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Modification of phospholipids in erythrocyte membranes by phospholipase D. A fluorescence and ESR spectroscopic study

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The conversion of more than 65% of the phospholipids in human erythrocyte membranes to phosphatidylmethanol and phosphatidic acid by incubation with phospholipase D and methanol increased the dissociation constant of the fluorescence probe ANS compared to untreated membranes, but did not affect the number of binding sites and the limiting fluorescence enhancement at maximal binding (I_{max}). On the contrary, the cationic fluorescence probe dansylcadaverin showed additional binding sites without a change in K_d and an increase of I_{max} upon incubation with phospholipase D treated erythrocyte membranes compared to incubations of membranes with the original phospholipid pattern. The characteristic temperature-dependence of the quenching of the membrane protein fluorescence by a membrane-bound nitroxide-labeled stearic acid was not influenced by the modification of the phospholipids. A slight reduction of the order parameter, S, determined by ESR-spectroscopy with the same nitroxide spin-labeled fatty acid incorporated into modified membranes compared to controls was found at 40°C, but not at 25°C. The results were interpreted as an indication of membrane domains that retained their physical properties and lipid composition during the incubation with phospholipase D.

Introduction

The use of phospholipases has turned out to be a valuable tool for investigations of the function of membrane phospholipids and their distribution in the membrane structure [1–3]. A well-known example is the evaluation of the asymmetric phospholipid distribution in the two halves of the erythrocyte membrane bilayer [4–6].

Phospholipase D (EC 3.1.4.4) has rarely been applied for this purpose [4,7–14]. This enzyme catalyzes not only the hydrolysis of the phosphate ester bond in glycerophospholipids to yield phosphatidic acid and the respective alcohol component but also the transfer of the phosphatidyl residue to other alcohol acceptors [15,16]. A large number of alcohols have been described as suitable substrates for this transphosphatidylation reaction [16].

Most biological membranes are known to have

Abbreviations: ANS, 1-anilinonaphtho-8-sulfonic acid; ESR, electron spin resonance; F(12/3), 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl; I_{max}, limiting fluorescence enhancement at maximal binding; K_d, dissociation constant; N, number of binding sites; Palkanol, 1,2-diacyl-*sn*-glycero-3-phosphoalkanol; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; PS, 1,2-diacyl-*sn*-glycero-3-phosphoserine; SM, sphingomyelin.

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a specific phospholipid pattern which is preserved at different external conditions. A certain composition of phospholipids with different headgroups seems to be necessary to maintain membrane structure and functions, but the implications of phospholipid diversity in biological membranes are not well understood. The modification of the phospholipid pattern by transphosphatidylation with external phospholipase D should provide experimental access to this problem.

In order to investigate to what extent the phospholipid headgroups are susceptible to the exchange of the alcohol component, erythrocyte membranes were incubated with phospholipase D purified from cabbage and with different alcohols. We have further investigated the properties of the modified membranes via ESR and fluorescence spectroscopic methods.

Materials and Methods

Purification of phospholipase D

Phospholipase D was purified from the yellow heart of savoy cabbage as described by Davidson and Long [17] up to the acetone precipitation step. The precipitate was dissolved in the starting buffer of the subsequent ion exchange chromatography (50 mM Tris-HCl/1 mM EDTA/0.25 mM mercaptoethanol (pH 7.2)). Undissolved material was removed by centrifugation. The clear supernatant was applied to a DEAE-cellulose column (4 × 46 cm). The column was washed with starting buffer until no ultraviolet absorption was detectable in the effluent. The bound proteins were eluted by a linear gradient of NaCl (0–1 M) in starting buffer. Fractions of 9.5 ml were collected at a flow rate of 50 ml/h and assayed for phospholipase activity. Phospholipase D activity eluted at 0.4–0.6 M NaCl. The fractions were pooled and dialyzed against a 20-fold volume of 2 mM EDTA (pH 7.2) and three times against a 20-fold volume of water. The clear and nearly colorless solution was freeze-dried and stored at −18°C.

Determination of phospholipase D activity

0.5–4 μmol phosphatidylcholine from egg yolk purified by preparative thin-layer chromatography was emulsified in acetate buffer (50 mM acetic acid-NaOH/40 mM $CaCl_2$ (pH 5.6)) by sonication (GTS 22/125, Lehfeldt, Heppenheim, F.R.G.; 20 kcycles, 3 × 10 s, stage 5). Phospholipase D dissolved in the same buffer or an aliquot of the fractions from the ion-exchange chromatography were added to obtain a total volume of 1.5 ml. The reaction was started by addition of 0.5 ml diethyl ether and the mixture was stirred for 10–30 min at 25°C. The reaction was stopped by addition of 0.2 ml 1 M HCl and the lipids were extracted. When the transphosphatidylation activity was tested, methanol or ethanol was added in concentrations of 5–20%. Unconverted substrate and the reaction products were separated by thin-layer chromatography with silica gel HR and chloroform/methanol/25% ammonia (60:30:10, v/v) as solvent and were quantified by phosphate determination.

The evaluation of the thin-layer plates in routine determinations was carried out by densitometry. The plates (0.25 mm) were sprayed with 10% sulfuric acid to transparency and charred for 60 min at 160°C. The spots were scanned with a double beam densitometer (Schoeffel Instruments SD 3000) with a slitwidth of 1 mm and a wavelength of 350 nm. Calibration curves of phosphatidylcholine and phosphatidic acid with the same fatty acid content as the substrate were linear up to 50 nmol.

Preparation of erythrocyte membranes

Membranes of human erythrocytes were prepared by the procedure of Dodge et al. [18]. Blood in ACD stabilisator was received from the blood transfusion service of the University Hospital Eppendorf, Hamburg, F.R.G., and stored not longer than 1 week. The membranes were resuspended in Tris buffer (50 mM Tris-HCl/110 mM NaCl (pH 7.2)) after the last step of the Dodge-procedure and washed two times in this solution by resuspending and centrifugation at 20000 × *g* for 20 min.

Incubation of erythrocyte membranes with phospholipase D

Erythrocyte membranes (12 mg membrane protein) were incubated at 37°C for 60 min in a total volume of 8 ml of a medium comprising 50 mM Tris-HCl (pH 7.2)/110 mM NaCl/10 mM $CaCl_2$/8 units phospholipase D and methanol,

ethanol, propanol, or butanol in concentrations of 10%, respectively. The alcanols were replaced by ethanolamine or glycerol (0.8–1.6 M) in some incubation. The composition of the different blanks and other variations of the incubation conditions are indicated in the legends of the tables. The reaction was stopped by chilling on ice. The membrane suspensions were transferred to centrifuge tubes and diluted with Mg-sorbitol buffer (2.7 mM $MgCl_2$/166 mM sorbitol/68 mM imidazole-HCl (pH 7.2)) for lipid analysis or with tris buffer for ESR and fluorescence measurements to a total volume of 50 ml and centrifuged at $20000 \times g$ for 20 min. The membranes were resuspended and washed three times in the same buffers.

Lipid extraction

Freshly prepared erythrocyte membranes and the membranes incubated with phospholipase D were extracted as described by Ways and Hanahan [19] in their extraction procedure 2. The lipids were finally dissolved in a small volume of chloroform/methanol (2 : 1, v/v) and stored at −18°C.

ESR spectroscopic procedures

Membrane suspensions (3 mg membrane protein/ml) in Tris buffer (50 mM Tris-HCl (pH 7.2)) were incubated for 10 min at room temperature with small volumes of the nitroxide-labeled fatty acid, F(12/3), dissolved in methanol (10 mM) to incorporate F(12/3) into the erythrocyte membranes. The membranes were cooled to 4°C and centrifuged for 15 min at $250000 \times g$. The supernatants were removed and 75 μl of the pellets were transferred into flat (0.1 mm) ESR tubes and flushed with nitrogen. All spectra were recorded with a Bruker ESR spectrometer (Bruker Physik, Karlsruhe, F.R.G.) at 3300 G. The tubes were inserted in a quarz dewar flushed with nitrogen for temperature regulation. The temperature was measured inside the dewar with a thermocouple.

The amount of F(12/3) incorporated into the membranes was determined by the procedure of Barnett and Grisham [20]. Briefly, a 10-fold volume of ethanol was added to aliquots of the erythrocyte membrane suspensions. These samples were transferred into ESR tubes and analyzed as described above. Only the isotropic spectrum was detectable, indicating that the probe was released completely from its membrane-binding sites. The concentrations of F(12/3) were determined from the height of the low-field line by comparison with standard solutions in ethanol. The ratio of the nitroxide probe to phospholipid in the membranes was kept below 1 : 50.

The parallel and perpendicular components of the hyperfine splitting tensors, $T'_{\parallel}$ and $T'_{\perp}$, were derived from the spectra and the order parameter, S, was calculated by the equations [21]:

$$S = \frac{T'_{\parallel} - T'_{\perp}}{T_{zz} - \frac{1}{2}(T_{xx} + T_{yy})} \times \frac{a_N}{a'_N}$$

$$a'_N = \tfrac{1}{3}(T'_{\parallel} + 2T'_{\perp})$$

The elements of the hyperfine tensor in the three principal directions, T_{zz}, T_{xx} and T_{yy}, were taken from Jost et al. [22]. $a_N = \frac{1}{3}(T_{zz} + T_{xx} + T_{yy})$ is the isotropic coupling constant.

Fluorescence spectroscopy

All determinations were carried out with an Aminco SPF 125 fluorescence spectrometer (American Instrument Co., Silver Spring, MD) equipped with a water-jacketed cuvette holder. The cuvette temperature was monitored with a thermocouple. ANS was added to the membrane suspensions as a solution in the appropriate buffers and dansylcadaverin dissolved in methanol. The concentration of methanol in the incubations was kept below 0.5%. Excitation and emission wavelengths were respectively set as follows: ANS 380, 460 nm; dansylcadaverin 330, 505 nm; protein 285, 330 nm. Blanks (without membranes or fluorescence probes) were run in parallel to correct for the emission of unbound probes and scattering.

The membrane protein concentration of erythrocyte membrane suspensions in Tris buffer (10 mM Tris-HCl (pH 7.2)) was varied from 0 to 400 μg/ml at a fixed concentration of the probes (10 μM) to determine the limiting fluorescence enhancement when all probe is bound to the membrane (I_{max}). I_{max} was calculated by extrapolating reciprocal plots to infinite protein concentration. Subsequently, the concentrations of the probes

were varied from 0 to 40 μM at otherwise unchanged conditions and the fluorescence intensities were measured at fixed protein concentrations of 100 or 200 μg/ml. The concentrations of the bound and free probes were obtained by comparison of the fluorescence intensities at each concentration with I_{max} as described by Azzi [23]. The number of binding sites (N) and the dissociation constants (K_d) were derived from Scatchard plots.

The nitroxide-labeled fatty acid was also applied to analyze the quenching of the membrane protein fluorescence. For this purpose, F(12/3) in methanol solution (10 mM) was added to erythrocyte membranes suspended in Tris buffer (50 mM Tris-HCl (pH 7.2)). The concentrations of membranes and spin probe were 100 μg membrane protein/ml and 50 μM F(12/3), respectively. After a preincubation for 15 min at 25°C the suspension was cooled and the fluorescence intensity of the membrane proteins was determined at temperatures ranging from 8 to 56°C. Controls without quencher were run in parallel.

Other analytical methods

Protein was determined with bovine serum albumin as standard by the procedure of Lowry et al. [24].

In order to determine the cholesterol content of erythrocyte membranes, chloroform/methanol solutions of lipid extracts containing up to 0.8 μmol cholesterol were evaporated to dryness. The lipids were resuspended in 0.4 ml isopropanol and cholesterol was quantified by an enzymatic test kit (Boehringer, Mannheim, F.R.G.).

Phospholipids were separated by two-dimensional thin-layer chromatography (silica-gel HR, 0.5 mm). Chloroform/methanol/25% ammonia (60 : 30 : 10, v/v) was used as solvent in the first direction and chloroform/methanol/acetic acid/water (60 : 30 : 4 : 2, v/v) in the second direction. The phospholipids were stained by iodine and quantified by phosphate determination as described previously [25].

Materials

DEAE-cellulose (DEAE-SS, capacity 0.6) was obtained from Serva, Heidelberg, F.R.G. Bovine serum albumin was purchased from Behringwerke AG, Marburg, F.R.G. and phospholipase D from Sigma Chemical Co., St. Louis, MO. The nitroxide-labeled fatty acid F(12/3) was obtained from Syva, Palo Alto, CA. Dansylcadaverin was prepared as described by Narayanan and Balaram [26] and purified by preparative thin-layer chromatography with chloroform/methanol/25% ammonia (60 : 30 : 10, v/v) as solvent. ANS was purchased from E. Merck, Darmstadt, F.R.G., and was applied without further purification. All other chemicals were of reagent-grade quality from E. Merck, Darmstadt, F.R.G.

Results

Digestion of erythrocyte membrane phospholipids by phospholipase D

The phospholipids in the erythrocyte membrane were extensively modified by incubation with alcanols, Ca^{2+} and phospholipase D from cabbage (Table I). More than 50% of the membrane phospholipids were converted to phosphatidylalkanols in the presence of methanol or ethanol. The reaction products of the transphosphatidylation and the hydrolysis catalyzed by phospholipase D – phosphatidic acid and phosphatidylmethanol – amounted to 68.1% of the membrane phospholipids.

Marked differences in the susceptibility of the single membrane phospholipids to the attack of phospholipase D were observed. Phosphatidylcholine was converted almost completely in incubations with methanol or ethanol, phosphatidylethanolamine was reduced to less than 20% of its original amount, while phosphatidylserine was digested only by 57% in the presence of methanol.

Obviously, the alkanol component also influenced the extent of the hydrolysis and alcoholysis of the membrane lipids (Table I). The overall digestion was reduced and the ratio of hydrolysis to alcoholysis was increased with increasing chainlength of the alkanols. Since initial experiments showed that the alkanol concentration had a relatively small effect on the reaction, this observation can probably not be explained by the lower molar concentration of the higher alkanol homologs. Furthermore, no indication for the incorporation of glycerol and ethanolamine by phospholipase D into the erythrocyte membrane

TABLE I

PHOSPHOLIPIDS OF ERYTHROCYTE MEMBRANES INCUBATED WITH PHOSPHOLIPASE D AND DIFFERENT ALKANOLS

Erythrocyte membranes were incubated with phospholipase D and alkanols and washed and the phospholipids were analyzed. The incubations contained methanol (Ome), phospholipase D and methanol (Pme), ethanol (Pet), propanol (Ppro), or butanol (Pbu). Values in mol%, mean ± S.D.

	Ome	Pme	Pet	Ppro	Pbu
Start	2.8 ± 1.4	0.3 ± 0.4	0.0 ± 0.3	1.6 ± 1.4	0.8 ± 1.4
SM	24.1 ± 2.9	21.0 ± 2.3	21.9 ± 2.6	19.2 ± 2.4	17.9 ± 2.6
PC	30.5 ± 1.9	0.9 ± 0.5	1.1 ± 1.3	2.4 ± 1.5	11.4 ± 2.6
PS	13.8 ± 1.3	5.9 ± 2.2	8.4 ± 1.3	11.2 ± 1.7	11.4 ± 1.3
PE	25.0 ± 2.1	3.8 ± 1.0	4.9 ± 2.2	13.6 ± 1.0	18.1 ± 4.0
PA	2.0 ± 0.7	14.8 ± 7.2	11.8 ± 3.7	12.2 ± 4.2	19.8 ± 1.4
Palkanol	1.8 ± 2.5	53.3 ± 8.2	51.3 ± 9.7	19.9 ± 1.6	22.5 ± 6.3

phospholipids under the conditions applied here were obtained. No changes in the phospholipid patterns of membranes incubated with these alcohols, which easily undergo transphosphatidylation with emulgated phosphatidylcholine as substrate [15,16], were detectable by thin-layer chromatography.

In order to eliminate the possibility that membrane components were lost during the incubation, the phospholipid/protein ratio and the cholesterol content was analyzed. No changes in the membrane composition (0.32 μmol lipid phosphorus/mg protein and 0.82 mol cholesterol/mol lipid phosphorus) occurred when methanol and ethanol were applied, while the incubation with propanol and butanol decreased the phospholipid content of the membranes to 0.23 μmol/mg protein. Consequently, these membranes lost their typical disk shape and formed large clots. All subsequent determinations were carried out with membranes incubated with methanol or ethanol to avoid the modification of the membrane composition by other mechanisms than the digestion by phospholipase D.

ESR analysis of erythrocyte membranes incubated with phospholipase D

The nitroxide-labeled ESR probe, F(12/3), was applied to evaluate the effects of the incubation with phospholipase D on the membrane fluidity. The spectra recorded from erythrocyte membranes labeled with this probe showed that sufficient incorporation was achieved by the rather simple labeling procedure applied here. The isotropic spectrum of the unbound label was hardly detectable (not shown). The order parameter, S, was derived from these spectra as described in Material and Methods (Table II). Either small or no differences were detected between membranes with the modified phospholipid pattern and control preparations. A small reduction of S by 1.5% was found in the phospholipase D-treated membranes at 40°C but not at 25°C. $CaCl_2$ in a concentra-

TABLE II

ORDER PARAMETER, S, DETERMINED WITH THE NITROXIDE-LABELED FATTY ACID F(12/3) INCORPORATED INTO ERYTHROCYTE MEMBRANES

Erythrocyte membranes were incubated with phospholipase D and methanol (P) in order to modify the membrane phospholipids. Controls were incubated without phospholipase but with methanol (O). The membranes were washed repeatedly with Tris buffer (50 mM Tris-HCl (pH 7.2)) and labeled with F(12/3). The ESR spectra were recorded in the presence of $CaCl_2$ in the indicated concentrations and S was calculated as described in Materials and Methods. Mean ± S.D.

	$CaCl_2$ (mM)	S	
		25°C	40°C
O	0	0.710 ± 0.011	0.623 ± 0.003
P	0	0.709 ± 0.004	0.614 ± 0.002
O	2	0.709 ± 0.003	0.638 ± 0.007
P	2	0.712 ± 0.006	0.629 ± 0.005
O	10	0.727 ± 0.008	0.639 ± 0.005
P	10	0.724 ± 0.010	0.637 ± 0.004

tion of 10 mM increased the order parameter by 3.7% in phospholipase D-treated membranes and 2.6% in control preparations at 40°C, and by 2.1% and 2.4%, respectively, when the spectra were recorded at 25°C.

Binding of fluorescence probes to erythrocyte membranes with a modified phospholipid pattern

The analysis of the temperature-dependence of the fluorescence of the membrane bound fluorescent label ANS and dansylcadaverin revealed no break points in plots of the fluorescence intensity and temperature, but strong differences in the fluorescence intensities in the presence of modified membranes compared to controls were found (not shown). These differences may be explained by changes in the binding capacity of the membranes resulting from an increased negative surface charge caused by the modification of the phospholipid polar head-groups. The fluorescence of the anionic probe ANS in phospholipase-treated membranes was decreased, while the fluorescence intensity of dansylcadaverin carrying a positive charge was increased. However, the phospholipid pattern of the membranes may also influence the quantum yield of the probes.

I_{max}, which is directly related to the quantum yield [23], was calculated from double-reciprocal plots (Fig. 1 and Fig. 2) to resolve this question. The dissociation constant and the number of binding sites were derived from Scatchard plots (Fig. 3 and Fig. 4). All data obtained in this way are presented in Table III. I_{max} of ANS was not affected by the modification of the membrane phospholipids, while I_{max} of dansylcadaverin was increased more than 2-fold. The reduced fluorescence intensity of ANS in modified membranes was caused only by the increase in the dissociation constant. On the contrary, the number of binding sites of dansylcadaverin was increased, while the dissociation constant was not changed. Consequently, the incubation of the membranes with phospholipase D resulted not only in an increased quantum yield of dansylcadaverin, but also in the formation of new binding sites. This result agrees with the assumption [26] that this probe preferentially occupies anionic binding sites. The observation that K_d of dansylcadaverin was not affected by the modification of the membrane

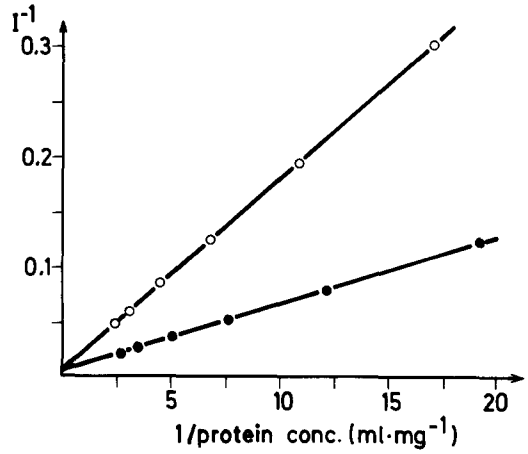

Fig. 1. Double-reciprocal plots of the binding of ANS to erythrocyte membranes. The fluorescence intensity of ANS in the presence of erythrocyte membranes preincubated with phospholipase D and methanol (○) or methanol (●) was determined as described in the Methods section at 25°C in Tris buffer (10 mM Tris-HCl (pH 7.2)).

phospholipids and the concomitant enhancement of the surface potential is unexpected and cannot be explained in the moment.

Several investigations demonstrated that the ion concentration in the medium changed the affinity of charged fluorescence probes like ANS to biological and model membranes by neutralization of the surface charge (e.g. Refs. 27–29). Fig. 5 and

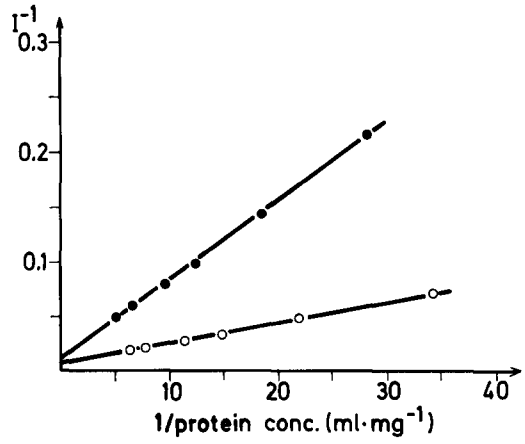

Fig. 2. Double-reciprocal plots of the binding of dansylcadaverin to erythrocyte membranes. The same procedures and symbols as in Fig. 1 were used, except that dansylcadaverin was applied.

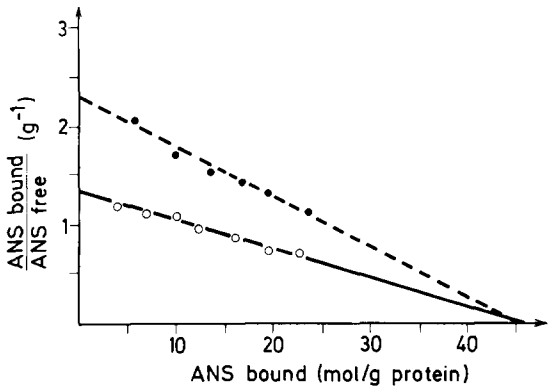

Fig. 3. Scatchard plot of the binding of ANS to erythrocyte membranes. Erythrocyte membranes were preincubated with methanol (●) or phospholipase D and methanol (○), washed in Tris buffer (10 mM Tris-HCl (pH 7.2)) and the fluorescence intensity of ANS was determined at varying concentrations of the fluorescence probe and fixed concentration of membrane protein at 25°C. For further details of the procedure and for the calculation of bound and free ANS see Materials and Methods section.

Fig. 6 show the effect of the $CaCl_2$ concentration on the fluorescence intensities of the probes used here in the presence of erythrocyte membranes. In addition to the expected effects that the binding of ANS was increased by neutralization of the surface charge and that dansylcadaverin was replaced from its binding sites by Ca^{2+}, the results of Fig. 5 and Fig. 6 reveal some differences in the response of both probes to the modification of the phospholipids by preincubation with phospholipase D and

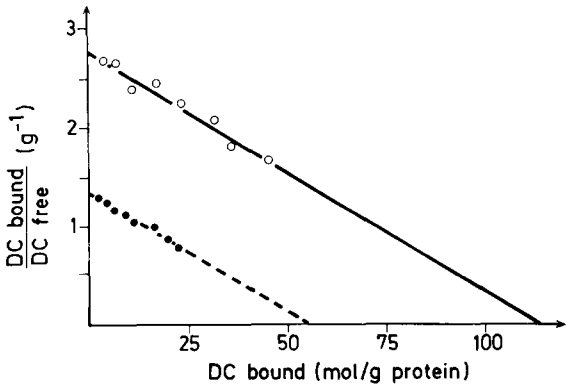

Fig. 4. Scatchard plot of the binding of dansylcadaverin to erythrocyte membranes. The same procedure and symbols as in Fig. 3 were applied.

TABLE III

BINDING CONSTANTS AND I_{max} OF ANS AND DANSYLCADAVERIN IN THE PRESENCE OF ERYTHROCYTE MEMBRANES

The procedures of incubating erythrocyte membranes with methanol (O) or phospholipase D and methanol (P), the labeling of the membranes with the fluorescence probes and the evaluation of the fluorescence data in order to determine the limiting fluorescence enhancement at maximal binding (I_{max}), the dissociation constant (K_d), and the number of binding sites (N) were carried out as described in the Materials and Methods section. The membranes were washed after the incubation with phospholipase D in Tris buffer (10 mM Tris-HCl (pH 7.2)). (Mean ± S.D.).

		I_{max}	K_d (μmol/l)	N (μmol/g protein)
ANS	O	193 ± 9	30 ± 9	50 ± 15
	P	170 ± 9	60 ± 20	49 ± 16
Dansylcadaverin	O	72 ± 1	29 ± 10	51 ± 7
	P	147 ± 21	32 ± 7	119 ± 21

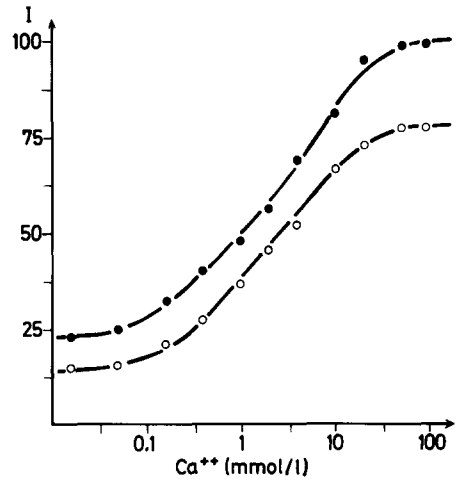

Fig. 5. The effect of $CaCl_2$ on the fluorescence intensity of ANS in the presence of erythrocyte membranes. The phospholipids in erythrocyte membranes were digested by incubating with phospholipase D and methanol. The membranes were washed in Tris buffer (10 mM Tris-HCl (pH 7.2)), incubated with ANS (10 μM) in the same buffer containing $CaCl_2$ in concentrations indicated in the figure and the fluorescence intensity was recorded at 25°C as described in Materials and Methods. Both phospholipase D-treated membranes (○) and membranes treated only with methanol (●) were applied in a concentration of 100 μg membrane protein/ml.

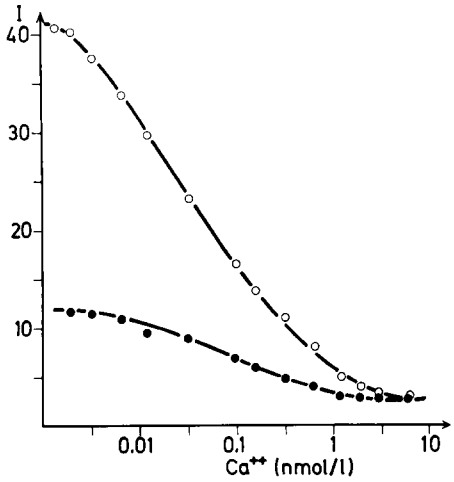

Fig. 6. Fluorescence intensity of dansylcadaverin in the presence of erythrocyte membranes as a function of the $CaCl_2$ concentration. The same symbols and procedures as in Fig. 5 were used, except that the determinations were carried out with dansylcadaverin as fluorescence probe.

to the addition of $CaCl_2$. The fluorescence of dansylcadaverin was already affected at an about 100-fold lower concentration of $CaCl_2$ than ANS. The fluorescence intensity of this probe decreased from very different levels in modified and unmodified membranes in the absence of $CaCl_2$ to the same intensity at high $CaCl_2$ concentrations (about 5 mM). This observation supports the assumption that the increase in the binding of dansylcadaverin upon incubating the membranes with phospholipase D is the result of the negative surface charge produced by the conversion of the natural membrane phospholipids to phosphatidylmethanol and phosphatidic acid and that dansylcadaverin mainly occupies binding sites in membrane domains containing the modified phospholipids. The fluorescence intensity of ANS was increased by the addition of $CaCl_2$ to the assays, but this effect was hardly influenced by the modification of the membrane phospholipids by phospholipase D. The difference of the fluorescence intensities of ANS in the presence of modified and unmodified membranes remained more or less constant at all concentrations of $CaCl_2$.

Quenching of the membrane protein fluorescence by a nitroxide-labeled fatty acid

Another approach to investigating membrane properties by fluorescence spectroscopy – especially membrane domains containing intrinsic membrane proteins – was introduced by Wallach and co-workers [30–32]. The fluorescence of intrinsic membrane proteins is quenched by membrane bound nitroxide spin-labeled lipid probes provided that the distance of the nitroxide and the fluorophore is in the range of 0.4–0.6 nm. Bieri and Wallach [31] found that the extent of the

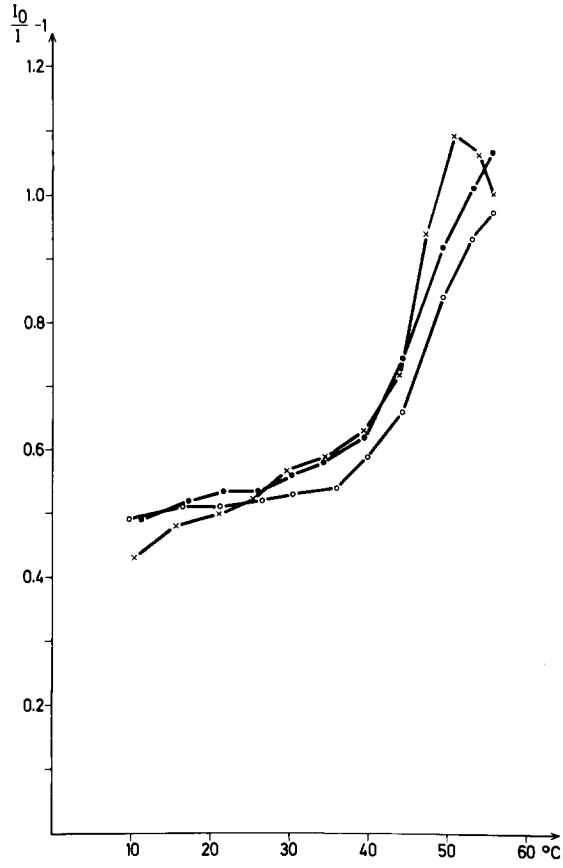

Fig. 7. The effect of temperature on the quenching of the fluorescence of erythrocyte membrane proteins by the nitroxide spin-labeled fatty acid F(12/3). Erythrocyte membranes were preincubated with methanol and phospholipase D (×) to modify the membrane phospholipid pattern. Control incubations with (●) or without methanol (○) were run in parallel. The membranes were washed in Tris buffer (50 mM Tris-HCl (pH 7.2)), resuspended and labeled with F(12/3) in the same buffer and the fluorescence intensity (I) of the membrane proteins was determined at various temperatures as described in detail in Materials and Methods. The fluorescence intensity without quencher (I_0) was determined under the same conditions to calculate I_0/I.

fluorescence quenching in erythrocyte membranes was in a characteristic way dependent on the temperature. At about 40 °C a rapid increase of quenching was observed. This effect was explained by the assumption that the conformation of the membrane proteins including the surrounding lipids was affected at this temperature resulting in a better access of the applied nitroxide-labeled fatty acid to the protein fluorophores [31]. This procedure was applied here to the erythrocyte membranes modified in their phospholipid pattern by incubation with phospholipase D and methanol. Figs. 7 and 8 show plots of the relation of I_0 (fluorescence intensity without quencher) and I (fluorescence intensity in the presence of quencher) as a function of the temperature. The increase of the protein fluorescence quenching was detectable to the same extent with modified and unmodified membranes. The effect was abolished by addition of $CaCl_2$ in concentrations of up to 10 mM to the fluorescence assays (Fig. 8). Apparently, the nitroxide-labeled fatty acid was rearranged or immobilized in the membrane, so that the close contact of the probe and the membrane proteins was prevented.

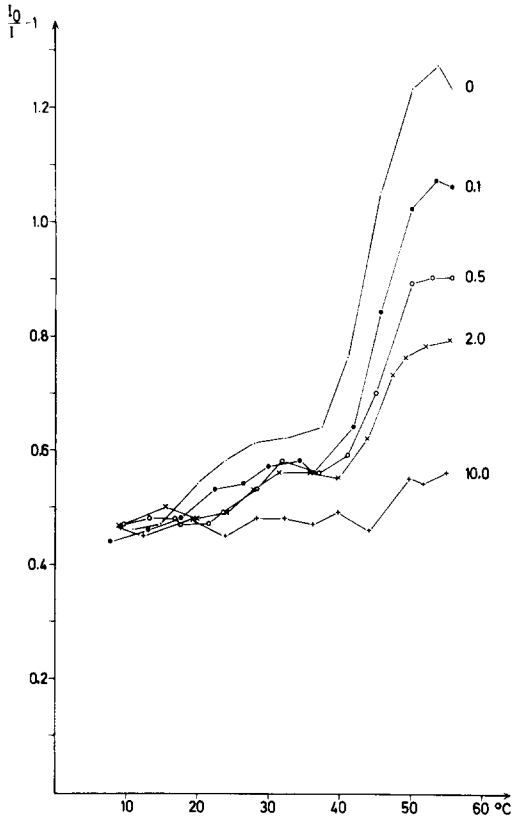

Fig. 8. Quenching of the erythrocyte membrane protein fluorescence by the nitroxide-labeled fatty acid (F(12/3) in dependence on the $CaCl_2$ concentration and the temperature. The determinations were carried out at the same conditions as the experiment of Fig. 7, except that $CaCl_2$ in the concentrations indicated in the present figure (mmol/l) was added to the fluorescence assays. Erythrocyte membranes preincubated with phospholipase D and methanol were applied.

Discussion

Some phospholipids in the erythrocyte membrane (mainly phosphatidylserine, but also phosphatidylethanolamine) were only partly modified by transphosphatidylation and hydrolysis during the incubation with phospholipase D, while phosphatidylcholine was almost completely digested. This observation may be explained by the hiding of these phospholipids inside of right-side-out partially sealed membranes. But the asymmetric distribution of phospholipids in the native membrane probably cannot explain the preferential modification of phosphatidylcholine observed in this study for several reasons. The asymmetric arrangement and the physical state of the membrane lipids change during the preparation of the plasma membrane. This was shown by covalent labeling [33], and the application of fluorescence [34] and spin probes [35,36]. De Kruijff and Baken [37] found that phosphatidic acid generated in the outer layer of phospholipid vesicles by phospholipase D exchanged rapidly with the phospholipids of the inner layer. This process – perhaps accelerated by the known fluidizing effect of short-chain alcohols on biological membranes [38,39] – probably occurred during the incubation with phospholipase D. Hexanol in low concentrations was found to cause an increased accessibility of phosphatidylethanolamine to phospholipase A_2 in erythrocytes [11]. All these observations suggest that the asymmetric distribution of the membrane phospholipids was lost during the membrane preparation and the incubation with phospholipase D and alcanols.

An other reason for the resistance of some phospholipids to the digestion by phospholipase

D could be the partial shielding by temporary interaction with membrane proteins and the preservation of certain membrane domains during the incubation with phospholipase D.

Some of the results presented in this communication support the second explanation, although the precise localization of the probes used here is not known and perturbation of the membrane structure by the probes may have occurred to a certain extent. Only the affinity of the fluorescence probe ANS to the membrane was reduced by the modification of the phospholipids, but not the number of binding sites and the limiting fluorescence enhancement at maximal binding. The same increase of the fluorescence intensity by the neutralization of the surface charge by Ca^{2+} was observed in modified and unmodified membranes. Since the negative surface charge of the modified membranes was at least partly due to the accumulation of anionic phospholipids, we assume that this probe sampled membrane domains that did not contain phosphatidylmethanol and phosphatidic acid, but membrane areas with the original protein and lipid composition. The observation that the maximal fluorescence intensity of ANS was not altered by the incubation with phospholipase D indicates that the physical properties of the membrane at the binding sites of this probe were not affected either. The binding of ANS to proteins may have also contributed to this effect.

On the contrary, dansylcadaverin is known to occupy binding sites at anionic phospholipids [26], probably phosphatidic acid and phosphatidylmethanol in membranes treated with phospholipase D. Consequently, this probe gained additional binding sites by incubating the membranes with phospholipase D and methanol with a concomitant increase in the maximal fluorescence intensity and was easily removed from the membranes by neutralization of the surface charge with Ca^{2+}. Obviously, dansylcadaverin binds to complete different membrane domains than does ANS. The physical properties as well as the phospholipid composition of these domains changed during the incubation with the phospholipase.

The shielding of phospholipids to the hydrolysis and alcoholysis may have resulted from the interaction with extrinsic membrane proteins. The preferential binding of spectrin to phosphatidylserine and phosphatidylethanolamine has been reported [40,41]. In addition, the selective interaction with intrinsic membrane proteins may have also contributed to the shielding effect. The fluorescence quenching experiments using a nitroxide-labeled fatty acid showed that the characteristic dependence of the quenching efficiency on temperature was preserved during the incubation with phospholipase D, indicating that the conformation of the intrinsic membrane proteins and probably also their interaction with membrane lipids was intact at least to a large extent in the membranes with the modified phospholipid pattern. A disadvantage of this procedure is the rather high concentration of labeled fatty acid necessary to achieve diffusion-limited quenching [32]. Effects of the bound label on the membrane structure cannot be excluded completely. The reduced quenching above 50°C may have resulted from partial disintegration of the membrane.

No consistent evidence for a change of the membrane fluidity caused by the modification of the phospholipids by phospholipase D was obtained by the applied procedures. The ESR and fluorescence spectroscopy experiments led to contradictory results. The higher I_{max} detected with dansylcadaverin at 25°C in modified membranes compared to controls can be interpreted as a reduction of the fluidity at the binding sites of this probe. On the other hand, no differences in the order parameter, S, between modified membranes and controls were detected with the nitroxide-labeled fatty acid F(12/3) at this temperature, while a slight increase in fluidity at 40°C indicated by a reduced order parameter was observed in modified membranes. Since these probes carry opposite charges, they probably occupied different binding sites and sampled membrane domains with different physical properties.

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