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CROSS-LINKING OF SH-GROUPS IN THE ERYTHROCYTE MEMBRANE ENHANCES TRANSBILAYER REORIENTATION OF PHOSPHOLIPIDS

EVIDENCE FOR A LIMITED ACCESS OF PHOSPHOLIPIDS TO THE REORIENTATION SITES

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Oxidation of erythrocyte membrane SH-groups and concomitant cross-linking of spectrin, which induce a partial loss of phospholipid asymmetry (Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32) are now shown to result in a remarkable increase of the rates of transbilayer reorientation of exogenously incorporated lysophospholipids. Reorientation of both, neutral lysophosphatidylcholine and of negatively charged lysophosphatidylserine is enhanced. A decrease of the activation energy of the reorientation process as well as quantitative changes of the dependence of reorientation on the lysophosphatidylcholine and cholesterol content of the membrane indicate formation of new reorientation sites or modification of existing sites. A common mechanism may underly the formation of reorientation sites and the occurrence of leaks for small solutes (Deuticke, B., Poser, B., Lütkemeier, P. and Haest, C.W.M. (1983) Biochim. Biophys. Acta 731, 196–210) subsequent to oxidation of membrane SH-groups. Whereas exogenous lysophospholipids completely equilibrate between the two lipid layers regardless of the extent of oxidation of SH-groups, endogenous inner layer phospholipids become available for reorientation in a graded way. Native phospholipid asymmetry is therefore not the result of a low transbilayer mobility of phospholipids, but probably due to a lack of access of inner layer phospholipids to the reorientation sites.

Introduction

The preferential orientation of phosphatidylethanolamine and phosphatidylserine to the cytoplasmic membrane interface [1] is stable [2] in circulating erythrocytes. Mild oxidation by diamide of membrane SH-groups to disulfide bonds, which is paralleled by selective cross-linking of the skeletal protein, spectrin, to oligomers, however, results in a complete loss of asymmetry of phosphatidylethanolamine and a partial loss of asymmetry of phosphatidylserine [3]. Data obtained recently in our [4,5] and other [6] laboratories have provided evidence that this loss of asymmetry is related to an enhancement of the transbilayer mobility of certain phospholipids. Endogenous phosphatidylcholine [6] as well as lysophosphatidylcholine [7] incorporated experimentally into the outer membrane layer were shown to reorient much faster to the inner layer in diamide-treated than in native cells.

On the other hand the distribution of sphingomyelin is not changed by diamide and the loss of asymmetry of phosphatidylserine is only partial [3]. These observations demonstrate that the treatment of erythrocytes with SH-oxidizing

agents does not result in a general transbilayer equilibration of phospholipids. Besides its effect on transverse lipid mobility diamide produces structural defects in the erythrocyte membrane [8], having the properties of aqueous pores, but also sensitive to perturbations of the membrane lipid domain.

In the present paper the reorientation process induced by SH-oxidizing agents and its site are characterized in more detail. Correlations between loss of asymmetry of phosphatidylethanolamine, increase of transbilayer reorientation of lysophosphatidylcholine, and pore formation induced in the membrane by diamide are discussed.

Materials

Fresh human blood, anticoagulated by heparin, was obtained from the local blood bank. Freshly collected blood samples from different animals were anticoagulated with heparin (Rabbit, rat) or citrate (ox). Erythrocytes were isolated by centrifugation and washed three times in 154 mmol · l^{-1} NaCl. L-1-[1-^{14}C]Palmitoyllysophosphatidylcholine (spec. act. 47.5 mCi/mmol) was purchased from Amersham-Buchler, non-radioactive palmitoyllysophosphatidylcholine from Sigma. ^{14}C-labelled lysophosphatidylserine was prepared from dioleoylphosphatidyl[U-^{14}C]serine (Amersham-Buchler) by cleavage of the fatty acid at position 2 with phospholipase A_2 from bee venom. The lysophospholipid was then purified by thin layer chromatography. Purity was checked with a radiochromatographic scanner.

Methods

Diamide treatment of erythrocytes

Erythrocytes washed with isotonic saline were resuspended in 10 vol. of a medium containing (concentrations in mmol · l^{-1}): KCl (90), NaCl (45), Na_2HPO_4/NaH_2PO_4 (12.5) and sucrose (44) (Medium A). The pH of the suspension was adjusted to pH 8.0, except in studies on the pH dependence, by the addition of 0.1 M NaOH. Subsequently, diamide was added to a final concentration of 5 mmol · l^{-1}, unless otherwise indicated, and the cells incubated at 37°C. After various times of incubation cells were isolated by centrifugation and washed three times with medium A of pH 7.4.

Measurement of the transbilayer reorientation of lysophosphatidylcholine

100 µl of a suspension of 1 vol. of pretreated erythrocytes in 1 vol. of Medium A were added to 30 nmol of dry ^{14}C-labelled lysophospholipid to incorporate the lysophospholipid into the outer membrane layer. Reorientation to the inner layer was quantified by the decrease of extractability of lysophosphatidylcholine by albumin as described elsewhere [9]. Briefly, after different times of incubation at 37°C, 50 µl aliquots were transfered to small vials. The cells were spun down, washed once with 1 ml of Medium A and taken up with 400 µl of Medium A. One 200 µl sample was centrifuged and the total amount of radioactivity in the membrane quantified. Another 200 µl sample was used to determine the radioactivity in the inner membrane lipid layer. To this end the cells were incubated twice (1–3 min, 4°C) with 1 ml of a solution of 1.5% albumin in Medium A to extract outer layer lysophosphatidylcholine. The rate constant (k_{in}) and the half time ($t_{1/2}$) of reorientation of lysophosphatidylcholine from the outer to the inner layer were then calculated (see Appendix) using an equilibrium ratio for the distribution of lysophosphatidylcholine between the inner and the outer lipid layer of 0.5 for diamide-treated cells (see Results) and a value of 0.3 for control cells (unpublished data).

Results

The SH-oxidizing agent diamide is known to oxidize membrane SH-groups to disulfide bonds. Both, intramolecular as well as intermolecular disulfide bonds, mainly between spectrin monomers, are formed [3]. This disulfide bond formation has previously been shown to result in a time-dependent loss of the asymmetric arrangement of phosphatidylethanolamine and phosphatidylserine [3].

Besides the loss of asymmetry of endogenous phospholipids [3], an enhancement of the transbilayer reorientation of exogenously incorporated lysophospholipids occurs in cells treated with diamide (Fig. 1). Rates of reorientation to the inner layer of both, neutral lysophosphatidylcholine as

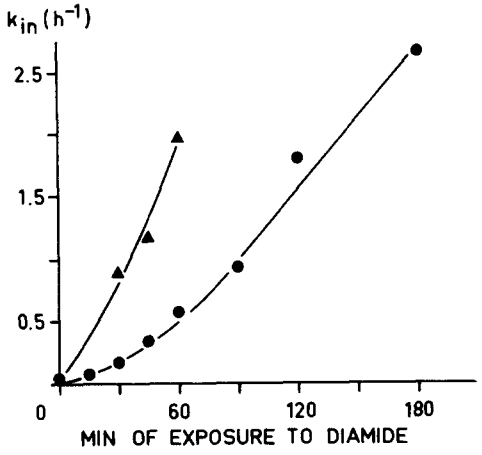

Fig. 1. Time-dependent increase of the rate (k_{in}) of transbilayer reorientation of lysophospholipids in erythrocytes pretreated with diamide. Cells were treated with diamide (5 mmol · l^{-1}) for different times and washed. [^{14}C]Lysophosphatidylcholine (●) or [^{14}C]lysophosphatidylserine (▲) were incorporated into the outer lipid leaflet and rates of reorientation of the lysophospholipid to the inner layer measured (see Methods).

well as negatively charged lysophosphatidylserine, increase with the duration of the exposure of cells to diamide. The enhancement of the flip is related in a strongly nonlinear way to the diamide-induced modification of membrane SH-groups (Fig. 2). Oxidation of the first 50% of the SH-groups only results in a small increase of the reorientation rate, while oxidation of further SH-groups goes along with a steep rise of the reorientation rate. No saturation of the reorientation rate occurred upon oxidation of essentially all of the diamide-sensitive SH-groups, i.e. of about 80% of the total membrane SH-groups [10]. The residual 20% react at best only very slowly with SH-reagents.

The effect of diamide on transbilayer reorientation exhibits saturation kinetics (half maximal effective concentration of 1.6 mmol · l^{-1}, data not shown), which were also observed for diamide's effect on membrane permeability [8]. Such saturation kinetics indicate that the rate of the reaction of diamide with SH-groups is probably not limited by the concentration of the oxidant, but determined by the collision frequency of pairs of SH-groups.

The reactivity of SH-groups [11] is known to be highly enhanced in their ionized state (pK of cysteine = 8.39, Ref. 12). In line with this general rule only minor effects of diamide were observed up to pH values of 7.7, whereas a steep increase of flip rates occurred above pH 8.0 (fig. 3). Oxidative cross-linking of SH-groups is probably directly involved in the effect of diamide. This is indicated by the easy reversibility of most of the enhancement of the flip upon reduction of the induced

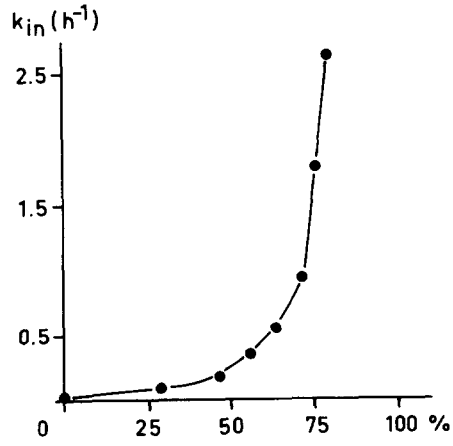

Fig. 2. Relationship between the increase of transbilayer reorientation of lysolecithin and the decrease of membrane SH-groups in diamide-treated cells. k_{in}-values (Fig. 1) are plotted against the percentage of membrane SH-groups oxidized by diamide under the same conditions. SH-groups were quantified by reaction with 4,4'-dithiodipyridine as described before [10].

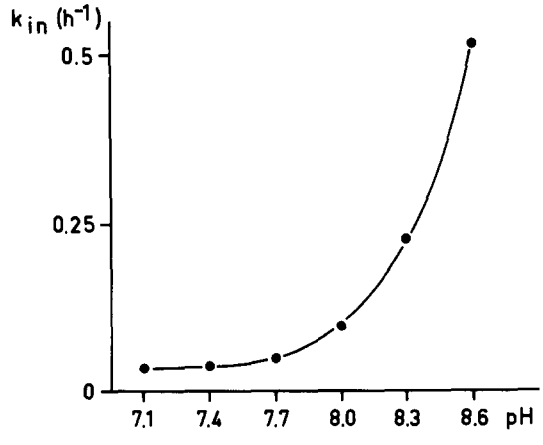

Fig. 3. pH-dependence of the effect of diamide on the transbilayer reorientation of lysophosphatidylcholine. Erythrocyte suspensions were titrated to pH-values between 7.1 and 8.6, treated with diamide (5 mmol · l^{-1}, 37°C, 30 min) and washed. Transbilayer reorientation of lysophosphatidylcholine was quantified at pH 7.4.

TABLE I

REVERSIBILITY OF THE DIAMIDE-INDUCED ENHANCEMENT OF TRANSBILAYER REORIENTATION OF LYSOPHOSPHATIDYLCHOLINE

Erythrocytes were treated with diamide for 0–60 min at 37°C and pH 8.0. After washing the cells were treated with 10 vol. of Medium A containing 5 mmol·l^{-1} dithioerythritol (DTE) for 30 min at 37°C and pH 8.0. An inner layer fraction of lysophosphatidylcholine at equilibrium of 0.5 was used to calculate k_{in} (see Results).

Time with diamide (min)	k_{in} (h^{-1})		% reversibility
	before DTE	after DTE	
0	0.022	0.016	
15	0.090	0.040	74
30	0.260	0.070	79
60	0.615	0.215	67

disulfide bonds (Table I). Such reversibility makes it rather unlikely that irreversible denaturation or aggregation of membrane proteins are the underlying reasons for the effect. Minor contributions of such non-specific secondary effects might be suspected in view of the incomplete reversibility (70–80%) of the diamide effect. Since, however, phospholipid asymmetry is partly abolished by the diamide treatment [3], a contribution of the altered arrangement of phospholipids between the two leaflets could also be responsible for the enhancement of transbilayer reorientation persisting after cleavage of the induced disulfide bonds.

TABLE II

INHIBITION OF THE EFFECTS OF DIAMIDE ON TRANSBILAYER REORIENTATION BY A SHORT PRETREATMENT WITH *N*-ETHYLMALEIMIDE

Erythrocytes were pretreated with iodoacetate to block intracellular GSH, washed and incubated with 10 vol. of Medium A containing 0.4 mmol·l^{-1} *N*-ethylmaleimide (37°C, pH 8.0). After 10 and 20 s the reaction was terminated by the addition of GSH and centrifugation. Washed cells were then treated with diamide (5 mmol·l^{-1}, 45 min, 37°C) and reorientation of lysophosphatidylcholine measured (see Methods for details).

Sequence of treatments	k_{in} (h^{-1})
Controls	0.022
Diamide	0.409
Diamide after	
N-ethylmaleimide, 10 s	0.078
N-ethylmaleimide, 20 s	0.051

A very small fraction of highly reactive membrane SH-groups is probably of crucial importance for the effect of diamide. This is demonstrated by an almost complete suppression of the effect of diamide by a very brief pretreatment of the cells with a low concentration of *N*-ethylmaleimide (Table II). Such a pretreatment also prevents the loss of asymmetric distribution of phospholipids after diamide [3] and suppresses the formation of aqueous pores [8]. While this highly reactive small fraction may have a trigger function, the time-dependence of the diamide effect indicates, on the other hand, that disulfide bond formation in a slowly reacting fraction of membrane SH-groups contributes very considerably to the effect of diamide (Figs. 1 and 2). Previously, we could demonstrate [3] that *N*-ethylmaleimide under the conditions of its suppressive effects reacts preferentially with SH-groups of the membrane skeletal protein spectrin and thereby blocks SH-groups that are involved in the cross-linking of this protein. Thus, disulfide bond formation of both, slowly and rapidly reacting SH-groups is necessary to obtain the effects.

Equilibrium distribution of lysophosphatidylcholine in diamide-treated erythrocytes. Symmetry of the reorientation process

Since the rate of its transbilayer reorientation becomes highly enhanced by a diamide treatment (30–90 min), the distribution ratio of lysophosphatidylcholine between the two lipid leaflets of the erythrocyte membrane could easily be followed over several half times of reorientation. The change of the distribution ratio with time, before equilibrium is reached, can be evaluated using an iterative procedure given in the Appendix. This procedure provides an equilibrium distribution ratio (inner : outer layer) for lysophosphatidylcholine of $(54 \pm 6)\% : (46 \pm 6)\%$, or, after correction for acylation of lysophosphatidylcholine in the inner layer of 49 : 51.

The rapid movement of lysophosphatidylcholine into the inner membrane leaflet, which occurs in diamide-treated cells without considerable acylation (< 10%, data not shown), also offers the possibility to measure the backward reorientation

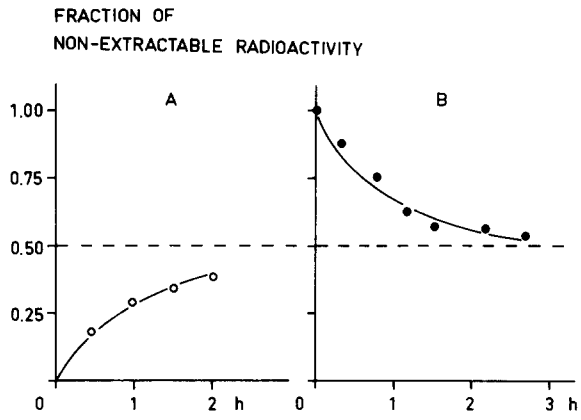

Fig. 4. Comparison of reorientation of lysophosphatidylcholine from the outer to the inner layer (flip) to that in the reverse direction (flop). Erythrocytes were treated with diamide (5 mmol·l^{-1}, pH 8, 37°C, 45 min), and washed. [^{14}C]Lysophosphatidylcholine was then incorporated into the outer leaflet and its reorientation to the inner layer quantified by following the increase of the non-extractable fraction of lysophosphatidylcholine (A). After accumulation of about 40% of the lysophosphatidylcholine in the inner layer, outer layer lysophosphatidylcholine was extracted by 1.5% albumin and cells incubated at 37°c. Reorientation of lysophosphatidylcholine from the inner to the outer layer was quantified by the decrease of the non-extractable fraction of lysophosphatidylcholine (B). Half times of flip and flop are approx. 0.8 h.

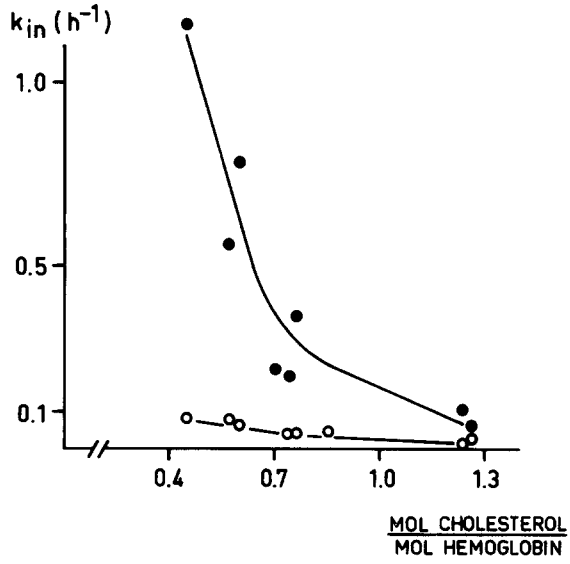

Fig. 5. Cholesterol dependence of the effect of diamide on transbilayer reorientation. Erythrocytes were depleted of or enriched with cholesterol by incubation (37°C, 24 h) with a dispersion of dipalmitoylphosphatidylcholine, containing various amounts of cholesterol [13]. After three washings, cells were treated with diamide (5 mmol·l^{-1},30 min, 37°C, pH 8.0). Diamide-treated cells (●) are compared with untreated cells (○).

of lysophosphatidylcholine from the inner to the outer layer (i.e. a 'flop'), after prior extraction of outer layer lysophosphatidylcholine. From Fig. 4 it becomes evident that both, the flip and the flop process occur at very similar rates ($k_{in} \approx k_{out}$). This demonstrates the symmetry of the induced flop process.

Properties of the diamide-induced flip process

Further studies served to characterize the diamide-induced flip process. To this end the activation energy of the flip in diamide-treated cells was compared with that in control cells. The activation energy of the reorientation of palmitoyllysophosphatidylcholine was much lower (79 kJ/mol) than that in control cells (134 kJ/mol; Ref. 9). Moreover, the effects of variations of the lysophosphatidylcholine as well as of the cholesterol content of the membrane on the reorientation process were studied. While the reorientation rate in control cells is essentially independent of the lysophosphatidylcholine content of the membrane [7], reorientation was about twice higher in diamide-treated cells containing 300 nmol lysophosphatidylcholine per ml of cells (6 mol% of the membrane phospholipids) as compared to cells containing only 20 nmol/ml cells (data not shown). Reorientation of lysophosphatidylcholine in diamide-treated cells was highly enhanced by cholesterol depletion, whereas cholesterol enrichment had only minor effects (Fig. 5). In control cells, on the other hand, cholesterol dependence is linear. All these results suggest the formation, by diamide, of a new type of flip sites instead of an increase of the number of native flip sites.

In view of the marked cholesterol dependency of the lysophosphatidylcholine reorientation in cholesterol-depleted cells it might be argued that diamide treatment, like cholesterol depletion, increases membrane fluidity. To test this hypothesis fluorescence depolarization of diphenyl-

TABLE III

DIAMIDE EFFECT ON ERYTHROCYTES OF VARIOUS MAMMALIAN SPECIES

Erythrocytes were washed and treated with diamide (5 mmol·l^{-1}, 30 min, pH 8 and 37°C). After three washings the reorientation of palmitoyllysophosphatidylcholine was measured.

Treatment	k_{in} (h^{-1}) in erythrocytes of			
	Rabbit	Human	Rat	Ox
Control	0.018	0.019	0.029	< 0.002
Diamide	0.062	0.173	0.106	< 0.002

hexatriene was measured in membranes prepared from normal cells treated with the SH-oxidizing agents, diamide and tetrathionate [3]. No significant difference in the polarization could be demonstrated, the polarization being 0.30 for control cells and 0.29–0.30 for modified cells. This may be taken as evidence that the changes responsible for the enhanced reorientation of lysophosphatidylcholine in diamide-treated cells in fact occur at discrete sites, but do not alter properties of the membrane lipid phase in general.

To investigate whether the enhancement of phospholipid reorientation by diamide is unique for the human erythrocyte, its effects were tested in erythrocytes of various species (Table III). Although less pronounced than in human erythrocytes, enhancement of reorientation was also observed in rabbit and rat erythrocytes. The higher content of unsaturated fatty acids and the lower content of sphingomyelin of rat erythrocytes as compared to human erythrocytes does obviously not greatly influence the effect of diamide on the lipid domain. In ox erythrocytes, on the other hand, no increase of reorientation of lysophosphatidylcholine was observed after diamide treatment of the cells. This lack of effect may be due to the impossibility to detect such an increase against the at least 10-fold lower native transbilayer reorientation rate in ox erythrocytes compared to those of human, rat and rabbit. Alternatively, the high sphingomyelin content could suppress formation of flip sites in membranes of ox erythrocytes.

Discussion

In recent years a number of laboratories has adduced evidence that the natural asymmetry of phospholipids in the erythrocyte membrane may partly get lost in a number of experimental situations [3] as well as under pathological conditions [14]. The loss of asymmetry is most prominent for the aminophospholipids residing in the inner layer, less pronounced for phosphatidylcholine, and undetectable for sphingomyelin [3] located in the outer layer. We have advocated the hypothesis that in some of these cases at least, the establishment of a more symmetrical distribution of the phospholipids is due to the loss of a stabilizing influence normally exerted on the inner layer phospholipids by the spectrin network or the membrane skeleton [3]. The data presented here and recent findings of other [5,7] provide further evidence for this concept (see Discussion below).

Diamide treatment enhances the rate of transbilayer reorientation of lysophosphatidylcholine and of negatively charged lysophosphatidylserine up to 150-fold. The oxidation of membrane SH-groups is a necessary prerequisite for the enhancement of the flip, since removal of the oxidant immediately stops the development of the effect [8]. Moreover, the suppression of the effect by *N*-ethylmaleimide (Table III), which preferably binds to spectrin under these conditions, suggests that SH-group oxidation in this skeletal protein plays a key role for the flip enhancement. On the other hand, however, this enhancement is not a simple function of the number of SH-groups oxidized, as no saturation of the effect occurs upon oxidation of essentially all of the reactive membrane SH-groups (Fig. 2). This feature clearly points to interposed reactions with more complicated kinetics. The increase of transbilayer mobility is certainly not a consequence of, but the reason for the partial loss of asymmetry of endogenous phospholipids, since reductive cleavage of the induced disulfide bonds does not re-establish phospholipid asymmetry, while rapidly normalizing the transbilayer mobility of exogenous lysophosphatidylcholine. Moreover, our fluorescence polarization studies indicate that the increased flip occurs at discrete reorientation sites.

Mechanism of the diamide-induced transbilayer reorientation of phospholipids

Differences in the activation energies and in the cholesterol dependence of the reorientation process in native and diamide-treated cells indicate the formation of a new type of flip sites by diamide. Conceptually, flip sites for phospholipid in a lipid-protein mosaic membrane may be situated in the lipid domain or at the interface between lipids and intrinsic proteins. Aggregation of extrinsic skeletal [7], and intrinsic membrane-intercalated proteins in diamide-treated cells was demonstrated to produce lipid domains free of proteins [15]. It was proposed that constraints against formation of flip sites normally imposed on the lipid bilayer by the skeleton might be removed by this aggregation [7]. Alternatively, lipid areas perturbed at their cytoplasmic surface by the aggregates of extrinsic proteins could be the sites of enhanced reorientation. In either case a lateral reorganization of phospholipids might be envisaged to result in a local phase separation, a concomitant increase of lateral compressibility of phospholipids at the phase boundary and a thinning of the bilayer in this region. These changes might ultimately end in the temporary formation of structural defects such as hydrophobic pores bound by the alkyl chains of the phospholipids, or hydrophilic pores bound by their headgroups [16,17], or non-bilayer, e.g. hexagonal, phases [18].

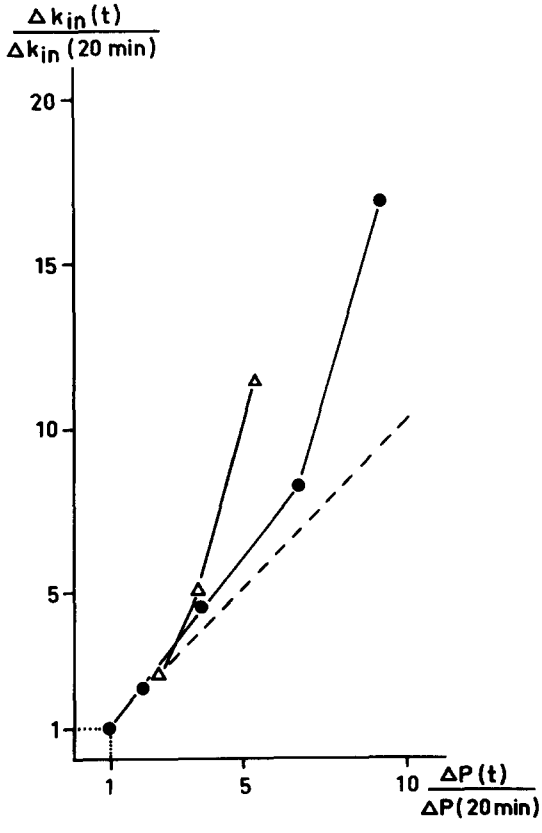

Fig. 6. Correlation between enhancement of transbilayer reorientation of lysophosphatidylcholine and leak formation by diamide. Data for time-dependent increase of leak permeability (ΔP), of *S*-acetamidothioglycol (●) or Cl^- (△), adapted from Ref. 8, are plotted against the increase of the reorientation rate of lysophosphatidylcholine (Δk_{in}), calculated data in Fig. 1. The diamide-induced leak and reorientation rate after 20 min treatment were taken as references (1.0).

In model membranes an enhancement of the transbilayer mobility of lipids by phase separations in the lipid bilayer or by introduction of protein into the lipid phase, which produces a 'mismatch' between lipid and protein [19], is often paralleled by an increased leakiness [20–22]. In line with this observation the enhancement of the transbilayer mobility of phospholipids by diamide is accompanied by formation of leaks for hydrophilic solutes in the erythrocyte membrane [8]. Fig. 6 shows the relationship between the increase of flip rates and the increase of the leak permeability for anions (Cl^-) and a model non-electrolyte, *S*-acetamidothioglycol. For small increases of reorientation rate and leak permeability the ratio of both is approximately unity. This 1 : 1 relationship suggests that the increase of the flip is due to an increase in the number of flip sites also acting as pores. For higher increases of leak permeability, i.e. after a more extensive modification by diamide, flip rates increase overproportional, a finding not yet interpretable.

Relationship between the enhancement of transbilayer mobility of exogenous phospholipids and the loss of asymmetry of endogenous phospholipids

The transbilayer mobility of exogenous lysophospholipids rises linearly with the time of exposure to diamide (Fig. 1). If an increase of transbilayer mobility is the only crucial step in the loss of phospholipid asymmetry in diamide-treated cells a prolongation of the exposure of the cells to diamide should increase the rate at which asymme-

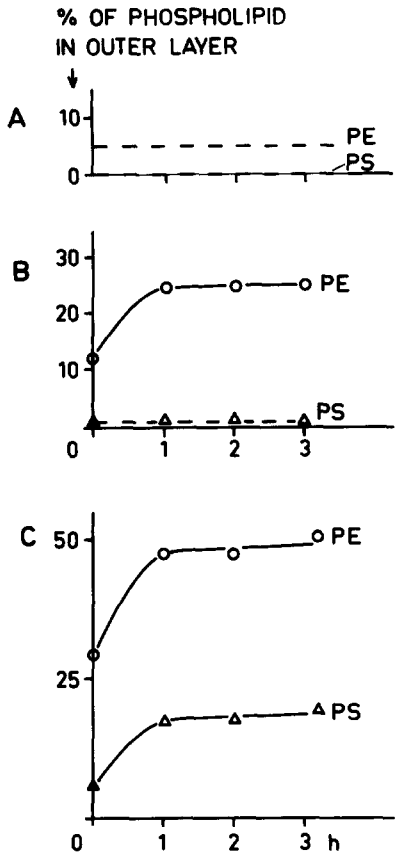

Fig. 7. Graded loss of phospholipid asymmetry induced by diamide. Erythrocytes were treated with diamide (5 mmol·l^{-1}, 37°C, pH 8.0) for 20 (B) or 30 min (C), washed and treated with phospholipase A_2 from bee venom [3] for 1, 2 and 3 h to quantify phospholipids in the outer lipid layer. Values for control cells are given for comparison (A). ○, phosphatidylethanolamine; △, phosphatidylserine.

try is lost, but not its extent. As shown in Fig. 7, however, this is not the case. Loss of asymmetry is graded in its extent. After a treatment with diamide for 20 min not more than 25% of the endogenous phosphatidylethanolamine and none of the phosphatidylserine become accessible to cleavage by phospholipase A_2, irrespective of the time length of the exposure to the enzyme. A 30 min treatment with diamide increases the fraction accessible to phospholipase A_2 to 45% in case of phosphatidylethanolamine, indicating equilibration of this phospholipid, while still only 20% of phosphatidylserine are cleaved by the enzyme. In contrast to exogenous lysophospholipids, for which the final distribution ratio in diamide-treated cells is essentially independent of the time length of exposure to diamide, endogenous phospholipids appear to become available for a transbilayer movement in a graded process depending on the time length of exposure to diamide. The asymmetric arrangement of phosphatidylethanolamine and phosphatidylserine in the erythrocyte membrane may therefore not only be due to a low transbilayer mobility but to a limited access of these phospholipids to flip sites which are clearly present even in the native membrane, as indicated by the measurable flip rates of exogenous lysophospholipids. Suppression of access to the flip site may be the results of direct or indirect interactions of inner layer phospholipids with skeletal proteins.

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Appendix

Mathematical model for the net reorientation of lysophospholipids to the inner membrane lipid layer after its insertion into the outer layer

The net movement of lysophospholipid between the two lipid layers of the membrane depends on the number of molecules in the outer layer (C_o), that in the inner layer (C_i) and on rate constants of unidirectional reorientation from the outer to the inner layer (k_{in}) and vice versa (k_{out}). A net reorientation to the inner layer can be described by:

$$\frac{dC_i}{dt} = k_{in}C_o - k_{out}C_i = k_{in}C_{tot} - (k_{in} + k_{out})C_i \qquad (1)$$

where $c_{tot} = C_i + C_o$, the total number of mole-

cules in the membrane, is assumed to be constant with time.

At equilibrium ($t = \infty$) the fraction of lysophospholipid in the inner layer will be

$$q = \frac{C_i(t \to \infty)}{C_{tot}} \tag{2}$$

and

$$\frac{dC_i}{dt} = 0.$$

Considering that at zero time all of the radioactivity is in the outer lipid layer ($C_{o(t=0)} = C_{tot}$), integration of Eqn. 1 results in

$$C_{i_{(t)}} = q \cdot C_{tot}\left(1 - \exp - \frac{k_{in}}{q} \cdot t\right) \tag{3}$$

As a result of the marked enhancement of transbilayer reorientation by diamide, the reorientation process can be measured without interference of acylation and hydrolysis nearly up to the equilibrium distribution of the lysophospholipid. Under these conditions both, q and k_{in}, can be calculated using an iteration procedure involving determination of the minimum of the function (S):

$$S = \sum_{n=1}^{n}\left[C_i(t_n) - q \cdot C_{tot}\left(1 - \exp - \frac{k_{in}}{q} \cdot t_n\right)\right]^2$$

For short periods of reorientation of lysophosphatidylcholine k_{in} can only be calculated assuming a certain q-value, namely 0.5 for diamide-treated cells (see Results) and 0.3 for controls (unpublished data).

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