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Influence of Enzymatic Phospholipid Cleavage on the Permeability of the Erythrocyte Membrane: III. Discrimination between the Causal Rôle of Split Products and of Lecithin Removal

B. Deuticke, M. Grunze*, B. Forst, and P. Luetkemeier

Abteilung Physiologie, Medizinische Fakultät, Rheinisch-Westfälische Technische Hochschule Aachen, D-5100 Aachen, West Germany

Summary. Cleavage of 55% of the lecithin in intact human erythrocytes by phospholipase A_2 (bee venom) markedly inhibits the mediated transport of L-lactate (via the monocarboxylate carrier) and of L-arabinose (via the monosaccharide carrier), while the major anion exchange system (probed by oxalate) and diffusion via the lipid domain (probed by erythritol) remain essentially unaltered. The causal role of the split products, unsaturated fatty acids and saturated lysolecithin, and of lecithin removal were now studied by sequential extraction of split products with serum albumin and by their controlled insertion into normal membranes. Careful choice of the albumin-to-cell ratio allowed the extraction of more than 95% of the fatty acids and up to 80% of the lysolecithin without hemolysis.

Extraction of fatty acids abolished inhibition of lactate and arabinose transfer, but induced inhibition of anion exchange and translipid permeation. Subsequent extraction of lysolecithin produced no further effects except on lactate transfer, which was inhibited.

Exogenous oleic and linoleic acid, at intramembrane concentrations equal to those produced by phospholipase A_2, inhibit lactate and arabinose transfer, while accelerating oxalate and erythritol movements, in agreement with effects of endogenous fatty acids. Exogenous lysolecithin inhibits all mediated transfer processes but does not alter translipid permeation. This pattern differs from that obtained for endogenous lysolecithin.

The action of exogenous lysolecithin can be suppressed by loading of the cells with cholesterol. Insertion of exogenous lysolecithin into cells depleted of endogenous lysolecithin does not restore the functional state before depletion, indicating that exogenous and endogenous lysolecithin may act differently.

* *Present address:* Medizinische Universitätsklinik, D-6900 Heidelberg, West Germany

Modification of membrane phospholipids by cleavage with phospholipases has been used by many investigators to study the relevance of lipids for protein-related functions of biomembranes. In many instances pronounced effects could be demonstrated. With the exception, however, of electrical characteristics of neurons [21] and axons [39], the properties investigated only comprised the binding of toxins, drugs [4, 28], transmitters [1], and hormones [2, 48] to their receptors, or enzymatic reactions [5, 10, 11, 13, 36, 37, 43].

In previous investigations [49, 50] of this series we have analyzed the effect of enzymatic cleavage of exofacial membrane phospholipids (phosphatidylcholine, sphingomyelin) on simple "translipid," and on facilitated, protein-mediated diffusion processes across the human erythrocyte membrane. Rates of nonelectrolyte movements via the lipid domain and of mediated exchange of inorganic anions remained essentially unaltered after hydrolysis of up to 60% of the phosphatidylcholine, corresponding to about 18% of the membrane phospholipids or 36% of those in the outer leaf of the lipid bilayer. In contrast, the movements of L-arabinose, catalyzed by the monosaccharide carrier system, and of L-lactate, transported by a specific monocarboxylate carrier, were markedly inhibited by phospholipid cleavage. In similar studies, inhibition of the active extrusion of Na^+ has recently been demonstrated in human erythrocytes treated with phospholipase A_2 [14]. These results obtained on erythrocytes provided first evidence for effects of phospholipid cleavage on solute translocation across biomembranes in intact cells.

Inhibitory effects of phospholipid cleavage can in principle be due either to the production of the split products, lysolecithin and fatty acid, which remain bound to the membrane, or to the disappearance of a particular phospholipid. In order to distinguish between these possible mechanisms, two procedures can

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be used. First, the split products of lecithin, although tightly bound to the membrane core, can be removed by treatment with serum albumin. Second, split products can be introduced into the membrane of normal cells. If the former procedure abolishes and the latter one mimics the effects of phospholipase A_2 treatment, split products are likely to be responsible for the effects of phospholipase A_2. Otherwise, the disappearance of a native phospholipid has to be considered.

Testing the removal of split products is easily accomplished in isolated membranes [10, 11, 13, 37, 43], but has met problems in intact erythrocytes, which lysed after extraction of part of the split products in earlier studies [17]. Comparisons between the actions of exogenous and endogenous fatty acid and lysolecithin, on the other hand, were mostly qualitative as yet, since effects were related to bulk concentrations of the exogenously added substances and not to those *within* the membrane.

The following attempt to further clarify the effects of phospholipase A_2 treatment on erythrocytes is based on a stepwise, controlled extraction of endogenous split products and a quantitative evaluation of the action of exogenous split products. From the results it will become evident that transport processes in the same membrane may differ markedly with respect to the mechanisms by which cleavage of phosphatidylcholine exerts its effects.

Materials and Methods

Materials

Phospholipase A_2 (E.C. 3.1.1.4) from bee venom (Boehringer, Mannheim) was used without further purification. Bovine serum albumin (fatty acid-free), linoleic and oleic acid (approximately 99%), and lysolecithin (egg yolk, type I, approx. 98%, No. L-4129) were obtained from Sigma, Munich. Labeled compounds (L-[U-^{14}C] lactic acid, sodium salt, [U-^{14}C] oxalate, [U-^{14}C] erythritol and L-[1-^{14}C] arabinose) were from Amersham Buchler, Braunschweig. Standard chemicals and biochemicals were of the highest purity available.

Methods

1) Treatment of erythrocytes with phospholipase A_2 and preloading with unlabeled test permeants. Freshly collected human erythrocytes were washed and suspended at a Hct. of 10% in a medium (Medium *A I*) of the following composition (concentrations in mM): KCl, 75; NaCl, 70; $CaCl_2$, 0.25; $MgCl_2$, 0.25; glycylglycine, 10; glucose, 2; sucrose, 44; and, alternatively: Na-L-lactate, 6; L-arabinose, 5; *m*-erythritol, 5; or no additive in experiments designed to study oxalate exchange.

After adjustment of pH to 7.4, phospholipase A_2 (4 μg/ml) suspension) was added for 60 min at 37 °C. Enzyme activity was stopped by addition of EDTA[1] (1 mM), the suspensions were centrifuged (10 min, 6000 × *g*), and the cells washed twice in medium *A II* which corresponded to medium *A I*, except that $CaCl_2$ was replaced by additional 0.25 mM $MgCl_2$ and by 1 mM EDTA and that 3.5 mM oxalate were present in experiments dealing with this anion. Cells treated correspondingly but without phospholipase A_2 served as controls.

2) Treatment of cells with albumin after phospholipid cleavage. Cells treated with phospholipase A_2 as described above were suspended (Hct. 5%) in medium *A II* containing albumin in amounts ranging from 6 to 30 mg per ml of cells, equivalent (at the standard Hct. of 5%) to concentrations between 35 and 175 mg/100 ml medium. The usual time of exposure to albumin was 10 min at 22 °C, but longer periods (up to 30 min) did not influence the results. In experiments on oxalate transfer, exposure to albumin was preceded by a 45-min incubation period at 37 °C in order to equilibrate the cells with this anion. After treatment with albumin the cells were again centrifuged and washed once in medium *A II*. Cells treated correspondingly, but without albumin, served as controls.

3) Incorporation of exogenous lysolecithin and fatty acids into fresh cells during preloading with unlabeled test substances. Cells were first equilibrated (60 min, 37 °C) with the unlabeled test permeant in 17 vols of a medium of the following composition (Medium *B*, concentrations in mM): KCl, 90; NaCl, 50; Na_2HPO_4/NaH_2PO_4, 6.25; glucose, 4.5; sucrose, 44; and alternatively: L-arabinose, 5; di-sodium oxalate, 3.5; Na-L-lactate, 5, or *m*-erythritol, 5. After this preloading, aliquots of the suspensions were cooled to 10 °C and mixed with aliquots of stock dispersions of either lysolecithin (2.5 mg/ml) or oleic or linoleic acid (10–40 μl of a solution of 40 μl 99% acid in 200 μl ethanol (96% vol/vol), dispersed in 0.5 ml 150 mM NaCl). The suspensions (final molar concentrations of the agents are given in the results) were incubated for 10 min at 10 °C, and then centrifuged at low temperature. The cells were washed three times with 5 vols of medium *B* prior to further use. In other experiments cells were exposed to lysolecithin and fatty acids during the efflux measurement periods.

4) Loading of the cells with labeled test substances and efflux measurements. 1.3 ml cells preloaded with unlabeled test permeant and treated with either phospholipase A_2, or phospholipase A_2 and albumin, or with either lysolecithin or long-chain fatty acids were suspended in 3 ml medium *B* containing the respective test substance. After adjustment of pH to 7.4 and of temperature to 20 °C, 0.5 μCi of labeled test permeant were added and the samples incubated for 60 min at 20 °C. Thereafter, the cells were separated by centrifugation (10 min, 6000 × *g*) and the supernatants removed carefully. Tracer back efflux into medium *B* was measured and evaluated as described previously [8].

5) Determination of phospholipids. Erythrocytes treated with phospholipase A_2 or exposed to exogenous lysolecithin were extracted [38] and phospholipids determined in an aliquot of the extract by one-dimensional thin-layer chromatography on silica plates (Merck, Darmstadt, No. 5715) using chloroform/methanol/acetic acid/H_2O (60:30:12:2.0, vol/vol) as a solvent. After development, the fractional contents of the various phospholipids and of degradation products were determined [18]. The percent cleavage of lecithin (PC) to lysolecithin (LPC) was calculated from the ratio (LPC/(LPC+PC)·100. Absolute concentrations of endogenous phospholipids were obtained from their fractional contents and the absolute content of lipid phosphorus, using a mean value of 5 μmol per ml of cells [15].

Fractional membrane concentrations of residual lysolecithin, after treatment of the cells with albumin, were calculated from the ratio $(LPC/PC)_{+alb}/(LPC/PC)_{-alb}$, assuming constant lecithin levels during the procedure. These fractional contents were con-

[1] *Abbreviations:* EDTA, ethylene diamine tetraacetic acid; BSA, bovine serum albumin; 18:1, oleic acid; 18:2, linoleic acid; 14:0, myristic acid.

verted into absolute values using the original lysolecithin content after treatment with phospholipase A_2, determined as described above. Intramembrane concentrations of exogenously applied lysolecithin were calculated from the ratio LPC/PC and the absolute concentration of endogenous lecithin.

Determination of fatty acids. An aliquot of the lipid extract [38] was evaporated, redissolved in approximately 50 µl isopropanol and subjected to gas-liquid chromatography (Packard Becker 419, glass column 1.5 m × 2 mm i.d., oven temp. 235 °C, flame ionization detector) on 5% Free Fatty Acid Phase on Chromosorb W AW (WGA, Düsseldorf). This procedure allowed quantitative analysis of fatty acids (and cholesterol) in a single-step procedure without derivatization. Peak areas were computed on-line by an automatic integrator (MINIGRATOR, Spectra Physics).

Relative membrane concentrations of fatty acids were calculated by relating their peak areas to peak areas for cholesterol, taking into account differences in detector response as derived from standards. Data for exogenous fatty acids pertain to the peak of the fatty acid applied (18:1 or 18:2). Data for endogenous fatty acids refer to the sum of fatty acids from the 14:0 peak to the last detectable peak eluting before the cholesterol peak.

Absolute concentrations were calculated from these fatty acid/cholesterol ratios in two ways:

1. In studies with exogenous fatty acids the mean cholesterol concentration of 3.5 µmol per ml of erythrocytes [16] served as a reference value.

2. In studies with endogenous fatty acids the total fatty acid peak area for cells treated with phospholipase only was taken to represent fatty acid contents equal, on a molar basis, to the lysolecithin content. This procedure appeared justified in view of our finding of a complete recovery of fatty acids produced by phospholipase A_2 even after repeated saline washings of the cells. The absolute residual contents of fatty acids in cells treated with phospholipase A_2 *and* albumin were computed from fatty acid/cholesterol ratios relative to the ratio for cells treated with phospholipase only and the absolute fatty acid content in these latter cells.

Results

Reversibility of Phospholipase A_2-Induced Effects

A 60-min treatment of erythrocytes with bee venom phospholipase A_2 (4 µg per ml of a 5% cell suspension in medium *A I*, 37 °C) brings about a hydrolytic cleavage of 54 ± 4% (mean ± SD from 16 experiments) of the membrane lecithin. This corresponds to the disappearance of 0.75 µmol lecithin per ml cells and the formation of equivalent concentrations of each, lysolecithin and fatty acids, as calculated on the basis of 5 µmol lipid phosphorus per ml cells and a mean fractional content of lecithin of 28%. In agreement with our earlier results [49, 50], this modification of membrane lipids goes along with a decrease of the self-exchange rates of L-arabinose to 57 ± 4% ($n=5$) and L-lactate to 43 ± 4% ($n=4$) of their original value. These inhibitory effects are partly (L-arabinose, Fig. 1a) or completely (L-lactate, Fig. 1*b*) abolished, if bovine serum albumin (BSA) is added to the medium during efflux measurements at a concentration of 12 mg/ml of cells (60 mg/100 ml medium). The effect of albumin reaches its full extent within < 30 sec after the cells have come into contact with the protein. This normalization of fluxes, which is not accompanied by hemolysis, provided first evidence that split products of phospholipid hydrolysis are involved in the transport inhibition produced by phospholipase A_2.

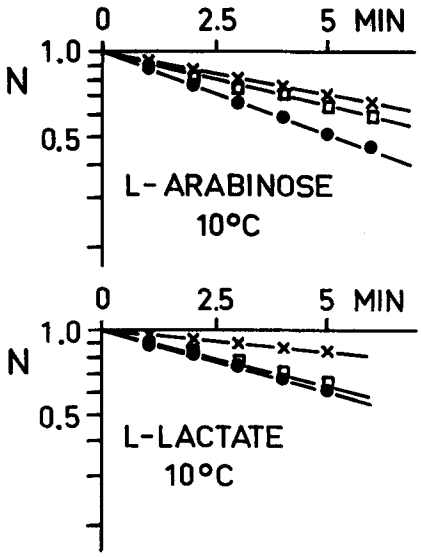

Fig. 1. Reversibility of effects of phospholipase A_2 treatment by exposure to albumin. The graph shows the time course of equilibrium exchange of L-arabinose and L-lactate in control cells (●), in cells treated with phospholipase A_2 (×), and in phospholipase-treated cells exposed to albumin (60 mg/100 ml medium) during flux measurement (□). For further details, *see Methods.* $N = \log [(cpm_\infty - cpm_t)/(cpm_\infty - cpm_0)]$, where cpm = radioactivity in the medium for times 0, ∞ and for various sampling times t

Gradual Removal of Products of Phospholipid Hydrolysis

In order to clarify in more detail which of the two split products, lysolecithin or fatty acid, acts inhibitory, we set up a regime to gradually deplete the membrane of the phospholipid degradation products. This procedure takes advantage of the very different affinity and number of binding sites of BSA for fatty acids [42] and lysolecithin [27]. By careful choice of the albumin concentration (per volume of cells), conditions could be established under which most (> 80%) of the fatty acids (*see also* ref. [17]) but only a minor (< 15%) amount of the lysolecithin was removed from phospholipase-treated erythrocytes (Table 1). No hemolysis occurred under these conditions. A further increase of the albumin concentration removed essentially all of the fatty acids (> 93%) and extracted increasing quantities of lysolecithin, up to a maximum of 75% of the total amount produced under our experimental conditions. This maximal depletion could be achieved by treating the cells with 30 mg BSA/ml

Table 1. Partial delipidation of the erythrocyte membrane by phospholipase A_2 and albumin[a]

	Albumin	Lysolecithin		Fatty acid	
	(mg/ml cells)	μmol[b]/ml cells	Relative[c]	μmol/ml cells	Relative
Control		≧0.015	–	ND	
Phospholipase A_2	0	0.75±0.06	1.0	0.75	1.0
	6	0.68	0.91	0.15	0.2
	12	0.62±0.05	0.82±0.06	≦0.05	≦0.07
	30	0.38±0.05	0.50±0.06	≦0.05	≦0.07
	2×30	0.20±0.05	0.26±0.06	ND	
	3×30	0.20±0.05	0.25±0.06	ND	

[a] Erythrocytes were treated with phospholipase A_2 (bee venom), as described previously [49, 50]. After blockage of enzyme activity (EDTA, 3 washings) the cells were exposed to bovine serum albumin (20 °C, 10 min). Mean values from 4–6 experiments ± SD.

[b] Calculated on the basis of a total phospholipid concentration of 5 μmol/ml cells and a fractional lecithin content of 28%.

[c] Relative to content in phospholipase-treated cells.

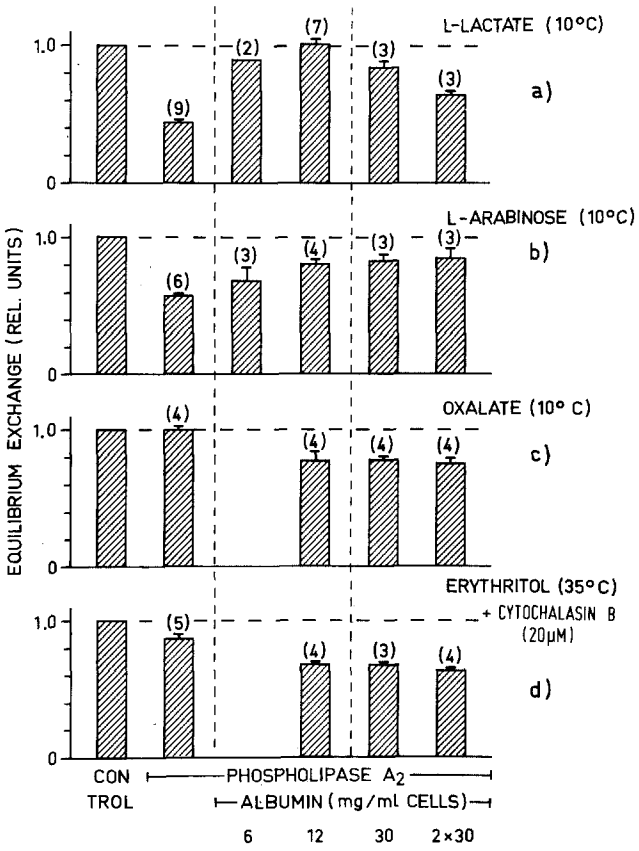

Fig. 2. Influence of sequential extraction of phospholipid split products on transport processes in human erythrocytes. Cells were treated with phospholipase A_2 as described in *Methods* and then exposed to albumin under the conditions given in Table 1. For further details, *see Methods*. Mean values ± SEM; number of experiments in brackets

cells for 1 to 3 times in succession. In view of the established 1:1 stoichiometry of lysolecithin binding to albumin [27], these amounts of BSA should suffice to bind the 0.75 μmol lysolecithin/ml of cells produced under our conditions. Part of the lysolecithin is apparently inextractable by albumin under the conditions of our experiments.

Cells depleted in this way of all their fatty acids and most of the lysolecithin do not lyse. Only at higher concentrations of albumin, which do not remove more lysolecithin, hemolysis finally occurs due to effects other than lysolecithin extraction (*see* Discussion).

Changes of Transport Rates during Stepwise Removal of Split Products

According to Fig. 2*a* inhibition of lactate exchange by phospholipid cleavage is relieved when the cells are treated with concentrations of albumin that remove almost all of the fatty acids but little lysolecithin (*cf.* Table 1). Subsequent removal of lysolecithin restores inhibition to some extent. Inhibition of arabinose exchange (Fig. 2*b*) is only partly relieved by removal of fatty acid and unaffected by subsequent extraction of 75% of the lysolecithin. Lactate and arabinose self-exchange in normal cells, serving as controls, are insensitive to albumin treatment over the range of concentrations used in our experiments (data not shown).

The simple diffusion of nonelectrolytes via the lipid domain of the erythrocyte membrane as well as the mediated exchange of inorganic anions are almost insensitive to enzymatic cleavage of lecithin [49, 50]. To clarify whether this lack of inhibition is due to an intrinsic irresponsiveness of the two pathways, we

studied the fluxes of erythritol, which penetrates via the lipid domain in the presence of inhibitors of the monosaccharide carrier system (*see* [6, 16] for references), and of oxalate, a substrate of the inorganic anion exchange system [7, 50] which – in contrast to sulfate – can conveniently be examined at low temperatures. Both transport processes are insensitive to albumin treatment of normal erythrocytes (data not shown).

Extraction of endogenous fatty acids by albumin produces a 25 to 35% inhibition of the two pathways (Fig. 2*c* and *d*). Subsequent removal of lysolecithin causes no further changes. This pattern indicates an accelerating effect of fatty acid moieties in the membrane, becoming evident upon their removal.

Effects of Exogenously Inserted Split Products

Due to the specificity of phospholipase A_2, which cleaves the ester bond at carbon-2 of the glycerol moiety of lecithin, and to the differences in saturation of the fatty acids linked to carbon-2 and carbon-3, the split products arising in the presence of phospholipase A_2 are predominantly stearoyl- and palmitoyl-lysolecithin and oleic and linoleic acid [45]. In order to examine their intrinsic effects, increasing concentrations of lysolecithin and oleic acid, which easily enter the erythrocyte membrane when applied exogenously [9, 26, 30, 35, 47], were added to cell suspensions during flux measurements. As shown in Fig. 3, exogenous lysolecithin inhibits L-lactate, L-arabinose and, to a lesser extent, oxalate transfer, while translipid permeation of erythritol remains essentially unaltered. Exogenous oleic acid, on the other hand, inhibits L-lactate and L-arabinose transfer, and accelerates erythritol permeation. Transfer of oxalate is enhanced at low concentrations and inhibited at higher concentrations of the fatty acid.

Qualitatively, the results for low concentrations (0.15–0.3 mM) of long chain fatty acids coincide with predictions from phospholipase A_2 experiments. Results for lysolecithin seem to contradict phospholipase studies at least in case of lactate and arabinose. However, in order to be quantitatively comparable with results from phospholipase A_2 studies, effects of exogenous split products have to be related to their *intra*membrane concentrations. Only those intramembrane concentrations of exogenous split products should be included in a comparison which can also be obtained by phospholipase A_2 treatment. We therefore worked out the conditions under which these levels of exogenous split products can be obtained in the erythrocyte membrane. The amount of fatty acids produced by phospholipase A_2 treatment under our standard conditions (0.75 μmol/ml cells) could be inserted by treatment of cells with 160 μM fatty acid (*see* Table 2 for detailed conditions). Lysolecithin could be incorporated only at concentrations lower than those produced by phospholipase A_2. Equivalent concentrations caused hemolysis.

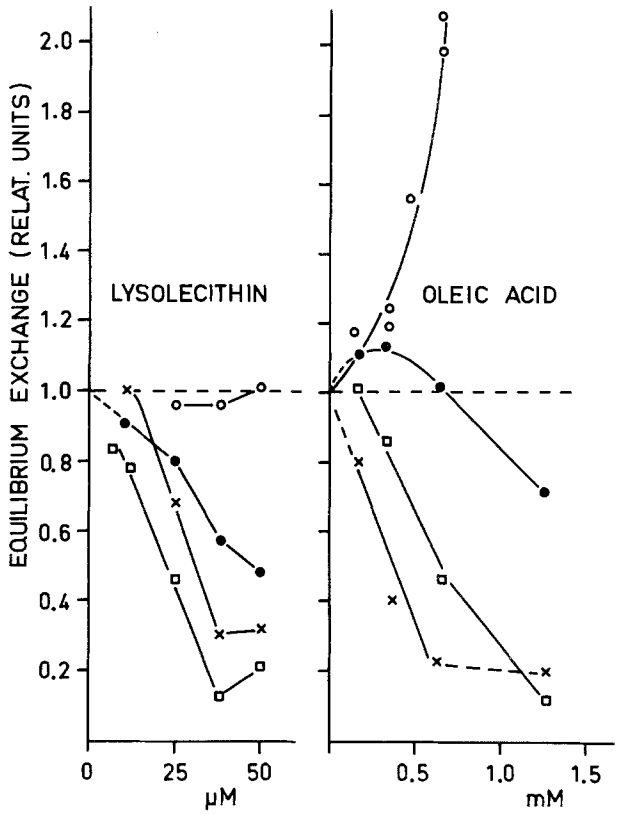

Fig. 3. Influence of exogenous lysolecithin and oleic acid on the equilibrium exchange of: (○) erythritol (in the presence of cytochalasin B, 20 μM), 35 °C; (●) oxalate 10 °C; (□) L-arabinose, 10 °C; (x) L-lactate, 10 °C. Cells were loaded with labeled test permeant and tracer efflux measured as described elsewhere [8]. The agents were added at the start of the efflux measurement

Table 2. Comparison of the levels of endogenous (after phospholipase A_2) and exogenous lysolecithin and long chain fatty acids in human erythrocytes[a]

	Endogenous	Exogenous			
	Phospholipase A_2	Lysolecithin		Fatty acids (160 μM)	
		(38 μM)	(25 μM)	18:1	18:2
Lysolecithin[b]	0.75 ±0.06	0.55 ±0.08	0.42 ±0.04		
Fatty acids[b]	0.75			0.73 ±0.16	0.69 ±0.25

[a] Treatment with phospholipase: 60 min, as described in *Methods*. For the insertion of exogenous compounds, cells were exposed to egg lysolecithin and to oleic and linoleic acid, at the concentrations given in brackets, for 10 min at 10 °C, then washed and extracted. Mean values ± SD; $n=4$.

[b] μmol/ml cells.

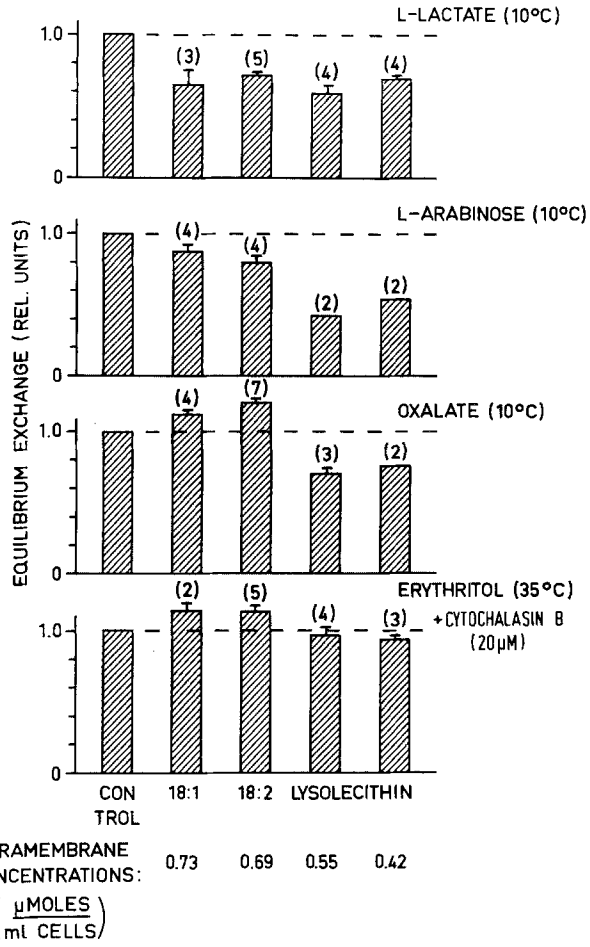

Fig. 4. Effects of exogenous split products of lecithin on simple and mediated transfer processes at concentrations also obtained by the treatment of cells with phospholipase A_2. Intramembrane concentrations of the exogenous split products are given in the lowest line. After preloading with labeled test permeant, the cells were either exposed to egg lysolecithin (0.38 and 0.25 μM) or to oleic or linoleic acid (160 μM) for 10 min at 10 °C. After thorough washing of the cells at 0 °C, fluxes were measured in solutions free of the lipids. Lysolecithin and fatty acids were determined by thin-layer and gas chromatography as described in *Methods*. Mean values ± SEM; number of experiments in brackets

Transport changes induced by these intramembrane concentrations of exogenous split products are summarized in Fig. 4. Both, oleic and linoleic acid have a marked inhibitory influence on lactate, a lesser one on arabinose transfer, and slightly enhance oxalate exchange as well as the simple diffusion of erythritol. No significant differences between the mono- and the di-unsaturated acid could be established. These findings well agree with predictions from phospholipase A_2 experiments. Exogenous lysolecithin, on the other hand, inhibits movements of lactate, arabinose, and oxalate, but has essentially no influence on simple nonelectrolyte diffusion. This difference is not due to the higher temperature in the erythritol experiments, since in preliminary studies with a new type of model nonelectrolyte [49], permeating via the lipid domain of biomembranes, we obtained the same result at 10 °C (*unpublished results*).

The effects of the two exogenously applied split products of lecithin, which are reversible if albumin is added during the efflux measurements (data not shown), thus tend into the same direction in case of lactate and arabinose exchange. They are opposite to each other for oxalate exchange, as could also be substantiated in experiments in which cells were treated with linoleic acid and lysolecithin simultaneously (Table 3).

Effects of Exogenous Lysolecithin on Cells Depleted of Endogenous Lysolecithin

Insertion of exogenous lysolecithin inhibits mediated transfer processes, while, in contrast, removal of endogenous lysolecithin has either no influence (oxalate, arabinose) or also inhibits (lactate). Since these two effects are opposite in direction, we examined whether insertion of exogenous lysolecithin could reverse the effects of a removal of endogenous lysolecithin. This did not prove to be the case (Table 4). Exogenous

Table 3. Compensation of effects of simultaneously inserted fatty acid and lysolecithin on oxalate exchange[a]

Rate coefficient of equilibrium exchange in absolute (min^{-1}) and relative units				
Expt.	Control	Lysolecithin[b]	Linoleate[c]	Lysolecithin + linoleate
1	0.0965 (1.0)	0.0746 (0.77)	0.1254 (1.30)	0.0954 (0.99) [0.77 × 1.30 = 1.0][d]
2	0.0958 (1.0)	0.0682 (0.71)	0.1123 (1.17)	0.0794 (0.83) [0.71 × 1.17 = 0.83][d]

[a] The agents were inserted into the membrane by a 10-min exposure at 10 °C. Subsequently, the cells were washed and fluxes measured at 10 °C as described in *Methods*.
[b] 38 μM. [c] 160 μM. [d] Expected values based on the effect of each single agent.

Table 4. Lack of normalization of inhibited lactate transfer in erythrocytes treated with phospholipase A_2 and albumin, by insertion of exogenous lysolecithin[a]

	Control	Lysolecithin (25 μM)	Phospholipase A_2			
			Albumin (mg/ml cells)			
			0	12	2×30	2×30, then lysolecithin (25 μM)
Lactate flux (relat. units)	1.0	0.69	0.42 ±0.05	0.97 ±0.03	0.63 ±0.06	0.39 ±0.08
Lysolecithin[b] contents of cells (μmol/ml)	0.01	0.42	0.75	0.62	0.23	0.78

[a] Cells were treated with phospholipase A_2 and the split products of lecithin extracted by albumin as demonstrated in Table 1 and Fig. 2. Cells depleted of all the lysolecithin extractable by albumin were then exposed to exogenous lysolecithin (25 μM, Hct. 5%, 10 min, 10 °C) during or before the measurement of equilibrium exchange. Fluxes were in arbitrary units relative to the controls. Data for the effect of lysolecithin on normal cells are taken from Fig. 3. Mean values ± SEM from 3 experiments.
[b] Calculated, as described in *Methods*, under the assumptions given in Table 1.

lysolecithin inhibits lactate transfer to the same extent in lysolecithin-depleted as in normal cells.

Influence of Membrane Cholesterol on the Effects of Exogenous Lysolecithin

Cholesterol and lysolecithin have been shown to interact in artificial membranes [25, 34]. In order to obtain indications whether a similar interaction also plays a role in cells treated with exogenous lysolecithin, transports were measured in normal cells and in cells loaded with cholesterol *in vitro* [16]. Increased cholesterol has previously been shown to inhibit translipid permeation and anion exchange in the erythrocyte membrane while accelerating arabinose transfer [16]. Inhibitory effects of exogenous lysolecithin in fact are suppressed in cholesterol-loaded erythrocytes (Table 5). On the other hand, the effects of cholesterol on nonelectrolyte transfer via the lipid domain and on the mediated transfer processes were not relieved by lysolecithin insertion.

Table 5. Suppression by cholesterol of the effect of exogenous lysolecithin on transfer processes[a]

		Control	Lysolecithin (25 μM)	
		Rate coefficient k (min^{-1})		
Erythritol (+20 μM cytochalasin B)	Normal[b]	0.0405	0.0385	(0.95)
	Cholesterol-loaded[c]	0.0335 (0.83)	0.0325 (0.84)	(0.96)
Oxalate	Normal	0.0821	0.0605	(0.74)
	Cholesterol-loaded	0.0270 (0.33)	0.0284 (0.46)	(1.05)
L-Arabinose	Normal	0.0721	0.0357	(0.50)
	Cholesterol-loaded	0.0786 (1.09)	0.0622 (1.74)	(0.79)

[a] Cells were loaded with cholesterol *in vitro* as described in refs. 15 and 16. Lysolecithin was present during measurements of equilibrium exchange. Fractional changes of equilibrium exchange in cholesterol-loaded relative to normal cells are *below* the k values; changes in lysolecithin-treated cells relative to respective controls are in brackets *behind* the k values.
[b] Cholesterol/phospholipid (mol/mol) = 0.7.
[c] Cholesterol/phospholipid (mol/mol) = 1.3.

Discussion

The use of phospholipases as tools in clarifying the significance of phospholipids for the function of membrane proteins has so far been largely restricted to nonvectorial processes. The binding of effectors and inhibitors and of catalytic properties of enzymes were shown to be affected by phospholipid cleavage [1, 2, 4, 5, 10, 11, 13, 28, 36, 37, 43, 48].

Insight into the underlying processes was provided by the removal of split products by serum albumin [10, 11, 13, 37, 43] and their insertion into normal membranes [1, 2, 10, 28, 29, 32, 41, 46, 48, 51). By these strategies it could be shown that in some instances the effects of phospholipase A_2 are caused by cleavage products and not by the disappearance of the native phospholipid [1, 10, 28]. Other systems proved to be insensitive to phospholipid hydrolysis as long as the unsaturated fatty acids were still present [11, 13, 37, 43]. Inactivation only occurred upon removal of split products (=delipidation). Both, phospholipase A_2 treatment and insertion of split products may act accelerating [11, 32, 33, 37, 41] or inhibitory [1, 41, 48], even in the same membrane [11, 41].

From the present study it becomes evident that differences in mechanism and direction also characterize the effects of phospholipase A_2 on transport, i.e., vectorial processes. As an essential prerequisite for the investigation of this problem we could demon-

strate that not only fatty acids, as already shown by Gul and Smith [17], but also lysolecithins produced by phospholipase A_2 can be removed almost completely from the erythrocyte membrane without hemolysis. Lysis of cells treated with phospholipase A_2 and albumin [17] only occurs at albumin concentrations exceeding those required for extracting the 75 to 80% of the total lysolecithin that can be removed under our experimental conditions. As reported elsewhere [19], albumin-induced lysis of phospholipase A_2 treated cells is most likely not due to "chemical disintegration" of the membrane but to a highly increased mechanical fragility of the cells resulting from a loss of surface area induced by membrane internalization. These lytic side effects can be avoided by proper choice of the albumin concentration.

The reason for the incomplete extraction of lysolecithin by albumin is obscure as yet. Transmembrane reorientation of lysolecithin during the 60-min exposure to phospholipase A_2 at 37 °C [30] might shield part of the lysolecithin. Attempts to increase extractability by raising temperature and prolonging the exposure time, however, were unsuccessful.

Effects of Extraction of Split Products

Fatty acids. Extraction of the unsaturated fatty acids predominantly formed upon cleavage of lecithin in human erythrocytes [45] normalizes two mediated transport processes (L-lactate, L-arabinose) inhibited by phospholipase A_2 (*see* Table 6 for a compilation of our findings). This pattern indicates that the function of the transport protein is inhibited by unsaturated fatty acids, but not (lactate) or only slightly (L-arabinose) affected by the conversion of 55% of the lecithin into lysolecithin. A similar pattern was previously described for the acetylcholine-activated Na^+ channel in the electroplax from *Torpedo californica* [1].

In contrast, the inorganic anion exchange system as well as the translipid pathway, which remain essentially unaltered after phospholipase A_2, become inhibited when the fatty acids are removed. This pattern is comparable to that observed with the Ca^{++} ATPase of the sarcoplasmic reticulum [43]. In that system, the inhibitory effect of fatty acid removal is much more pronounced than in the intact erythrocyte membrane. The difference is probably due to dissimilarities not between the two systems, but between the experimental situations: In the membrane vesicles used for studies on the ATPase and other enzymes all phospholipids are cleaved by a suitable enzyme, while in the membrane of the intact cell only a fraction of the exofacial phospholipids is hydrolyzed. The effects are thus "masked" by the diacylphospholipids still present.

Transport inhibitions which only become evident (erythritol, oxalate) after extraction of the endogenous unsaturated fatty acids, are indicative of a stimulating effect of these membrane components in addition to their above-mentioned inhibitory actions on lactate and arabionose transfer. Both effects can also be produced by exogenous application of fatty acids to the normal membrane (*cf.* Table 6).

Lysolecithin. The insensitivity of three of the four transport processes studied to the removal of lysolecithin produced from lecithin indicates that endogenous lysolecithin does not affect these systems. As an exception, removal of endogenous lysolecithin inhibits lactate transfer, suggesting an accelerating action. Both, lack of effect on oxalate and arabinose transfer and acceleration of lactate movements, however, are at variance with the inhibitory action of exogenous lysolecithin observed in all three systems. Moreover, insertion of exogenous lysolecithin into membranes depleted of endogenous lysolecithin does not reactivate lactate transfer, but produces additional inhibition (Table 4). Possible reasons for this discrepancy will be considered below.

Table 6. Pattern of effects of phospholipid cleavage and phospholipid split products on transfer processes in erythrocytes

	Phospholipid cleavage	Extraction of		Insertion of	
		Unsaturated fatty acids	Saturated lysolecithin	Unsaturated fatty acids	Saturated lysolecithin
Translipid permeation of					
Erythritol	No effect	Inhibition	No effect	Acceleration	No effect
Mediated transfer of					
Oxalate	No effect	Inhibition	No effect	Acceleration (→ inhibition)	Inhibition
L-Lactate	Inhibition	Normalization	Inhibition	Inhibition	Inhibition
L-Arabinose	Inhibition	Partial normalization	No effect	Inhibition	Inhibition

Effects of Exogenously Applied Split Products

Exogenous fatty acids, if applied in the adequate range of concentration, generally act like endogenous fatty acids (Fig. 4 and Table 6). In contrast, results obtained with exogenous and endogenous lysolecithin only coincide in the case of the simple translipid diffusion of erythritol. The divergent response of the mediated transports to endo- and exogenous lysolecithin (*cf.* Table 6) cannot be due to differences in the fatty acid patterns, since egg yolk as well as human red cell membrane lysolecithin contain palmitic and stearic acid as the major components and only minor amounts of oleic acid [45].

An important difference between membranes containing exogenous or endogenous lysolecithin concerns cholesterol. Endogenous lysolecithin most likely interacts with cholesterol [34], since it replaces lecithin originally present. Exogenous lysolecithin, however, is present in excess of the cholesterol available in the normal cell. The significance of this difference is underlined by our observation that the inhibitory effects of lysolecithin are suppressed in cholesterol-enriched membranes (Table 5). It may be speculated that endogenous lysolecithin is withheld from interaction with certain transport proteins by cholesterol, while exogenous lysolecithin is free to interact.

The persistence of the effects of cholesterol in the presence of lysolecithin can easily be explained by quantitative considerations: The concentration of inserted cholesterol amounts to 3.0 μmol/ml of cells while lysolecithin is incorporated under the conditions of our experiments only at about 0.4 μmol/ml cells. This amount is complexed by the extra-cholesterol without affecting the amount of cholesterol available for interaction with native membrane phospholipids.

The impossibility to compensate for the effects of a removal of endogenous lysolecithin by insertion of exogenous lysolecithin (Table 4) indicates irreversible changes in the membrane after the depletion, sensed by the lactate system. The effects of exogenous lysolecithin, on the other hand, are fully reversible, which excludes a causal role of membrane protein extraction by lysolecithin micelles, claimed to be involved in the lysolecithin-induced increase of cation permeability and consecutive hemolysis [3].

In the light of these considerations the changes of lactate transfer upon removal of endogenous lysolecithin have to be interpreted independent of those induced by exogenous lysolecithin.

Molecular Basis of the Effects of Split Products

Unsaturated fatty acids. Permeability changes of the lipid domain upon insertion or removal of unsaturated fatty acids can be related to their membrane-fluidizing action, also reported for other systems [33, 41, 44, 45]. Since this effect of endogenous fatty acids only becomes apparent when they are removed from the membrane and not when they are formed by cleavage of lecithin, it seems to be the presence or absence of an unsaturated alkyl chain that plays a role. Whether this chain is attached to a glycerol moiety in lecithin or mobile as free acid is probably not important. The formation of clusters of free fatty acids, described in artificial phospholipid membranes [20], thus appears not to affect permeability, if it also occurs in the erythrocyte membrane.

The inhibitory action of exogenous unsaturated fatty acids on the mediated transport of lactate and arabinose, not unexpected in view of their effects on enzyme activities in other membranes [1, 28, 29, 51], may also arise from an increase of membrane fluidity, in line with the response of these transport processes of cholesterol depletion [16]. The inhibitory influence of endogenous fatty acids, however, is probably not due to fluidity changes of the lipid domain. Such changes are unlikely to occur in view of the unaltered erythritol permeability after mere cleavage of phospholipids. The role of lateral phase separations of lipids, claimed to occur in erythrocyte membranes treated with phospholipase A_2 [22], remains to be elucidated. Disruption of the bilayer, however, does not seem to be involved since nonlamellar structures could not be detected by ^{31}P NMR in such membranes [31].

Alternatively, specific interactions of fatty acids with transport proteins must be considered. These interactions may occur in each leaf of the bilayer, although the fatty acids are formed or inserted only in the outer one, since lateral [12] and transmembrane [35] mobility of fatty acids is well established. The involvement of the head group charge is an open problem.

The complexity of the action of fatty acids is illustrated in particular by their dual, accelerating and inhibitory, effect on the inorganic anion exchange system. Interestingly, only the latter effect is shared by non-ionic detergents (e.g., Brij 96), having the same apolar moiety but a different head group (B. Deuticke, *unpublished results*).

Lysolecithin. Saturated lysolecithins might principally act by mechanisms similar to those of fatty acids. An effect on membrane fluidity, however, does not seem very likely, since at 35 °C neither endogenous nor exogenous saturated lysolecithin alter the permeability of the lipid domain (Table 6). The effects of exogenous lysolecithins on mediated transfer, analogous to effects on membrane enzymes [1, 41, 46, 48], very likely result from a direct interaction with pro-

teins. An interesting aspect concerns the problem of sidedness. Exogenous lysolecithins are introduced into the outer layer of the membrane. In contrast to the fatty acids, the major fraction probably remains there during the time period of our experiments, since transmembrane reorientation seems to be a slow process [30]. If one accepts this view, the transport inhibition either might occur in the outer layer by direct perturbation of the domain of the transport protein located there, or might be a consequence of the passive expansion of the inner leaf of the membrane following the insertion of lysolecithin into the outer leaf and the consecutive increase of the total membrane surface area [40]. Both concepts are in line with our observation that the effects of the exogenous lysolecithins are maximal instantaneously.

Lecithin removal. Consequences of lecithin depletion may be involved in the inhibitory effect of lysolecithin removal observed in case of lactate transfer. This inhibition is prevented by endogenous but not counteracted (Table 4) by exogenous lysolecithin. Therefore, a particular dependency of the transfer system on lecithin [23] or on the sphingomyelin/lecithin ratio, which increases from 0.83 to 1.48 after cleavage and removal of lecithin, must be discarded. Irreversible changes of membrane structure may play a decisive role.

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