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INFLUENCE OF ENZYMATIC PHOSPHOLIPID CLEAVAGE ON THE PERMEABILITY OF THE ERYTHROCYTE MEMBRANE

II. PROTEIN-MEDIATED TRANSFER OF MONOSACCHARIDES AND ANIONS

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Summary

In order to investigate the influence of membrane lipids on transport via the protein domain of the erythrocyte membrane, a number of facilitated diffusion processes was studied by tracer flux techniques in whole cells after cleavage of up to 65% of the phosphatidylcholine or the sphingomyelin by phospholipase A_2 from *Naja naja* or bee venom, or by sphingomyelinase, respectively.

The mediated fluxes of L-arabinose, which is transported by the glucose carrier, and of L-lactate, which uses a specific monocarboxylate carrier, were markedly inhibited by cleavage of either phosphatidylcholine or sphingomyelin. These phospholipid dependencies are in line with earlier data on cholesterol dependencies (Deuticke, B. (1977) Rev. Physiol. Biochem. Pharmacol. 78, 1—97). They can only in part be explained by changes of membrane fluidity. More specific interactions of the degradation products with the carrier proteins seem also to play a role.

Sulfate and oxalate transfer, which proceed via the inorganic anion-exchange system, are essentially unaffected by cleavage of phosphatidylcholine and less sensitive to sphingomyelin cleavage than the two other processes. This also agrees with earlier data on cholesterol independency of sulfate transfer. The inorganic anion-exchange protein thus seems to be less dependent on the surrounding lipids in its conformation and its mode of action than the two other carriers.

Abbreviations: PCMBS, *p*-chloromercuriphenylsulfonate; ANS, 1-anilino-8-naphthalene sulfonate.

Introduction

One of the fundamental implications of membrane models postulating intrinsic proteins imbedded in a fluid lipid phase [1] are lipid dependencies of protein-related membrane functions. For transport processes this has been demonstrated in a number of cases. Non-electrolyte and ion transport systems in various membranes are influenced by the fatty acid composition [2—4]. In erythrocytes of various mammalian species the rates of mediated anion transfer processes are closely correlated with the phospholipid composition [5,6]. Changes of membrane cholesterol levels have been reported to affect the facilitated diffusion of anions [7], monosaccharides (Refs. 7—9, and Wilbrandt, W. and Becker, C., personal communication), glycerol [7] and uridine [9]. To interpret these effects one assumes an influence, on protein conformation or conformation changes, of adjacent membrane lipids, which may form a particular annulus, stabilized by hydrophobic interactions in the membrane core, or by polar forces localized in the interfacial domain (cf. Ref. 10 for a review). In order to obtain further insight into the lipid-protein interactions modulating mediated transfer processes we have studied the influence of enzymatic cleavage of two major phospholipids, phosphatidylcholine and sphingomyelin, on the protein-mediated transfer of L-arabinose [11], L-lactate [6,7], sulfate [7] and oxalate [12] in human erythrocytes.

Materials and Methods

Materials. Phospholipase A_2 from *Naja naja* venom (Sigma) and sphingomyelinase from *Staphylococcus aureus* were purified as described in a previous paper [13]. Pure phospholipase A_2 from bee venom was obtained from Sigma. Labelled compounds (L-[U-^{14}C]lactic acid, sodium salt; [1-^{14}C]glycerol; L-[1-^{14}C]arabinose, [U-^{14}C]oxalic acid and sodium [^{35}S]sulfate) were from Amersham Buchler, Braunschweig.

Human blood was obtained from the local blood bank. Standard chemicals used were of the highest purity available, *p*-chloromercuriphenylsulfonate (PCMBS) was from Sigma.

Methods. Erythrocytes were washed three times in 154 mM NaCl. 1 vol. of erythrocytes was suspended in 10 vols. of a medium containing, besides the test substance at concentrations given in Results, the following constituents (mM): KCl (100), NaCl (50), sucrose (44), $MgCl_2$ (0.25), glycylglycine (10) (medium B). $CaCl_2$ was added to these media at a concentration of 0.25 or 10 mM, depending on the phospholipase to be used.

Cleavage of phospholipids, loading of the cells with labelled test substances and measurements of tracer efflux were carried out as described in the preceding paper [14].

In studies on the influence of phospholipase A_2 from *Naja naja* on sulfate and oxalate transfer a somewhat different procedure had to be used in order to avoid precipitation of insoluble calcium salts. Cells were first treated with phospholipase in medium B (pH 7.4) containing 10 mM $CaCl_2$ but no sulfate or oxalate, then washed and resuspended in a medium containing beside sulfate or oxalate (mM): sodium acetate (150), sucrose (44), $CaCl_2$ (0.25), $MgCl_2$

(0.25) and glycylglycine (10) (medium C, pH 6.8). Replacement of chloride by acetate and low pH served the purpose to obtain high rate coefficients of tracer efflux [7].

Hemolysis during the efflux period never exceeded 5% thus not affecting the validity of the rate coefficient of tracer appearance in the medium as a measure of transmembrane flux under the standardized conditions used in our experiments. The volumes of control and phospholipase-treated cells were determined by measurements of dry weight.

Results

L-Arabinose

L-Arabinose penetrates the human erythrocyte membrane by the monosaccharide transfer system [11]. Due to a very low affinity ($K_m > 100$ mM), transfer rates can be measured at 10°C by conventional tracer techniques. Treatment of erythrocytes with phospholipase A_2 produces an approximately linear decrease of the rate of equilibrium exchange. Cleavage of 50% of the phosphatidylcholine goes along with a 30% reduction of the transfer (Fig. 1a). Treatment with sphingomyelinase (Fig. 1b) produces even more drastic changes of a somewhat different type. Cleavage of the first 15—20% of this phospholipid has almost no effect, increasing hydrolysis produces pronounced inhibition. 50% cleavage go along with a mean of 50% reduction. These effects are not likely to be due to changes of cell volume, since the cellular water content (not corrected for intercellular water) only increased from 62.0 to 63.5% after 50% hydrolysis of the phosphatidylcholine and from 63.0 to 65.5% (mean values from five experiments) after cleavage of 60% of the sphingomyelin. Moreover, neither nonspecific adsorption of the enzyme nor time-dependent secondary changes of the membrane in presence of the enzyme can probably account for the phenomena observed, since the same relationship between phospholipid cleavage and inhibition was obtained, whether cells were incubated with a small amount of enzyme for a long, or with a large amount of

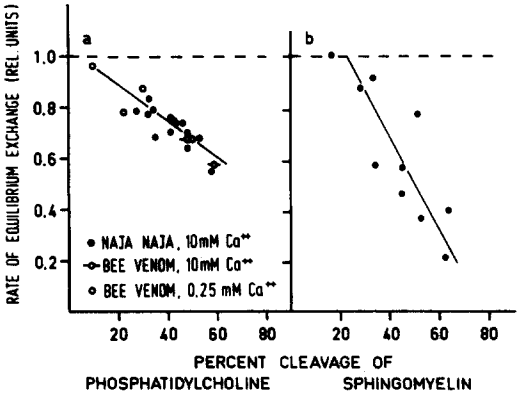


Fig. 1. Rates of equilibrium exchange of L-arabinose in human erythrocytes treated (a) with phospholipase A_2, or (b) with sphingomyelinase. Treatment with enzyme and efflux measurements (pH 7.35, 10°C, hematocrit 5%, 10 mM L-arabinose) as described under Materials and Methods. Note that effects of bee venom phospholipase are independent of the extracellular concentration of Ca^{2+}.

TABLE I

LACK OF INFLUENCE OF ENZYME CONCENTRATION AND EXPOSURE TIME ON THE RELATIONSHIP BETWEEN PHOSPHOLIPID CLEAVAGE AND INHIBITION OF ARABINOSE TRANSFER

Phospholipase A_2 (*Naja naja*, I.U./ml cells)	Exposure time (min)	Cleavage of phosphatidylcholine (%)	Rate of equilibrium exchange, relative to controls
2.5	30	48	0.71
5	10	46	0.75

enzyme for a short time (Table I). The alterations of transport are thus most likely due to the cleavage of the membrane lipid.

Anions

The mediated transfer of anions across the erythrocyte membrane occurs via two separate pathways: (1) an exchange system specialized on inorganic anions [7], but also accepting dicarboxylates [6,7,15,16], and certain polar monocarboxylates, and (2) a transfer system specific for aliphatic monocarboxylates, in particular L-lactate and pyruvate [6,7,16—18]. The discrimination of the two pathways largely rests on their different sensitivities to inhibitors [6,16, 19]. As is evident from Fig. 2, sphingomyelinase treatment affects the exchange of L-lactate to the same extent as that of L-arabinose. Treatment with phospholipase A_2, however, has a more pronounced effect on L-lactate than on L-arabinose. 50% cleavage of phosphatidylcholine leads to a 50% inhibition of lactate exchange, but reduces arabinose exchange only by 30%.

In contrast to the qualitative similarities between L-lactate and L-arabinose, sulfate exchange (Fig. 3) is almost insensitive to hydrolysis of up to 60% of the phosphatidylcholine, whereas sphingomyelinase treatment inhibits sulfate exchange, although to a lesser extent than that of L-lactate and L-arabinose (25% inhibition at 50% cleavage). The lack of effect of phospholipase A_2 was observed with the enzymes from *Naja naja* and from bee venom. The particular

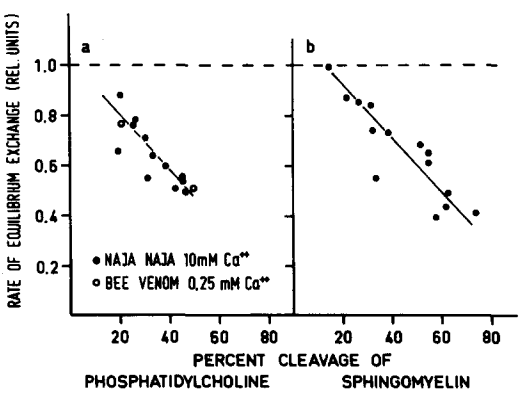
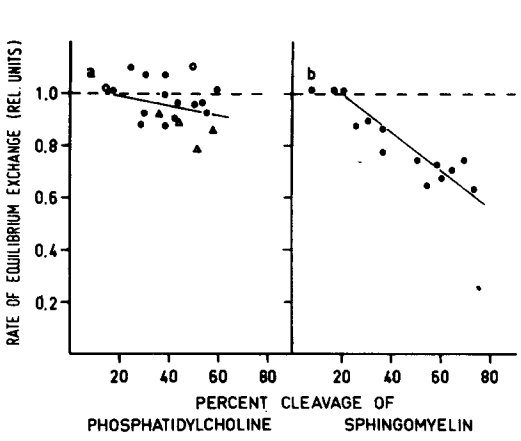


Fig. 2. Rates of equilibrium exchange of L-lactate in human erythrocytes treated with (a) phospholipase A_2 or (b) spingomyelinase. Efflux measurements (pH 7.35, 20°C, 5 mM L-lactate hematocrit 5%) as described under Materials and Methods.



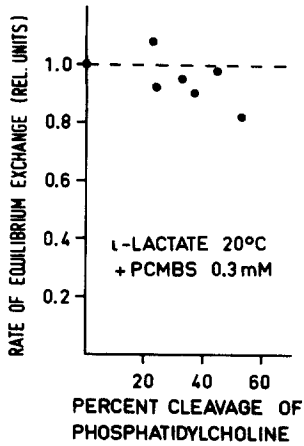


Fig. 3. Rate of equilibrium exchange of sulfate (○, ●) and oxalate (△) in human erythrocytes treated with phospholipase A_2 from *Naja naja* (●) or bee venom (○, △) or with sphingomyelinase (●). Enzyme treatment and efflux measurements as described under Materials and Methods. Sulfate: 3.5 mM, pH 6.80 and 7.35, 35°C. Oxalate: 3.5 mM, 10°C, pH 7.35.

Fig. 4. Lack of effect of cleavage of phosphatidylcholine on the PCMBS-insensitive fraction of L-lactate exchange. Fluxes measured after treatment with phospholipase A_2 (*Naja naja*) in the presence of PCMBS.

procedure of the enzyme treatment (which was different for the two enzymes (see Materials and Methods)), obviously does not influence the effect on anion transfer.

In these studies the fluxes of sulfate were measured at 35°C, those of lactate and arabinose at lower temperatures. In order to exclude that differences in temperature account for the different sensitivities to phospholipase A_2, the effect of the enzyme on oxalate transfer, which can be easily be measured at 10°C [19], was studied. From Fig. 3 it is obvious that the data points for oxalate exchange after treatment with phospholipase A_2 fall into the range of the points for sulfate exchange, excluding temperature as a reason for the low sensitivity of sulfate exchange to phosphatidylcholine cleavage and indicating a true difference in the responses of sulfate and lactate exchange to cleavage of this phospholipid.

Further evidence for this view could be obtained by comparing the influence of phospholipase A_2 on lactate exchange in the presence or absence of PCMBS. PCMBS inhibits the transfer of lactate via the monocarboxylate transfer system [6], but leaves unaltered the residual movement of lactate via the inorganic anion system *. According to Fig. 4, PCMBS-insensitive lactate exchange (k = 0.0151 min^{-1} as compared to 0.2344 min^{-1} in the absence of the mercurial) is essentially unaffected by cleavage of phosphatidylcholine as expected for a process involving the inorganic anion-exchange system.

Our experimental results, including the differences between effects of phospholipase A_2 and sphingomyelinase, could be due to the fact that sphingomyelinase only needs 0.25 mM Ca^{2+}, while phospholipase A_2 from *Naja naja*

* PCMBS-insensitive lactate exchange also includes a contribution of non-ionic diffusion via the lipid phase, amounting to about 40% of this residual flux (Deuticke, B., unpublished observations).

TABLE II

INDEPENDENCE OF THE EFFECT OF PHOSPHOLIPID CLEAVAGE ON ANION TRANSFER ON THE CALCIUM CONCENTRATION

Transport substrate	Enzyme source, Ca^{2+} concn. (mM)	Cleavage of phosphatidyl-choline (%)	Rate of equilibrium exchange (relative units)
Lactate	Bee venom, 0.25	46	0.54
	Naja naja, 10	45	0.53
Sulfate	Bee venom, 0.25	40	0.85
	Naja naja, 10	42	0.91

requires the presence of 10 mM Ca^{2+}. In order to exclude effects of high Ca^{2+} as a basis of artifacts, the influence of bee venom phospholipase A_2, which only requires 0.25 mM Ca^{2+}, was compared with that of the *Naja naja* enzyme. According to the results compiled in Table II and the data points for bee venom included in Figs. 1 and 3, a given extent of phospholipid cleavage produces the same effect on arabinose and lactate transfer, regardless of the calcium concentration in the medium.

Discussion

As shown in the preceding paper [14], enzymatic cleavage of up to 65% of the phosphatidylcholine or sphingomyelin in the intact erythrocyte does not affect the permeability of the lipid membrane domain. In contrast, a number of protein-mediated transfer processes is inhibited under these conditions. The magnitude of the effect excludes artifacts due, e.g. to loss of surface area or swelling of the cells [14]. An inhibitory action of the enzyme protein, independent of the cleavage of phospholipids, such as a tight binding to the transport sites, is also unlikely in view of the close correlation between the extents of cleavage and inhibition irrespective of the conditions by which cleavage was obtained (Table I). The differences between the effects on lactate and arabinose transfer on the one hand, and on sulfate and oxalate transfer on the other hand also provide evidence against an unspecific basis of the inhibition. The following considerations may shed some light on possible mechanisms.

Intrinsic, membrane-spanning proteins are supposed to be surrounded by an annulus of 'boundary lipids', differing in its physical properties from the 'free' bilayer domain [10,20—22]. In the erythrocyte membrane about 25% of lipid have been assigned to this type [23], in agreement with findings in other plasma membranes [24]. Chemical specificity in the boundary lipid, leading to a lipid composition of the annulus different from that in the bilayer domain, has been claimed in a number of systems [4,10]. However, exchange between annular and free membrane lipid seems to be possible [21,25].

Studies on the transport and enzymatic function of membrane proteins indicate the importance of their lipid environment. Discontinuities in the temperature dependencies of such functions have been related to phase transitions of the adjacent lipid or the boundary phase [26]. Modulation of protein-related

membrane functions by manipulation of the fatty acid composition or the cholesterol content points to the relevance of lipid fluidity [4,19,27]. On the other hand, relations between head group properties and protein-mediated functions have been observed [10].

The effects of phospholipid cleavage on transport processes in whole erythrocytes have to be discussed within the framework of these data and with regard to known molecular features and lipid dependencies of the transport proteins.

(a) L-Arabinose transfer

L-Arabinose is transported by the common monosaccharide carrier of the erythrocyte membrane which exhibits the properties of a mobile carrier [11]. Reconstitution experiments [28,29] indicate the involvement of a glycoprotein fraction of about 55 000 daltons (band 4.5 according to the enumeration of Steck in SDS-polyacrylamide gel electropherograms).

Lipid dependency of this carrier is indicated by inhibition of monosaccharide transfer after cholesterol depletion and activation by excess cholesterol (Refs. 7—9, and Wilbrandt, W. and Becker, C., personal communication). If these effects have anything to do with the accompanying changes of membrane microviscosity [30], the carrier is at least not affected in the direction one would anticipate intuitively, namely greater conformational mobility and hence higher activity in the more fluid environment of a cholesterol-depleted membrane. Inhibition of mediated transfer in a more fluid membrane could of course be explained by the assumption that optimal efficiency of an intrinsic membrane protein requires a certain conformational stabilization by its lipid environment.

According to the present data, which agree with earlier observations [31] on stereospecific glucose binding to phospholipase-treated erythrocyte membranes, inhibition of arabinose exchange is observed in two cases of phospholipid cleavage which probably go along with opposite changes of membrane fluidity: treatment with phospholipase A_2 has been claimed to rigidify the lipid domain to some extent [32], while removal of head groups by phospholipases of the C-type seems to act fluidizing [33,34]. If one brings these data in line with the results obtained in cholesterol-depleted cells, one could invoke changes of membrane fluidity in the explanation for the inhibitory effect of sphingomyelinase treatment. Additional assumptions, however, would have to be adduced to explain the inhibitory effect of phospholipase A_2. Among others one might consider replacement of annular phospholipids by degradation products (ceramide, fatty acid, lysophosphatidylcholine) or redistribution of cholesterol between free and annular phospholipid secondary to the phospholipid cleavage.

In order to characterize more precisely the underlying perturbations, effects of externally applied degradation products are presently studied (Grunze, M. and Deuticke, B., unpublished results). Palmitic and oleic acid, products of the action of phospholipase A_2, only slightly (less than 20%) inhibit arabinose exchange at membrane concentrations in the range observed after maximal enzymatic cleavage, i.e. one fatty acid/six phospholipid molecules. This makes a major contribution of free fatty acids unlikely.

Since the action of the impermeable phospholipases is restricted to the outer membrane layer in our experiments, the primary perturbation of the carrier protein will probably occur in its exofacial domain. An exclusive assignment is not possible in view of the probable transbilayer equilibration of the hydrolysis products [35,36]. In its localization, the inhibition of the arabinose (and the lactate) carrier thus differs markedly from the inhibition by phospholipase A_2, of the $(Ca^{2+} + Mg^{2+})$-ATPase of the human erythrocyte [37]. This transport enzyme is only sensitive to direct cleavage of endofacial phospholipids and consequently insensitive to a degradation of sphingomyelin which resides in the outer membrane layer. Such differences between a membrane enzyme utilizing an intracellular substrate, ATP, and a carrier-mediating transmembrane migration seem quite plausible.

(b) L-Lactate transfer

L-Lactate is transported across the erythrocyte membrane predominantly by a monocarboxylate-specific carrier, different from the inorganic anion-exchange system [6,16—18]. Inhibitor binding studies suggest the involvement of proteins in the 55 000 dalton (band 4.5) or the 30 000 dalton (band 7) region [18]. Lipid dependency of this carrier, which was already suggested by: (1) a correlation between transport rates and membrane phospholipid composition in various mammalian species [6]; (2) a discontinuous temperature dependency [6], and (3) an inhibitory effect of cholesterol depletion [7], is further substantiated by the present findings. Although the phospholipase sensitivity of lactate transfer parallels that of arabinose transfer, different mechanisms may contribute: according to preliminary results, palmitate and oleate incorporated into the erythrocyte membrane markedly inhibit lactate transfer, in contrast to arabinose transfer (Grunze, M. and Deuticke, B., unpublished results).

(c) Sulfate and oxalate exchange

Sulfate and oxalate transfer are mediated by the dominating 100 000 dalton protein fraction (band 3) of the erythrocyte membrane (for recent reviews cf. Refs. 7, 12, 15, 38). In line with the essential insensitivity of sulfate exchange to cholesterol depletion [7] and to alimentary changes of the phospholipid fatty acid pattern in the erythrocyte membrane (Haest, C.W.M. and Deuticke, B., unpublished results), our data indicate that the sulfate transfer system is less lipid dependent than the system discussed above. In agreement with this claim, exogeneous long chain fatty acids are not inhibitory (Grunze, M. and Deuticke, B., unpublished results), in marked contrast to the pronounced effects of their short chain homologues [7].

The cleavage of 60% of the membrane phosphatidylcholine by phospholipase A_2 should lead to the occurrence of negative charges in the membrane interface at a density * likely to change the surface potential by more than

* A theoretical charge density can be computed from the cleavage of 16% of the total phospholipids (5 μmol/ml cells), assuming random distribution of the fatty acids over both bilayers, a 50% ionization of the fatty acid at a bulk pH of 7.4 [41] and a surface area of 150 μm^2 cell. A charge density of $-1/600$ $Å^2$ is thus obtained. Restriction of the fatty acid to the outer lipid layer would even lead to $-1/300$ $Å^2$.

−50 mV (Ref. 39, p. 107). This change is obviously not sensed by the sulfate transfer system. Similar changes of the surface potential, induced in erythrocytes by the fluorescent anionic probe, ANS, have tentatively been invoked to explain the inhibitory effects of the probe on anion transfer [40]. The difference between the long chain fatty acids and ANS either points to differences in localization of the two (exclusion of the fatty acids from the environment of the anion carrier?) or argue against the contribution of surface charge to inhibition of anion transfer.

In conclusion, our results demonstrate the potential value of enzymatic phospholipid cleavage as a tool for studying the lipid dependency of mediated transport processes in the erythrocyte. Kinetic evaluation of the type of inhibition and its assignment to particular degradation products as well as corroborating physical studies are under way and will lead to a deeper understanding of the underlying molecular events.

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