is at least a 95% significance level of difference indicated. The appearance of a given label number in parentheses indicates a probable difference between the systems cited (95%–99% confidence limits), while entries outside of parentheses indicate a highly significant difference (> 99% confidence limits).

The data shown in Fig. 1 and in the significance table yield some results which are unexpected. For the order parameter *S* the red blood cell and the red blood cell ghost show higher values than the bladder and hexagonal-plaque membranes near the surface of the membrane as reported by the 5 and 7 labels. While the result that the intact red blood cell cannot be distinguished from the red blood cell ghost might be expected, the fact that the bladder membrane and the hexagonal-plaque membranes are virtually the same is not. The hexagonal bladder membranes contain both the highly ordered plaque areas as well as the protein-free hinge areas and thus might be expected to have an order intermediate between that of the isolated plaque membrane and the bladder liposomes assuming a relative increase of the motionally restricted lipid pool in the lattice domains [17]. The equality of order between the bladder and hexagonal-plaque membranes suggests that the highly ordered protein part of each system is not significantly perturbing the underlying lipid structure.

For both types of systems the membranes with protein generally have a higher order (when there is a difference) than the liposomes derived from the intact membranes. This difference here is always detected by the 12 label in agreement with the observation of Marsh and Watts [19] that the effect of protein-lipid interaction in native membranes is better seen close to the terminal methyl end of the phospholipids acyl chains. Because the red blood cells tend to have a higher order than the bladder systems, it is found that at the level of the 5 and 7 labels the red blood cell liposomes have a higher order than the bladder liposomes. Note that there is no 16 entry in the table, indicating that at this level of penetration of both types of membranes the possible structure differences at the surface of the membrane are not detected.

For the polarity factor as measured by the isotropic hyperfine constant A_N we note again that the intact red blood cells and ghost membranes tend to have a higher value than the bladder

and hexagonal plaque membranes, this time not only at the level of the 5 and 7 labels but also for the 12 label. In contrast it is noted that the hexagonal plaque and bladder membranes cannot be distinguished from their liposomes, a result indicative of the little effect of the protein lattice on the polarity profile of these membranes. This result is, however, consistent with that found for the order parameter S, suggesting that the highly ordered protein units in the bladder membrane are not significantly perturbing the underlying lipid structure.

One might have expected that the highly ordered protein system in the bladder membrane would have resulted in a more ordered lipid substructure as well as a more polar environment near the surface of the membrane as compared to the red blood cell membranes with a random arrangement of proteins. As we note, just the opposite is observed. This unusual result suggests that the highly ordered protein in the hexagonal plaque particles may have a different relationship to its associated lipid than that between the protein and lipid in the erythrocyte membrane.

There are two simple models of the hexagonal membrane which are consistent with the current data. In the first model the protein subunits of the hexagonal membrane extend deep into the external half of the lipid bilayer leaving most of the lipid found in the inner half of the bilayer where few proteins are to be found. The spin label probe would be expected to partition itself in a manner similar to the location of the lipids and, accordingly, would then reflect an essentially liposome-type environment on the average. Another model compatible with our results would have most of the lattice protein residing as a coating on top of the luminal leaflet of the membrane, with few and short polypeptide segments anchoring the subunits to the relative unperturbed lipid bilayer. In this case the spin label would find itself again in an essentially liposome-like environment either in the inner or outer half of the membrane, the highly ordered protein having a relatively minor effect on the lipid bilayer compared to other transmembrane proteins containing membranes. Although it would seem that these two possibilities would be ideally suited for investigation by freeze-fracture techniques [9], unfortunately results obtained to date do not resolve the issue due to plastic deformation and to uncertainties on the characterization of the fractured planes [19].

The protein coat model cannot be viewed as one involving no interactions between the proteins and the lipids since the subunits have the characteristics of an intrinsic membrane protein: they are not extracted with chelating or chaotropic agents and they remain attached to the membrane after incubation at high ionic strength solutions. Their solubilization requires detergent treatment. This clearly indicates a hydrophic interaction between the subunit polypeptide and the acyl chains of the phospholipids but one which is unable to produce the significant perturbation of the membrane lipids expected by such a highly organized protein coat.

Lewis and De Moura [20] have recently described a mechanism for the modulation of the number of the amiloride-sensitive Na^+ transport sites in the apical surface of the urinary bladder epithelium. It involves cycles of fusion and detachment of cytoplasmic fusiforme vesicles with the luminal lattice membrane. The lipid protein interaction described in this paper is compatible with this hypothesis. The low polarity facilitates the approach of the two interacting membranes and the mobility of the lipids in the bilayer favors the phospholipid exchange required for membrane fusion.

Our models postulate unrestricted rotational and lateral diffusion in the membrane lattice domains. Direct measurements of these physical parameters in isolated hexagonal plaques and bladder membrane liposomes are required to assess its validity.

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