Biochimica et Biophysica Acta 855 (1986) 325–336
Elsevier

BBA 72900

Bacterial cytotoxins, amphotericin B and local anesthetics enhance transbilayer mobility of phospholipids in erythrocyte membranes. Consequences for phospholipid asymmetry

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(Received August 23rd, 1985)

Key words: Cytotoxin-membrane interaction; Phospholipid asymmetry; Anesthetic; Polyene antibiotic; (Erythrocyte membrane)

(1) Incorporation of the channel-forming polyene antibiotic amphotericin B and of cytotoxins from *Staphylococcus aureus* (α-toxin) or *Pseudomonas aeruginosa* into erythrocyte membranes results in a concentration-dependent enhancement of the flip rates of exogenous lysophosphatidylcholine. The flip rate is also enhanced by incorporation of tetracaine and dibucaine. (2) Removal of tetracaine and amphotericin B from the cells normalizes the flip rates. (3) In parallel to the enhancement of flip rates, α-toxin produces a loss of transmembrane asymmetry of both phosphatidylethanolamine and phosphatidylserine. (4) Pretreatment of cells with amphotericin or high concentrations (over 2.5 mmol·l^{-1}) of tetracaine, followed by removal of the perturbing agent by washing, produces a selective loss of the asymmetric orientation of phosphatidylethanolamine to the inner membrane layer, as evaluated by the accessibility of the lipid towards cleavage by phospholipase A_2. The extent to which asymmetry is lost depends on the time of pretreatment with amphotericin or tetracaine, indicating a limitation by the rate of reorientation of phosphatidylethanolamine to the outer membrane surface. (5) Evaluation of the accessibility of phosphatidylethanolamine towards cleavage by phospholipase A_2 in the presence of local anesthetics indicates accessible fractions much higher than those obtained after removal of the perturbant. In the presence of tetracaine, endofacial phosphatidylethanolamine seems somehow to become accessible to phospholipase A_2. Phosphatidylserine does not exhibit this peculiarity. (6) The results indicate that various types of perturbation of the lipid domain of the erythrocyte membrane may enhance the transbilayer mobility of phospholipids as well as destabilize the asymmetric distribution of aminophospholipids. However, as in other instances reported previously (Haest, C.W.M., Erusalimsky, J., Dressler, V., Kunze, I. and Deuticke B. (1983) Biomed. Biochim. Acta 42, 17–21), there is no tight coupling between transbilayer mobility and destabilization of asymmetry of the transbilayer distribution of phospholipids.

Introduction

The slow rate of transbilayer reorientation rate of phospholipids in pure lipid bilayers ($t_{1/2}$ of several days) becomes highly enhanced upon the incorporation of erythrocyte membrane proteins, such as glycophorin and the anion exchange protein (band 3) [1,2]. The enhancement of the transbilayer reorientation in such lipid-protein systems is paralleled by an increase in their permeability for hydrophobic substances, suggesting the formation of aqueous leaks [3,4].

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Transbilayer reorientation (flip) rate and leak permeability depend on the structure of the lipid phase [3]. Sealing of the hydrophobic barrier is best obtained by a complex mixture of lipids. Leak permeability and enhanced transbilayer reorientation have been proposed to be due to packing defects at the lipid/protein interface [1]. Alternatively, the leak pathway could be formed by protein aggregates [3].

In the present study we checked whether such packing defects acting as flip sites are also produced by insertion of channel-forming proteins from bacteria and of the pore-forming polyene antibiotic amphotericin into the erythrocyte membrane. In parallel, the effects, on phospholipid transversal reorientation, of a more general perturbation of the lipid phase by the local anesthetic tetracaine were studied. The observed increases in transversal reorientation were compared with the enhanced accessibility of inner membrane layer phospholipids towards cleavage by phospholipase A_2.

Materials

Blood from rats and rabbits was freshly collected by venipuncture and anticoagulated with heparin. Fresh human blood, anticoagulated with heparin or citrate, was obtained from the local blood bank. Erythrocytes were isolated by centrifugation, the buffy coat was removed and cells were washed three times with 10 vol. 154 mmol $\cdot l^{-1}$ NaCl.

Bovine serum albumin (essentially fatty-acid-free) was obtained from Boehringer-Mannheim, dibucaine and tetracaine from Hoechst (Frankfurt), chlorpromazine and amphotericin B from Sigma (Taufkirchen), and Dextran 4 from Serva (Heidelberg). L-1-[1-^{14}C]Palmitoyllysophosphatidylcholine (spec. act., 45 mCi/mmol) and L-1-[1-^{14}C]oleoyllysophosphatidylcholine (spec. act., 57 mCi/mmol) were obtained from Amersham Buchler (Braunschweig) and New England Nuclear (Dreieich), respectively.

Purified cytotoxin from *Pseudomonas aeruginosa* and α-toxin from *Staphylococcus aureus* were gifts from Dr. R.N. Weiner (Giessen, F.R.G.) and Professor S. Bhakdi (Giessen).

Methods

Pretreatment of erythrocytes with cytotoxins. Washed erythrocytes, suspended in a medium comprising (mmol $\cdot l^{-1}$): KCl (90), NaCl (45), Na_2HPO_4/NaH_2PO_4 (12.5) (medium A; pH 7.4), and 25 mmol $\cdot l^{-1}$ Dextran 4 (M_r 4000–6000) to protect cells against colloid-osmotic lysis, were treated (5 min, 37°C) with various concentrations of *P. aeruginosa* cytotoxin and α-toxin at a hematocrit of 20 or 33%, respectively.

Measurement of lysophosphatidylcholine flip in cytotoxin-treated cells. After centrifugation, the cytotoxin-treated cells were resuspended in 10 vol. of medium A with 25 mmol $\cdot l^{-1}$ Dextran 4 at 22°C. [^{14}C]Lysophosphatidylcholine (25 nmol/ml of packed cells) was incorporated into the outer membrane layer of the erythrocyte and the reorientation (flip) of the lysophospholipid to the inner layer was measured at 37°C as described before [5].

Rates of hemolysis of erythrocytes after pretreatment with cytotoxins. Erythrocytes were suspended in medium A with 25 mmol $\cdot l^{-1}$ of Dextran 4 and treated with *P. aeruginosa* cytotoxin (hematocrit 20%) or α-toxin (hematocrit 33%) for 5 min at 37°C. After centrifugation, 20 μl of packed, α-toxin-treated cells were mixed with 20 μl of medium containing Dextran and 80 μl of 154 mmol $\cdot l^{-1}$ NaCl, containing 1 vol% of isotonic phosphate buffer. Hemolysis was registered continuously in a cuvette with 1 mm light-path by the decrease in turbidity of the cell suspension at 700 nm. In the case of *P. aeruginosa* cytotoxin-treated cells, hemolysis was tested in medium A containing 12 mmol $\cdot l^{-1}$ raffinose in order to retard hemolysis and facilitate its quantification.

Effects of amphotericin and local anesthetics on flip rates. In most of the experiments on the effects of amphotericin B and tetracaine or nupercaine, the cells were loaded with [^{14}C]lysophosphatidylcholine as usual. Subsequently, they were resuspended in medium A containing 44 mmol $\cdot l^{-1}$ sucrose. Antibiotic or anesthetic were added from stock solutions in dimethylsulfoxide or ethanol, respectively, and flip rates were measured in the presence of the agents. In the case of tetracaine, the lysophosphatidylcholine fraction not extractable by albumin was quantified after three washes

of the cells with medium containing sucrose (44 mmol · l^{-1}).

In experiments on the reversibility of the effects of amphotericin, erythrocytes were treated with the polyene (hematocrit 10%, 5 min, 37°C) and washed three times either with medium A containing sucrose or with this medium supplemented with 1.5% albumin. Flip rates of [^{14}C]lysophosphatidylcholine were measured as usual.

The reversibility of the effects of a 5–60 min pretreatment of erythrocytes with tetracaine was studied by removal of the agent by three washings of the cells with medium A containing sucrose.

Quantification of the incorporation of amphotericin into the erythrocyte membrane. Erythrocytes (hematocrit 10%) were treated with amphotericin (5 min, 37°C) in medium A with sucrose and pelleted by centrifugation. The supernatant and a yellow sediment of amphotericin were removed by suction. Erythrocytes were hemolyzed with 1 vol. of H_2O and mixed with 11 vols. of isopropanol followed by 7 vols. of chloroform [6]. After sedimentation of denaturated protein, the content of amphotericin in the organic phase was quantified by measuring its absorption at 408 nm using a calibration curve of amphotericin [7].

Accessibility of membrane phospholipids to cleavage by phospholipase A_2. 1 vol. of erythrocytes was suspended in a medium (pH 7.4) comprising (mmol · l^{-1}): KCl (90), NaCl (45), Hepes (10), $CaCl_2$ (0.25), $MgCl_2$ (0.25) and sucrose (44). Following addition of 20 IU of bee venom phospholipase A_2 per ml packed cells, the erythrocyte suspension was incubated for 40 or 60 min (see Results) at 37°C. Subsequently, enzyme activity was blocked by addition of EDTA (1 mmol · l^{-1} final concentration), phospholipids were extracted and phospholipid degradation was analyzed as described before [8]. Only experiments where hemolysis was no greater than 3% were evaluated.

Results

In the present study we investigated the effects of the incorporation of a polyene antibiotic, of cytotoxic proteins and of anesthetics into the hydrophobic domain of the erythrocyte membrane on the transbilayer mobility of lysophosphatidylcholine between the outer and the inner membrane layer, and on the asymmetric distribution of the inner membrane layer aminophospholipids, phosphatidylethanolamine and phosphatidylserine.

Effects of amphotericin B

Incorporation of amphotericin B into the membrane of human erythrocytes produces a concentration-dependent increase of the rate of transversal reorientation of palmitoyllysophosphatidylcholine from the outer to the inner layer. A measurable increase (2–3-fold) in the flip rate (k_{in}) is already obtained upon addition of 5 μg of amphotericin per ml of medium or $3 \cdot 10^6$ molecules per cell. By measurements of the fraction of amphotericin incorporated into the membrane (see Methods), we could show that at best 4–8% ($n = 4$) of the external amphotericin is incorporated at concentrations between 20 and 40 μg per ml of medium. This finding indicates that $1.5 \cdot 10^5$ molecules of amphotericin per cell, equivalent to 1 amphotericin per $1.5 \cdot 10^3$ phospholipid molecules, suffice to produce the first measurable increase of the flip.

In order to check whether this effect of amphotericin is reversible or irreversible, the antibiotic was removed from amphotericin-treated cells by washing with salt medium containing albumin (see Methods) prior to the measurement of flip enhancement. Such a washing procedure has been shown to reverse the antibiotic-induced leak permeability [9]. Pretreatment of cells for 1 h with 20 and 50 μg amphotericin per ml cells at 37°C increased the flip rate constant from 0.019 to 0.296 and 0.350 h^{-1} respectively. Subsequent washing of the cells reversed the flip rates to 0.052 and 0.092 h^{-1} respectively, indicating a reversibility of 88% and 78% respectively.

The enhancement of the transbilayer reorientation of lysophosphatidylcholine is accompanied by an increase of the accessibility of phosphatidylethanolamine towards cleavage by phospholipase A_2 (Fig. 2). In the concentration range of amphotericin in which the flip rates increase up to 10-fold, the fraction of accessible phosphatidylethanolamine rose from 5% (in control cells) to about 40%, when cells were treated with phospholipase A_2 in the presence of the polyene.

A further increase in the flip rate to about

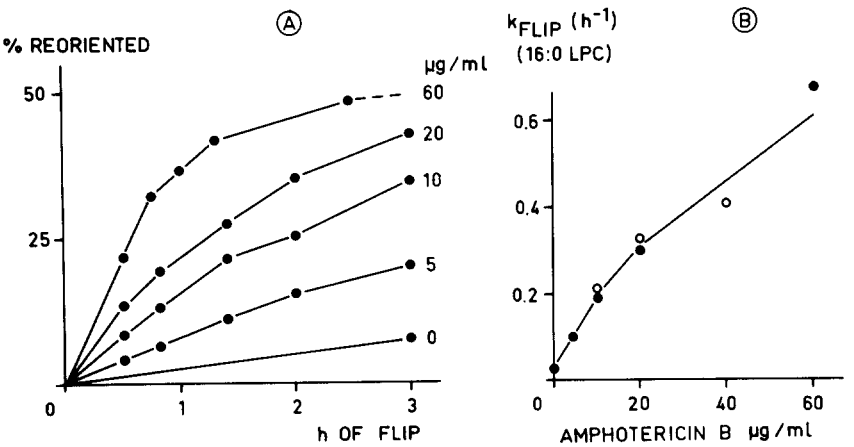

Fig. 1. Enhancement of the flip rate of lysophosphatidylcholine by amphotericin B. [^{14}C]Palmitoyllysophosphatidylcholine was incorporated into the outer membrane layer of human erythrocytes and its time-dependent reorientation to the inner layer (A) was measured in the presence of various amounts of amphotericin per ml of medium (see Methods for details). From the time-dependence of the reorientation, rate constants (k_{flip}) were calculated [5], assuming, after correction of acylation [31], a steady-state distribution ratio of lysophosphatidylcholine between the inner and the outer layer of 0.3 (B).

30-fold by higher concentrations of amphotericin resulted in an additional small increase in the cleavable phosphatidylethanolamine fraction to almost 50%. Even at this extreme increase of the flip rates and of the accessibility of phosphatidylethanolamine, essentially no phosphatidylserine could be cleaved by the phospholipase. In these experiments the cells were pretreated with amphotericin for 60 min before exposure to phospholipase A_2. Longer periods of pretreatments did not significantly augment the cleavage of phosphatidylethanolamine.

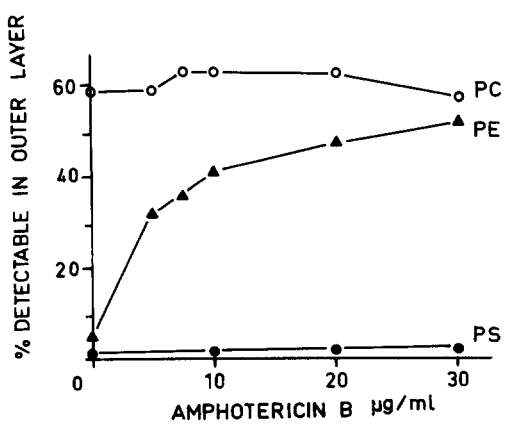

Fig. 2. Increase of accessibility of phosphatidylethanolamine towards cleavage by phospholipase A_2 in amphotericin-treated erythrocytes. Human erythrocytes pretreated (60 min, 37°C) with various amounts of amphotericin B per ml of medium were exposed to phospholipase A_2 for 60 min at 37°C (see Methods for details). Abbreviations: PC, PE, PS, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine.

In order to find out whether the increased accessibility of phosphatidylethanolamine in amphotericin-treated cells is in fact due to a true loss of its asymmetric distribution and not to artifacts resulting from a high transbilayer mobility of the phospholipid, amphotericin was removed from the cells by washing prior to testing the accessibility of phosphatidylethanolamine to phospholipase. Unfortunately, such reversibility studies could only be carried out for polyene concentrations that render about 30% of the phosphatidylethanolamine accessible to phospholipase. Modification by higher amounts of antibiotic was accompanied by hemolysis exceeding 5% during the phospholipase treatment. Under these conditions the accessibility of phosphatidylethanolamine remained increased to the same extent after removal of the polyene. These results clearly demonstrate that a true loss of asymmetry accounts for the increase of accessibility of phosphatidylethanolamine.

Effects of cytotoxins

Cytotoxins from *S. aureus* (α-toxin) and from *P. aeruginosa* also produce a concentration-depen-

dent enhancement of the flip rates of lysophosphatidylcholine in erythrocytes (Fig. 3). Studies on α-toxin were carried out with human and rabbit erythrocytes, studies on *P. aeruginosa* cytotoxin with rat erythrocytes. *P. aeruginosa* cytotoxin is a protein ($M_r = 25000$) which produces leaks [10] for small ions and nonelectrolytes of up to 1.5 kDa (pore radius over 1.2 nm [11]), whereas α-toxin (M_r 34000) is known to associate in a hydrophobic milieu to hexamers, producing pores with a radius of 1.5 nm [12]. For both toxins, a first increase of the flip rate is observed when they are added at a total concentration of $(1–2) \cdot 10^5$ molecules per cell. At these concentrations only 5–10% of the α-toxin is bound to the cell [13]. A minimum of about 10^4 molecules per cell is thus probably required to produce flip enhancement. The increase of the flip rate is closely correlated with the formation of pores by the cytotoxins (Fig. 3), as indicated by the concentration-dependent increase of the rates of colloid-osmotic lysis of erythrocytes pretreated with the toxin.

Since rabbit erythrocytes have been shown to hemolyze in salt media at concentrations of α-toxin much lower than required for human erythrocytes [13], effects of α-toxin in erythrocytes from the two species were compared. The enhancement of flip rates was slightly higher in rabbit than in human erythrocytes at the same concentration of α-toxin. At 20 μg/ml the flip rate was increased from 0.019 to 0.069 h^{-1} (i.e., 3.5-fold) in human erythrocytes and from 0.015 to 0.107 h^{-1} (i.e., 7-fold) in rabbit cells. The half-times of hemolysis of cells treated with the toxin (see Methods and Fig. 3) in this concentration range were also comparable, indicating that the same extent of leakiness corresponds to the same extent of flip enhancement in both erythrocyte species.

Rabbit erythrocytes, but not human ones, are claimed to have $5 \cdot 10^3$ high-affinity binding sites per cell for α-toxin [14]. These binding sites are most likely not involved in flip enhancement. At an α-toxin concentration of 4 μg/ml medium, at which all of these high-affinity sites may be expected to be occupied – as indicated by rapid hemolysis ($t_{1/2}$ 20 min) of all cells – no enhancement of the flip was observed.

The enhancement of flip rates of lysophospha-

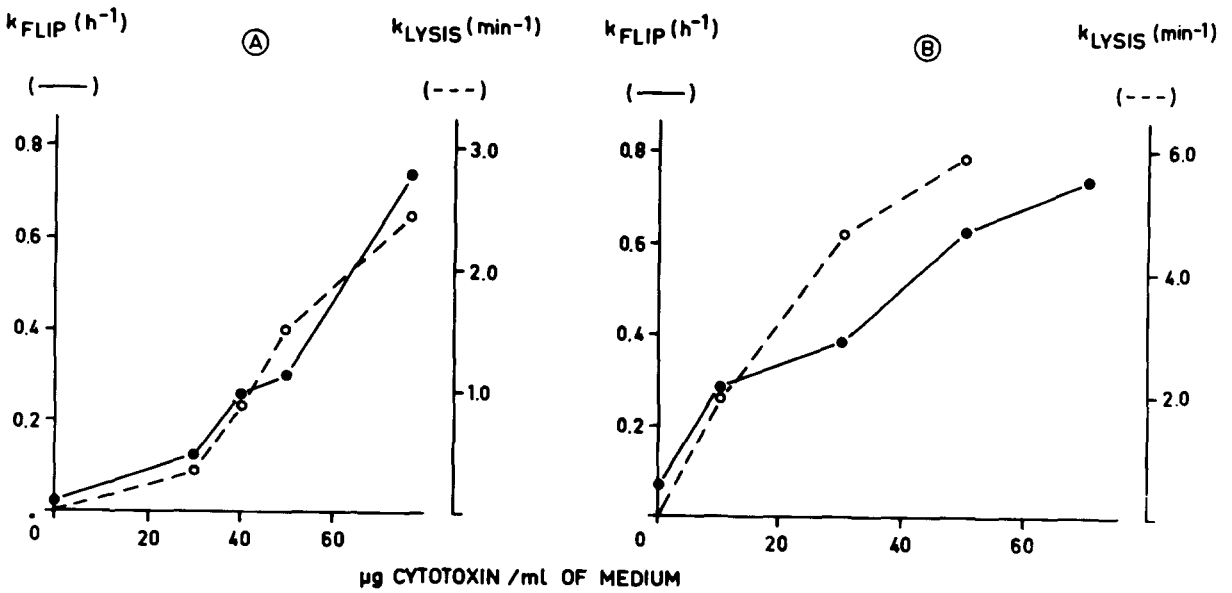

Fig. 3. Enhancement of the flip rates of lysophosphatidylcholine and of the membrane leak permeability by cytotoxins. [^{14}C]Lysophosphatidylcholines were incorporated into the outer membrane layer of erythrocytes and their orientation rates were measured in the presence of the cytotoxins. (A) *S. aureus* α-toxin in human erythrocytes, flip of palmitoyllysophosphatidylcholine. (B) *P. aeruginosa* cytotoxin in rat erythrocytes, flip of oleoyllysophosphatidylcholine. Rates of colloid-osmotic lysis as an indicator of induced leak permeabilities were determined as described in Methods.

tidylcholine by α-toxin is accompanied by a concentration-dependent loss of asymmetry of both aminophospholipids, phosphatidylethanolamine and phosphatidylserine (Fig. 4). Cleavage of phosphatidylethanolamine seems to saturate at about 50%, while that of phosphatidylserine does not exceed 30%. The extent of cleavage saturates when about 16–32 μg toxin per ml are added. At this concentration the flip rate is increased 5–10-fold.

Effects of anesthetics

(a) Enhancement of flip rates. Local anesthetics and related agents are known to perturb the lipid phase as well as proteins of membranes, either directly or by modification of lipid-protein interactions [15–18]. Such perturbations may accompany an enhancement of leak permeability [19,20]. Since in our previous studies we had established a close correlation between formation of membrane leaks and enhancement of flip rates of phospholipids, it seemed of interest to measure flip rates and phospholipid asymmetry in cells modified by a local anesthetic. Recent reports have already claimed that anesthetics may enhance the accessibility of phosphatidylethanolamine to amino reagents [21] and phospholipase A_2 [22].

At concentrations of tetracaine of up to 0.5 mmol · l^{-1} no significant increase of the flip rate was observed (Fig. 5). At higher levels a concentration-dependent increase of the flip rate of palmitoyllysophosphatidylcholine occurred. At 3.6 mmol · l^{-1} the flip was enhanced about 50-times relative to that in control cells. This increase almost completely (over 98%) disappeared after three washings of the cells with salt media at 0°C (see Methods). For example, the flip rate constant (k_{flip}) of cells in the presence of 2.5 mmol · l^{-1} tetracaine was 0.148 h^{-1}. Removal of tetracaine after 5 or 60 min pretreatment reversed k_{flip} to 0.027 and 0.034 h^{-1}, respectively (k_{flip} for control cells is 0.019 h^{-1}).

The kinetics of reorientation of lysophosphatidylcholine in the presence of tetracaine differed from that observed in presence of other perturbants, e.g., amphotericin (Figs. 1 and 6A). The fraction of lysophosphatidylcholine not extractable by albumin rapidly increased during the 10–15 min of incubation (Fig. 6A). This was followed by a slow further increase of the non-extractable fraction, ending up at the low level of 20% (as com-

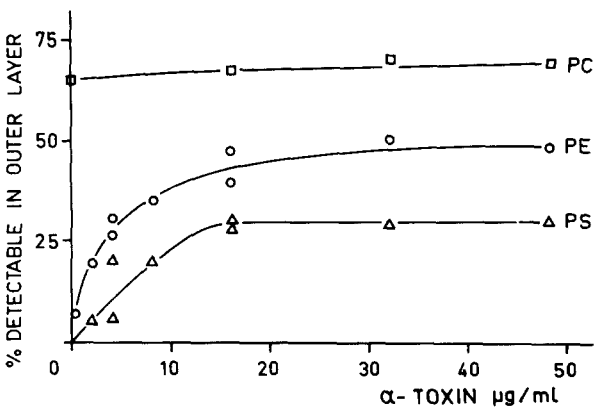

Fig. 4. Loss of asymmetry of aminophospholipids in human erythrocytes in the presence of α-toxin. Human erythrocytes were treated with increasing amounts of α-toxin per ml of medium (60 min, 37°C) in medium A with 40 mmol · l^{-1} Dextran 4. Accessibility of aminophospholipids towards phospholipase A_2 was checked as described in Methods. Abbreviations: PC, PE, PS, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine.

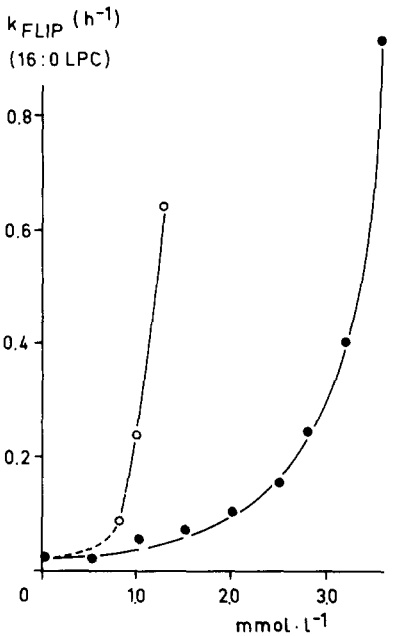

Fig. 5. Enhancement of the flip rates of lysolecithin by anesthetics. [^{14}C]Palmitoyllysophosphatidylcholine was incorporated into the outer membrane layer of human erythrocytes and its reorientation to the inner layer was measured in the presence of various amounts of tetracaine (●) or dibucaine (○) (see Methods for details).

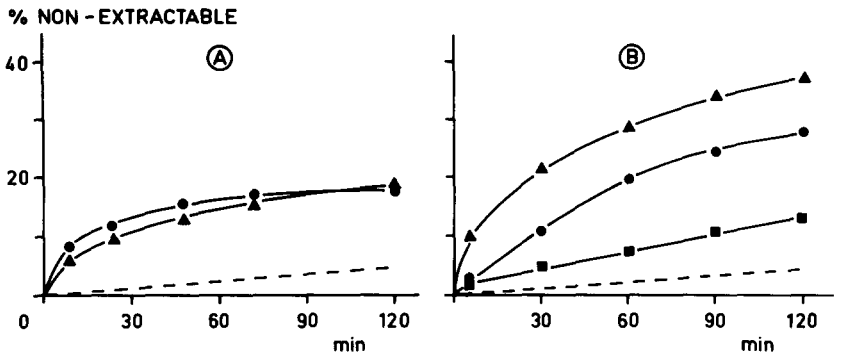

Fig. 6. Influence of membrane-bound tetracaine on time-dependent loss of extractability of exogenous lysophosphatidylcholine by albumin. [^{14}C]Palmitoyllysophosphatidylcholine was incorporated into the outer membrane layer of erythrocytes which were incubated in medium A containing sucrose and different concentrations of tetracaine. After various times aliquots were centrifuged. (A) Erythrocytes directly extracted with albumin. (▲) 2.5 and (●) 3.0 mmol·l^{-1} of tetracaine. (B) erythrocytes washed three times with medium A to remove tetracaine prior to albumin extraction. ■, 2.0; ●, 2.8; and ▲, 3.2 mmol·l^{-1} of tetracaine (see Methods for details). The dotted line represents the flip of control cells. Note that the presence of tetracaine augments the extractability of lysophosphatidylcholine.

pared to 50% in amphotericin-treated cells). Acylation of exogenous lysophosphatidylcholine in the tetracaine-treated cells proved that a reorientation of lysophosphatidylcholine to the inner surface had actually occurred.

In looking for an explanation for the low steady-state level of the non-extractable fraction, we checked the possibility that tetracaine interfered with the albumin extraction. To this end, cells were loaded with labelled lysophospholipid and flip measurements were started in the presence of tetracaine as usual. In contrast to the standard procedure, however, the samples taken after various time intervals were centrifuged and the cells were washed at 0°C with saline to remove tetracaine prior to quantification of lysophosphatidylcholine extractability by albumin. With this modified method, the flip kinetics was similar to that observed in cells treated with amphotericin and other modifying agents (Fig. 1 and Ref. 5). The non-extractable fraction gradually increased to more than 35% within 2 h in the presence of 3.2 mmol · l^{-1} of tetracaine (Fig. 6B). Thus, albumin-extraction in the presence of tetracaine obviously leads to erroneous results concerning the reorientation of exogenous lysophosphatidylcholine.

Dibucaine is an anesthetic known to produce membrane stabilizing (expanding) and hemolytic effects at concentrations about 5-fold lower than in the case of tetracaine [23]. Flip enhancement, which indicates perturbation of the membrane lipid domain, was also produced by dibucaine at about 3-fold lower concentration than by tetracaine (Fig. 5).

(b) Changes of accessibility to phospholipase A_2. As in the case of amphotericin and the cytotoxins, the accessibility of phosphatidylethanolamine towards cleavage by phospholipase A_2 is increased in the presence of tetracaine (Fig. 7). In contrast to amphotericin, however, the enhancement by tetracaine is not dependent on the time of pretreatment with the agent. This indicates that the observed cleavage of almost 50% of the phosphatidylethanolamine by phospholipase A_2 after a 5 min pretreatment with 2.5 mmol · l^{-1} of tetracaine (Fig. 7A) must be due to a very rapid increase of the accessibility of the phospholipid at the outer layer. Since the flip of lysophosphatidylcholine, although enhanced 10-fold, still has a half-time of about 1 h in the presence of 2.5 mmol · l^{-1} tetracaine, the question arises whether the very rapid increase of the fraction of the phosphatidylethanolamine accessible to phospholipase is in fact the result of a true, primary loss of asymmetry or results from a secondary, rapid reorientation of phosphatidylethanolamine from the inner to the outer membrane layer induced by the simultaneous presence of tetracaine and phospholipase A_2.

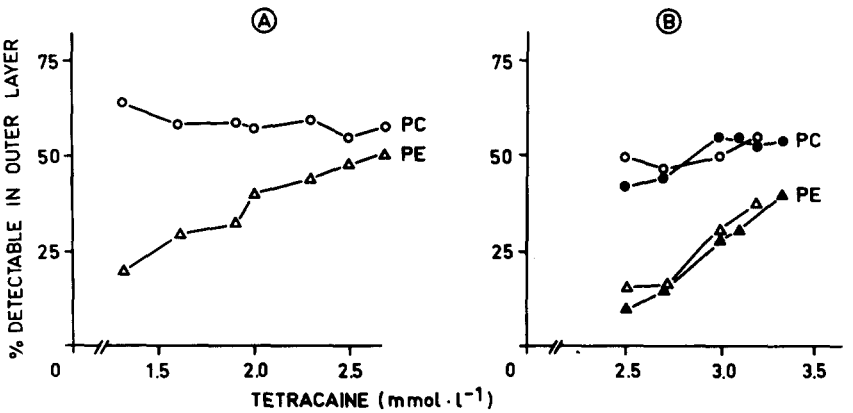

Fig. 7. Loss of phosphatidylethanolamine asymmetry in human erythrocytes induced by tetracaine treatment. (A) Cells pretreated for 5 min with low concentrations of tetracaine prior to exposure to phospholipase done in the presence of the anesthetic. (B) Cells pretreated with various concentrations of tetracaine for 60 min (closed symbols) and 120 min (open symbols) at 37°C, were washed three times prior to checking the accessibility of phosphatidylethanolamine towards cleavage by phospholipase A_2 as described in Methods. PC, phosphatidylcholine, PE, phosphatidylethanolamine.

The excellent reversibility of the effect of tetracaine on flip rates enabled us to distinguish between these two possibilities. After a 1–2 h pretreatment of erythrocytes with low concentrations of tetracaine and subsequent removal of the anesthetic by washing, the increase of phosphatidylethanolamine accessibility was only small (less than 15%, compare Fig. 7B). If, however, the cells were pretreated with higher concentrations for 1–2 h and washed, the accessible fraction of phosphatidylethanolamine increased to almost 40%. This indicates that the ready accessibility of phosphatidylethanolamine for phospholipase in cells exposed to high levels of tetracaine was indeed due to an irreversible loss of asymmetry of the phospholipid. At low concentrations of tetracaine, however, no true loss of asymmetry occurs. The enhanced accessibility of phosphatidylethanolamine towards cleavage by phospholipase A_2 in the presence of tetracaine (Fig. 7A) must therefore result from its rapid reorientation to the outer surface under these conditions (tetracaine + phospholipase A_2). Under all these conditions the fraction of cleavable phosphatidylserine was less than 10%. This demonstrates once again the tighter binding of phosphatidylserine to the inner surface as compared to phosphatidylethanolamine.

In this context, a recent observation of Tamura et al. [22] may have to be reinterpreted. These authors claimed that the asymmetric distribution of phosphatidylethanolamine is lost after a treatment of erythrocytes with chlorpromazine at 0.2–0.3 mmol · l^{-1} (10 min at 37°C). In our hands, however, such a pretreatment with chlorpromazine, followed by three washes with medium A containaing sucrose, only resulted in a minor irreversible enhancement of phosphatidylethanolamine cleavage by bee venom phospholipase A_2. The fractions of degraded phospholipid were 18 and 22% for 0.2 and 0.3 mmol · l^{-1}, respectively, as compared to 32 and 44% found by Tamura et al. [22]. At 0.4 mmol · l^{-1}, no significant further increase in cleavage was observed, whereas higher concentrations produced hemolysis during lipase treatment. The discrepancy between their results and ours could be due to incomplete removal of the anesthetic by washing in their studies and a consecutive enhancement of phosphatidylethanolamine accessibility by the mechanism outlined above.

Discussion

The flip-flop of phospholipids has been postulated to be a statistical event occurring at fluctuating defects in the membrane lipid domain

[24]. Such defects may result from a thinning of the lipid bilayer, ultimately ending in the formation of water-filled pores, enclosed by the polar head-groups of the phospholipids. Alternatively, the interfacial regions between lipids and intrinsic membrane proteins may act as flip sites. Indeed, introduction of isolated intrinsic membrane proteins into pure lipid bilayers enhances leak permeability as well as transversal reorientation of phospholipids [1–4]. The observed enhancements have been interpreted to be due to a mismatch between lipid and protein, decreasing the hydrophobic barrier for the polar head group.

In certain pathological cells, the transversal reorientation rate of phosphatidylcholine is enhanced. Erythrocytes from patients with hereditary pyropoikilocytosis or sickle-cell disease have 2–3-times faster flip rates (at 37°C) than control cells [40]. In hereditary spectrin-deficient mouse erythrocytes (*sph/sph*) (a kind gift from Dr. S. Bernstein, Bar Harbor, ME, U.S.A.) we found a 10-fold higher flip rate for lysophosphatidylcholine as compared to control cells (data not shown). Similar results were recently reported by others [25].

A much more dramatic increase in flip rate (more than 50-fold) could be obtained by chemical modification of erythrocytes by the SH-oxidizing agent, diamide [5], by less specific oxidants such as periodate and iodate, as well as by *t*-butylhydroperoxide [26]. Moreover, an extreme flip enhancement was also observed in cells subjected to electric breakdown of the permeability barrier at 0°C [27].

In the present work we demonstrate that not only modification of membrane proteins, but also a primary modification of the lipid domain by insertion of water-soluble cytotoxic proteins (*P. aeruginosa* cytotoxin and α-toxin) and of the polyene antibiotic amphotericin highly enhances the flip rate of exogenous lysophospholipids (30–40-fold).

An increase in flip rates first occurs when $1.5 \cdot 10^5$ molecules of amphotericin per cell are incorporated into the hydrophobic domain. Assuming its complete association to decamers [28], forming a water-filled pore, the number of perturbing channels would be $1.5 \cdot 10^4$ per cell. Studies on black lipid membranes have shown [29] that amphotericin produces a cation-selective leak when added to one side of a bilayer, suggesting formation of a single-length channel which spans a hydrophobic membrane segment of 2.2 nm. In contrast, addition of the polyene to both sides induces an anion-selective leak, presumably consisting of a double-length channel [29]. Recently we have been able to show that the leak induced in erythrocytes by amphotericin is cation-selective [30], which indicates formation of a single-length channel. This finding suggests that the channel may primarily modify the outer membrane layer. Since, however, the hydrophobic domain of the channel is shorter than that of the lipid bilayer, it may be expected that packing defects at the interface between channel and lipid will be produced. It seems likely, therefore, that these defects represent sites of (rapid) phospholipid reorientation in amphotericin-treated cells.

Since amphotericin forms complexes with cholesterol [7], it might also be speculated that the flip enhancement results from the formation of cholesterol-depleted membrane domains. This hypothesis, however, is not tenable, since we have shown previously that removal of cholesterol from unmodified membranes enhances flip rates of lysophosphatidylcholine only to a minor extent [31].

In the case of α-toxin 10^4 molecules bound per cell produce the first measurable increase of flip. Assuming their complete association into hexameric aggregates, this number could account for $1.6 \cdot 10^3$ pores. This number should be compared with the $4 \cdot 10^5$ intercalated particles [32] present in native erythrocytes. α-Toxin has been claimed to form a ring-like hexameric structure after its penetration into a hydrophobic environment [12]. Although the depth of insertion of the toxin into the membrane is not known, a major part of the complex remains at the outer surface [12]. This suggests, as in case of the amphotericin channel, a primary interaction with the outer membrane layer. Moreover, since specific protein receptors for this toxin are lacking on the human erythrocyte surface and toxin binding to high-affinity binding sites [13] of rabbit erythrocytes ($5 \cdot 10^3$/cell) does not enhance the flip rate, it can be speculated that the lipid domain is the primary site of modification by α-toxin. This hypothesis is sup-

ported by the alleged role of gangliosides in toxin binding [33]. Once again, the results with α-toxin (and *P. aeruginosa* cytotoxin) indicate that packing defects at the pore-lipid interface are responsible for the enhanced flip.

In addition to producing structural defects acting as flip sites, the perturbing agents destabilize the preferential orientation of aminophospholipid to the inner surface. Such a stabilization has been claimed to originate from the membrane skeleton [5,8,24,34]. Thus, in addition to the modification of membrane protein by disulfide bond formation, as in the case of diamide treatment of erythrocytes, perturbation of the lipid phase causes release of inner layer aminophospholipids from their stabilizing interactions. In this case, too, the native orientation of phosphatidylserine is much more resistant than that of phosphatidylethanolamine. The asymmetry of phosphatidylserine is maintained even after a 40–50-fold increase in the flip rate (Figs. 2, 7).

Enhancement of accessibility of phosphatidylethanolamine towards cleavage of phospholipase A_2 is still observed after normalization of flip rates by removal of tetracaine and amphotericin. The enhanced accessibility of phosphatidylethanolamine is therefore due to a true loss of its asymmetric orientation. This extends previous similar observations on the irreversible loss of phospholipid asymmetry in erythrocytes resealed after electric breakdown of the permability barrier [27] and in cells subjected to treatment with the SH-oxidizing agent, diamide, and subsequent reductive cleavage of induced disulfides [5]. In both situations, flip rates were normal, or at least enhanced only very little, during the measurement of phospholipid accessibility [5,27]. Enhancement of flip rate and loss of asymmetry are therefore two different phenomena, although presumably somehow linked to each other in almost all cases studied so far. For possible exceptions see Ref. 40 and 41. In any case, a high flip rate is probably a prerequisite for a detectable loss of asymmetry. At the normal, very low rates of the process any loss of asymmetry would become measurable only after very long incubation times. On the other hand, high flip rates are not a sufficient prerequisite for a loss of asymmetry. The weakening of interactions stabilizing the orientation of aminophospholipids to the inner membrane surface seems to be an independent additional requirement. It should be realized that a destabilization of asymmetry occurring at normal flip rates would become measurable only after very long incubation times.

Recently, Seigneuret and Devaux [35,36] interpreted studies on the transbilayer reorientation of spin-labeled analogs of membrane phospholipids in terms of an ATP-driven transport of aminophospholipids from the outer to the inner leaflet. This transport was proposed to be responsible for the asymmetric orientation of these phospholipids. They suggested that at high flip rates (as induced by diamide) the 'uphill' aminophospholipid transport would become ineffective due to the increased rate of a counteracting passive reorientation process. Our present and earlier data do not lend support to this idea. In the first place an enhancement of the flip rate of lysophospholipids to a certain level (say, 10-fold) by various treatments (e.g., diamide, amphotericin or tetracaine) does not result in the same increase of accessibility of phosphatidylethanolamine towards phospholipase A_2 (this study and Refs. 5, 37). Second, the orientation of phosphatidylserine to the inner surface is much more stable than that of phosphatidylethanolamine. Loss of asymmetry is a graded process which affects the two phospholipids to a different extent, depending on the degree of modification (Figs. 2, 4, 7 and Refs. 5). Finally, we found that analogs of inner membrane layer phospholipids, lysophosphatidylethanolamine and lysophosphatidylserine, spontaneously accumulate into the inner membrane layer [24,37]. This accumulation also occurs in the absence of energy supply and after blockage of glycolysis [24]. In view of these considerations, we prefer a model in which the membrane skeleton directly or indirectly stabilizes the orientation of aminophospholipids to the inner surface *. This model does not exclude

* An energy-dependent transbilayer uphill transport of aminophospholipids [34,35] would exhibit two peculiar features. First, it would be able to operate at 4°C, where most ATP-driven transport processes become ineffective. Second, it would have a surprisingly high rate of 'pumping' (k = 0.2–0.5 h^{-1} at 4°C [39]), much higher than would be necessary to compensate for a very small leak (k = 0.015 h^{-1} at 4°C [39]). It seems difficult to account for a steady-state distribution of phosphatidylethanolamine of about 4 : 1 in the native membrane on the basis of these rate coefficients.

the possibility that the transfer of diacylaminophospholipids, in contrast to that of their lysoderivatives, occurs by a fast but passive protein-mediated process.

In contrast to the irreversible enhancement of accessibility of phosphatidylethanolamine following a temporary exposure of cells to high concentrations of the anesthetic, even low concentrations of tetracaine (2 $mmol \cdot l^{-1}$) markedly enhance the accessibility of phosphatidylethanolamine to phospholipase A_2 if present during the enzyme treatment. The concentrations needed are considerably lower than those required for a similar enhancement by a mere pretreatment with anesthetic. This enhancement of cleavage of phosphatidylethanolamine in the presence of anesthetic cannot result from a stimulation of the lipase by anesthetic, since such compounds inhibit phospholipase A_2 at the higher concentrations used in this study [28]. On the other hand, the lack of cleavage of phosphatidylserine is not the result of such inhibition, since this phospholipid was cleaved in hemolyzed cells (data not shown). It seems also unlikely that the phospholipase obtains access to the inner membrane layer in the presence of tetracaine. As an alternative explanation, the accelerated degradation of phosphatidylethanolamine could be due to a focal perturbation of the membrane by a combined effect of phospholipase and tetracaine, leading to a pronounced enhancement of the transversal reorientation of phosphatidylethanolamine to the outer surface and its subsequent degradation by the lipase. These processes would maintain a gradient for the phospholipid and facilitate its gradual further breakdown.

An analogous mechanism would account for the finding that in the presence of tetracaine albumin apparently can remove lysophosphatidylcholine from the inner layer. Since flip rates are only moderately enhanced under these conditions (5-fold at 2 $mmol \cdot l^{-1}$ of tetracaine) and will even be lower at the extraction temperature of 0°C, a mere enhancement of the flip cannot be responsible for the observed effect. Focal perturbation, by albumin and tetracaine, of the membrane lipid phase leading to a pronounced enhancement of the transversal reorientation of endofacial lysophosphatidylcholine and its subsequent binding to albumin could be envisaged as an explanation.

A further problem resulting from our observations concerns the transbilayer mass balance of phospholipids in cells after loss of asymmetry induced by exogenous perturbants. Within the framework of the bilayer couple hypothesis [42], shape changes (echinocytosis) would be expected in cells upon the net transfer of phospholipid from the inner to the outer layer. Such changes were not detectable in our study, since the perturbants per se induce shape changes. A stomatocytogenic effect of tetracaine has long been known [43]. The same type of shape change occurs in cells treated with *P. aeruginosa* cytotoxin [11]) as well as with amphotericin B (data not shown). These shape effects with certainly mask shape changes resulting from a reorientation of phospholipids and make their evaluation impossible.

Acknowledgements

The authors are indebted to Dr. R.N. Weiner and Professor S. Bhakdi for their generous gifts of *P. aeruginosa* cytotoxin and α-toxin. We thank Mrs. H. Thomas for secretarial help and Mr. F.-J. Kaiser for photographic work. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 160/C3).

References

1 Van der Steen, A.T.M., De Jong, W.A.C., De Kruijff, B. and Van Deenen, L.L.M. (1981) Biochim. Biophys. Acta 647, 63–72
2 Greenhut, S.F. and Roseman, M.A. (1985) Biochemistry 24, 1252–1260
3 Van Hoogevest, P., Du Maine, A.P.M., De Kruijff, B. and De Gier, J. (1984) Biochim. Biophys. Acta 777, 241–252
4 Van Hoogevest, P., Du Maine, A.P.M. and De Kruijff, B. (1983) FEBS Lett. 157, 41–45
5 Bergmann, W.L., Dressler, V., Haest, C.W.M. and Deuticke, B. (1984) Biochim. Biophys. Acta 769, 390–398
6 Rose, H.G. and Oklander, M. (1965) J. Lipid Res. 6, 428–431
7 Norman, A.W., Demel, R.A., De Kruijff, B., Geurts Van Kessel, W.S.M. and Van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 290, 1–14
8 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32
9 Clark, M.R., Guatelli, J.C., White, A.T. and Shohet, S.B. (1981) Biochim. Biophys. Acta 646, 422–432
10 Frimmer, M. (1983) Trends Pharm. Sci. 3, 419–421
11 Weiner, R.N., Schneider, E., Haest, C.W.M., Deuticke, B., Benz, R. and Frimmer, M. (1985) Biochim. Biophys. Acta 320, 173–182

12 Bhakdi, S. and Tranum-Jensen, J. (1983) Trends Biochem. Sci. 8, 134–136
13 Bhakdi, S., Muhly, M. and Füssle, R. (1984). Infect. Immun. 46, 318–323
14 Cassidy, P. and Harshman, S. (1976) Biochemistry 15, 2348–2355
15 Seeman, P. (1972) Pharmacol. Rev. 24, 583–655
16 Bond, G.H. and Hudgins, P.M. (1977) Biochem. Pharmacol. 26, 2241–2245
17 Trudell, J.R. (1977) Anesthesiology 46, 5–10
18 Wang, H.H. and Limbacher, H.P. (1983) Proc. Natl. Acad. Sci. USA 80, 5297–5301
19 Lieber, M.R., Lange, Y., Weinstein, R.S. and Steck, T.l. (1984) J. Biol. Chem. 259, 9225–9232
20 Singer, M.A. and Jain, M.K. (1980) Can. J. Biochem. 58, 815–821
21 Bradford, P. and Marinetti, G.V. (1981) J. Membrane Biol. 61, 193–198
22 Tamura, A., Moriwaki, N. and Fujii, T. (1983) Chem. Pharm. Bull. 31, 1692–1697
23 Seeman, P. (1966) Biochem. Pharmacol. 15, 1753–1766
24 Dressler, V., Haest, C.W.M., Plasa, G., Deuticke, B. and Erusalimsky, J.D. (1984) Biochim. Biophys. Acta 775, 189–196
25 Rossi, M.E., Mohandas, N., Bernstein, S.E., Mentzer, W.C., Clark, M.R. and Shohet, S.B. (1982) Blood 60 (Suppl. 1) 26a (abstr.)
26 Haest, C.W.M., Heller, K.B., Schwister, K., Kunze, I., Dressler, V. and Deuticke, B. (1983) Biomed. Biochim. Acta 42, 127–129
27 Dressler, V., Schwister, K., Haest, C.W.M. and Deuticke, B. (1983) Biochim. Biophys. Acta 732, 304–307
28 Kleinberg, M.E. and Finkelstein, A. (1984) J. Membrane Biol. 80, 257–269
29 Marty, A. and Finkelstein, A. (1975) J. Gen. Physiol. 65, 515–526
30 Deuticke, B., Lütkemeier, P. and Sistemich, M. (1984) Biochim. Biophys. Acta 775, 150–160
31 Bergmann, W.L., Dressler, V., Haest, C.W.M. and Deuticke, B. (1984) Biochim. Biophys. Acta 772, 328–336
32 Weinstein, R.S., Khodadad, J.K. and Steck, T.L. (1980) in Membrane Transport in Erythrocytes. Alfred Benzon Symposium, Vol. 14 (Lassen, U.V., Ussing, H.H. and Wieth, J.O., eds.), pp. 35–46, Munksgaard, Copenhagen
33 Kato, I. and Naiki, M. (1976) Infect. Immun. 13, 289–291
34 Franck, P.F.H., Bevers, E.M., Lubin, B.H., Comfurius, P., Chiu, D.T.Y., Op den Kamp, J.A.F., Zwaal, R.F.A., Van Deenen, L.L.M. and Roelofsen, B. (1985) J. Clin. Invest. 75, 183–190
35 Seigneuret, M. and Devaux, P.F. (1984) Proc. Natl. Acad. Sci. USA 81, 3751–3755
36 Zachowski, A., Fellman, P. and Devaux, P.F. (1985) Biochim. Biophys. Acta 815, 510–514
37 Haest, C.W.M., Erusalimsky, J., Dressler, V., Kunze, I. and Deuticke, B. (1983) Biomed. Biochim. Acta 42, 17–21
38 Scherphof, G. and Westenberg, H. (1975) Biochim. Biophys. Acta 398, 442–451
39 Zachowski, A., Craescu, C.T., Galacteros, F. and Devaux, P.F. (1985) J. Clin. Invest. 75, 1713–1717
40 Franck, P.F.H., Op den Kamp, J.A.F., Lubin, B., Berendsen, W., Joosten, P., Briet, E., Van Deenen, L.L.M. and Roelofsen, B. (1985) Biochim. Biophys. Acta 815, 259–267
41 Kuypers, F.A., Van Linde-Sibenius Trip, M., Roelofsen, B., Op den Kamp, J.A.F., Tanner, M.J.A., and Anstee, D.J. (1985) FEBS Lett. 184, 20–24
42 Sheetz, M.P. and Singer, S.J. (1974) Proc. Natl. Acad. Sci. USA 76, 935–938
43 Deuticke, B. (1968) Biochim. Biophys. Acta 163, 494–500