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Enhancement of transbilayer mobility of a membrane lipid probe accompanies formation of membrane leaks during photodynamic treatment of erythrocytes

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In order to further characterize membrane alterations in human erythrocytes subjected to photodynamic treatment the passive transbilayer mobility of a phospholipid analogue was studied in cells illuminated for various lengths of time in the presence of the photosensitizer, aluminum chlorotetrasulfophthalocyanine These measurements were combined with the characterization of the membrane leaks for polar solutes occurring under the same conditions with respect to their apparent size, number and ion selectivity The time-dependent photodynamic enhancement of leaks for K^+ as well as choline or erythritol was paralleled by a marked increase of the transbilayer reorientation rate of the amphiphilic lipid probe, palmitoyllysophosphatidylcholine from 0.05% min^{-1} in native cells to 0.32% min^{-1} after 60 min illumination. The asymmetric orientation of native phospholipids was not affected by this treatment The leak permeability proved to be due to the formation of pores with apparent radii of about 0.45 nm after 60 min illumination, and of 0.75 nm after 90 min. The number of pores per cell was calculated to be < 1, the pores are slightly cation-selective ($P_K/P_{Cl} \approx 3:1$). Since photodynamic treatment did not induce lipid peroxidation under the prevailing experimental conditions, protein modification must be the primary cause of both, leak permeability and flip enhancement. Since it is also likely that the leak permeability arises from oxidation of intrinsic membrane proteins, the results raise the interesting possibility that oxidative alteration of intrinsic membrane proteins may lead to enhanced transbilayer mobility of lipids.

Introduction

Membrane aspects of photodynamic cell damage, which have been analysed in numerous tissues [1], are particularly well studied in the erythrocyte membrane It has been shown that illumination of native cells or resealed ghosts in the presence of various photosensitizers produces cell lysis [2,3] This lysis is of the colloid-osmotic type, i e , resulting from the uptake of salt and water driven by the osmotic drag of impermeable intracellular constituents [3–5] Lysis originates from the oxidatively induced formation of membrane leaks permeable to small ions, but also to nonelectrolytes and to macromolecules up to a limiting size [6,7] The extent of induced leakiness seems to depend on the experimental conditions, in particular on the duration of the light exposure Evidence has been provided that the formation of leaks is not primarily related to lipid peroxidation but results from protein damage [6,8] There is also evidence that oxidation of intrinsic membrane proteins per se and no crosslinking of membrane skeletal proteins or of band 3 protein produces the leaks responsible for cell swelling and lysis [9]

Formation of aqueous membrane leaks and colloid-osmotic lysis also occur as a consequence of red cell membrane damage resulting from exposure to SH-oxidizing [10,11] and radical-forming [12–15] agents and from exposure to short pulses of high voltage (electroporation [16]) For these types of membrane damage the molecular basis of the leaks is not yet completely defined

Besides the occurrence of leaks these types of damage share as a common property a local perturbation of the

Abbreviations AlClSPc, aluminum chlorotetrasulfophthalocyanine CCCP, carbonylcyanide *m*-chlorophenylhydrazone DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonate, r_{SE}, Stokes-Einstein radius

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membrane lipid domain indicated by a marked enhancement of the rates of transbilayer reorientation (flip-flop) of membrane-intercalated long-chain phospholipid analogs, such as lysophospholipids or palmitoylcarnitine [17–20] The passive, non-mediated flip-flop of these probes, which are not transported [21,22] by the recently discovered [23] ATP-dependent aminophospholipid flippase, can easily be followed by a procedure that takes advantage of their selective extractability from the outer leaf of the lipid bilayer by albumin [24,25] The temporal relationship between leak formation and enhancement of the transbilayer mobility of amphiphiles as well as some common features of both phenomena suggest the involvement of the same or related membrane alterations [10]

Whether this parallelism between leak formation and enhancement of flip-flop also holds for photodynamic membrane damage is yet unknown We have therefore addressed this problem in a study also comprising the analysis of the photodynamically induced red cell membrane leaks by the strategies applied earlier in our laboratory to other types of leak-forming membrane damage

Materials and Methods

Materials

Human blood from healthy donors was from the local blood bank. Photofrin II (a hematoporphyrin derivative) was obtained from Photofrin Medical Inc, Raritan, NJ, U S A Aluminum chlorotetrasulfophthalocyanine (AlClSPc) was a gift from Ciba-Geigy, Basel Dextran 1 (FD 1, M_r 800–1200), Dextran 4 (M_r 4000–6000) and Dextran 8 (M_r 8000–12000) were from Serva, Heidelberg Palmitoyllysophosphatidylcholine, carbonylcyanide *m*-chlorophenyl hydrazone (CCCP), cytochalasin B, protoporphyrin IX (disodium salt), glutathione peroxidase (bovine erythrocytes), glutathione reductase (bakers yeast) and NADPH were from Sigma, Munich DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonate) from Calbiochem Bovine serum albumin, fraction V, fatty acid free, and phospholipase A_2 (bee venom) were from Boehringer, Mannheim [^{14}C]Erythritol, [^{14}C]choline chloride and [^{14}C]palmitoyllysophosphatidylcholine were from Amersham-Buchler, Braunschweig

Methods

Freshly drawn human blood, anticoagulated with citrate, was stored at 4°C in a conventional storage medium containing glucose (20 mM) and adenine (25 μM) and used for experiments within 5 days Erythrocytes were isolated by centrifugation (5 min, 6000 × g), plasma and buffy coat were removed and the cells washed three times with isotonic saline at room temperature

Photodynamic treatment

Washed erythrocytes were suspended in 10 vols of the following medium (concentrations in mM) KCl (90), NaCl (40), NaH_2PO_4/Na_2HPO_4 (12 5), Dextran 4 (26) (= medium A) After addition of photosensitizer (usually AlClSPc) the suspensions (usually 70 ml) were illuminated at room temperature under continuous stirring in a 200 ml beaker (6 5 cm diameter) with white light from below and above, using slide projectors Light intensities as measured by a radiometer (Optometer 40a, United Detector Technology) were 46 mW/cm^2 from below and 4 mW/cm^2 from above at the suspension surfaces Unless stated otherwise, the final concentration of AlClSPc was 300 μg/ml Illumination times were varied between 10 and 90 min in order to obtain a variable extent of damage. After illumination the cells were usually washed twice in medium A and immediately used for the further procedures

Flip rates

Transbilayer movements of exogenously inserted probes were measured as described in detail elsewhere [25] Briefly, [^{14}C]palmitoyllysophosphatidylcholine was inserted into the outer membrane layer of erythrocytes (0 7 μCi (= 15 nmol) per ml packed cells) by incubation of a cell suspension in medium A (hematocrit 50%) for 5 min at 22°C with the probe spread by evaporation on the wall of the incubation tube Following centrifugation, the cells were washed twice with medium A (4°C) and resuspended in medium A (hematocrit 10%). Reorientation of the lipid probe to the inner membrane layer, at 37°C, was quantified by following the decrease of its extractability by albumin, Initial rates of reorientation were derived from the initial linear part of a plot of the nextractable fraction versus time

Phospholipid asymmetry

The asymmetry of phospholipid distribution between the two leaflets of the membrane was characterized using the accessibility of outer layer phospholipids to cleavage by phospholipase A_2 from bee venom, using established procedures [26] and avoiding hemolysis exceeding 4%

Leak permeabilities

Oxidatively induced leak permeabilities were quantified by evaluating rates of K^+ release into K^+-free media, of colloid-osmotic hemolysis and of tracer fluxes of appropriate hydrophilic test solutes.

(a) K^+-leakage Photodynamically treated cells (0 3 ml) were injected into 6 ml isotonic choline chloride adjusted to pH 7 4 by tetraethylammonium hydroxide K^+-release, at 37°C, was continuously followed by a K^+-selective glass electrode (Tacussel X 110, Solea-Villeurbanne, France)), combined with a double compartment reference electrode (K 701, Radiometer) In this

electrode, the reference electrolyte compartment containing saturated KCl is coupled to the solution to be analyzed by a bridge compartment containing Tris-sulfate. At the low cell density of the suspension the extracellular K^+ concentration after attainment of equilibrium was assumed to be equal to the total K^+ content of the suspension, which was obtained by finally lysing the cells with a small amount of Triton X-100. The reciprocal of the half-time of K^+ release served as a relative measure of the K^+ leak permeability.

(b) Hemolysis. Photodynamically treated cells were suspended at a hematocrit of 5% in phosphate-buffered (5 mM) isotonic NaCl and incubated at 37°C. Fractional hemolyses were determined after suitable time intervals by measuring hemoglobin contents in the supernatant and in the total suspension [11].

(c) Tracer fluxes. Photodynamically treated cells were suspended in 2–4 vols of medium A, containing Dextran 8 instead of Dextran 4 and in addition erythritol (2 mM) or choline chloride (0.5 mM). Cells were then loaded with ^{14}C-labelled erythritol or choline and efflux rates measured as described earlier [10,11].

(d) Size of induced leaks. Estimation of leak sizes was based on an equilibrium approach described earlier [13]. Briefly, photodynamically treated erythrocytes were incubated for 24 h in iso-osmotic $NaCl$ solutions containing 40 mosmol · l^{-1} of nonelectrolytes of varying molecular sizes as potential protectants against colloid-osmotic lysis. During this prolonged period of incubation all external nonelectrolytes that can enter the cells via the induced defect at a measurable rate will do so and therefore not act as protectants. The radius of the smallest nonelectrolyte providing full protection against lysis after this long incubation period should be a reliable indicator of the "pore radius".

(e) Ion selectivity of photodynamic leaks. In order to characterize the ion selectivity of the leaks induced by photodynamic treatment, membrane potentials created by an outward-directed salt gradient were determined using an indirect approach originally developed by Macey et al. [27]. The approach is based on measurements of the membrane potential-dependent distribution of H^+ in suspensions of erythrocytes made H^+ permeable by the protonophore CCCP. When the intracellular compartment is well-buffered, and the extracellular one unbuffered, changes in H^+ distribution will be fully reflected by changes of the extracellular pH provided that band 3-mediated Cl^-/OH^- exchange, which might counterbalance the potential-induced changes of intracellular pH, is blocked by DIDS [28]. The extracellular pH changes can easily be monitored by a glass electrode. In our study photodynamically treated cells (150 µl) were washed, treated with DIDS (0.33 µmol/ml cells) for 30 min at 37°C in medium A, and then suspended in 6 ml of unbuffered mixtures of isotonic sucrose and isotonic KCl solutions, mixed to obtain various concentrations of KCl. In addition the medium contained 80 µM CCCP and 10 µM DIDS. The intracellular pH value is obtained by final lysis of the cells since the high buffer capacity of the cellular contents will then determine the pH of the whole system. Absolute values for the membrane potential E can be obtained from the equation

$$E = (pH_e - pH_i)\,\frac{R\,T}{F}$$

(f) Analysis of lipid peroxidation. The absence or presence of lipid peroxidation in photodynamically treated cells was investigated by two procedures.

(1) Formation of fatty acyl hydroperoxides was checked following the analytical procedure of Thomas and Girotti [29]. In this procedure hydroperoxides occurring in phospholipids are quantified, after the liberation of the fatty acyl chain from the native phospholipid by bee venom phospholipase A_2 [30], by a coupled assay using glutathione peroxidase to reduce lipid hydroperoxides by GSH, and a GSH reductase/NADPH system to quantify the amount of GSSG formed. The assay was validated using *t*-butylhydroperoxide as a standard. Ghost membranes from the photodynamically treated erythrocytes were isolated and further processed in the presence of 40 µM desferrioxamine to prevent iron-catalyzed peroxide decomposition [29].

(2) Formation of thiobarbituric acid-reactive material (malondialdehyde etc.) upon photodynamic treatment of the cells was checked by subjecting the cell suspension to the assay of Stocks and Dormandy [31] immediately after illumination. Absorbances at 532 nm were obtained from the spectra of the colour complexes formed, using appropriate blanks, in order to ensure that the absorbance observed could in fact be assigned to the products of lipid peroxidation. Absorbances were converted into µmol malondialdehyde/ml packed cells using standards of freshly prepared malondialdehyde [13] which were subjected to the same assay, and the hematocrit value of the suspension. Formation of malondialdehyde in ghost suspensions was determined accordingly.

Results and Discussion

In order to characterize the extent of photodynamic membrane damage induced under our experimental conditions, membrane leakiness was determined after illumination of cells under conditions under which neither net movements of alkali cations nor cell swelling and colloid-osmotic lysis can occur (see Methods). Since leak formation does not proceed after the illumination, and the leaks already formed are irreversible [9], this procedure allows to determine defined leak permeabilities for any given extent of photodynamic damage.

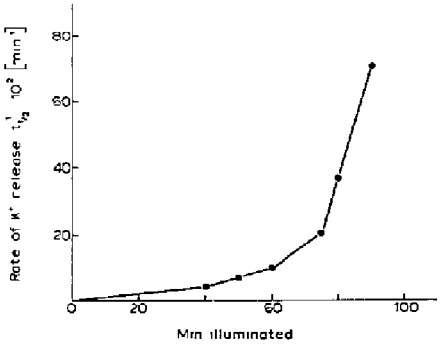

Fig 1 Increase of the rate of K^+ release from human erythrocytes as a function of the time of illumination in the presence of the photosensitizer, AlClSPc Cells were illuminated as described in Methods and washed After resuspension in K^+-free medium (see Methods), K^+ release was followed by a K^+-selective glass electrode at 37°C (Hct 5%, pH 7 4) The reciprocal of the half-time serves as a measure of the rate constant

Fig 1 demonstrates the time-dependent increase of the rates of K^+ loss of cells illuminated in the presence of 300 μg/ml AlClSPc for time periods up to 90 min After 60 min illumination, for instance, cellular K^+ is lost, during a subsequent dark period, with a half-time of 10 min After these 60 min illumination erythritol permeability, at 0°C, has reached a value of $1\,3\ 10^{-8}$ cm s^{-1} (as compared to $< 3\ 10^{-9}$ cm s^{-1} in controls), that of choline a value of $1\,4\ 10^{-8}$ cm s^{-1} (as compared to $< 10^{-9}$ cm s^{-1} in controls) and the cells undergo colloid-osmotic lysis with a half-time of about 90 min (see Fig 2) The extent of damage increases rapidly and overproportionally with the time of illumination (see Figs 1 and 2B)

Properties of the induced leaks

(1) Size

Since the induced leaks permit the passage of very polar compounds, they can be envisaged as aqueous pores to which apparent radii may be assigned In the present study such radii were derived from the Stokes radii of nonelectrolytes just protecting all cells from colloid-osmotic lysis for 'infinite' time As becomes evident from Fig 3, after 30 min illumination sucrose ($r_{SE} = 0\,46$ nm) is almost fully protective, while mannitol ($r_{SE} = 0\,36$ nm) protects only about 5% of the cells After 90 min illumination Dextran 1 ($r_{SE} = 0\,75$ nm) is required to achieve full protection, but nonelectrolytes of smaller size still protect a part of the cell population From these observations it follows, that (a) the size of the leaks increases with increasing time of illumination ($r_p \approx 0\,46$ nm after 60 min, 0 75 nm after 90 min), and (b) leak sizes seem to be distributed heterogeneously over the cell population

Using these leak radii one can also estimate approximate apparent numbers of leaks (pores) per cell on the basis of assumptions on the diffusion coefficient inside the pore (D_p) and its length (l_p) [13] Using $l_p = 5$ nm and $D_p = D_{bulk}$, the total area available for leak diffusion (A_{leak}) follows from

$$A_{leak} = P_{leak}\, A_{Ery}\, l_p / D_p$$

where P_{leak} = measured leak permeability, A_{Ery} = surface area of the erythrocyte ($1\,4\ 10^{-6}$ cm^2) For 60 min illumination and using a P_{leak} (to erythritol) of $1\,3\ 10^{-8}$ cm s^{-1} one arrives at $A_{leak} = 0\,18\ 10^{-4}$ cm^2, i e, a fraction of about 10^{-8} of the cell surface area After 90 min illumination ($P_{leak} = 5\,6\ 10^{-8}$ cm s^{-1}) A_{leak} amounts to $0\,8\ 10^{-14}$ cm^2 If we assume that $A_{leak} = n_p\ A_p$, where $A_p = \pi\ r_{p_2}$, we can derive values for n_p, the apparent number of pores

After 60 min we obtain 0 2, after 90 min 0 5 pores/cell Thus, in cells subjected to photodynamic treatment as in cells subjected to other types of leak-forming damage [10,13,14,16] the apparent number of defects per cell is smaller than 1, indicating either gross errors in the assumptions underlying the estimates or the involvement of short-lived defects fluctuating in time (and space) As discussed previously [13] the possible errors in our assumptions will only introduce small changes in n_p Formation of short-lived fluctuating barrier defects seems to be responsible for the membrane damage after photodynamic treatment

(2) Ion selectivity

In a first attempt to characterize the ion selectivity of the photodynamic leak we determined its anion/cation discrimination on the basis of bi-ionic membrane potentials developing when the leaky cells are suspended in sucrose media containing increasing concentrations of KCl The membrane potentials were derived from the intracellular pH and the pH of the erythrocyte suspension media as described in Refs 27, 28 and in the Method

Potentials obtained at different extracellular KCl concentrations provide a linear relationship when plotted semilogarithmically (Fig 4) From the slope one can calculate the ratio P_K/P_{Cl} for the leaks on the basis of the Goldman equation, assuming that only these two ions contribute to the membrane potential The validity of this approach can be tested by adding valinomycin to native erythrocytes suspended in the medium described above The slope of 57 mV per 10-fold increase of extracellular KCl indicates that the valinomycin-treated erythrocytes behave as a K^+-selective electrode as expected In the case of photodynamically treated erythrocytes, the mean slope from eight experiments

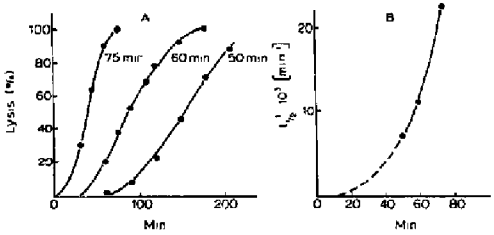

Fig. 2 (A) Time course of the colloid-osmotic lysis following photodynamic damage Erythrocytes were illuminated in the presence of AlClSPc for the time periods given at each curve washed and resuspended in a saline medium (B) Reciprocal half-times of lysis (from panel A) serving as rates, plotted against time of illumination in the presence of AlClSPc

was 28 ± 6 mV per 10-fold change of KCl This corresponds to a ratio P_K/P_{Cl} of only 2 5 1 This selectivity was the same for brief and for extended periods of photodynamic treatment and is thus not related to the leak size The ratio obtained can be compared with the ratio P_K/P_{Cl} of 4 1 reported for membrane leaks produced by chemical membrane modification with diamide [11] Preliminary results for other types of oxidatively induced leaks (Heller, K B and Deuticke, B, unpublished results) indicate similar selectivities

Enhancement of transbilayer mobility of lipids

Labelled palmitoyllysophosphatidylcholine inserted into the outer lipid leaflet of the erythrocyte membrane

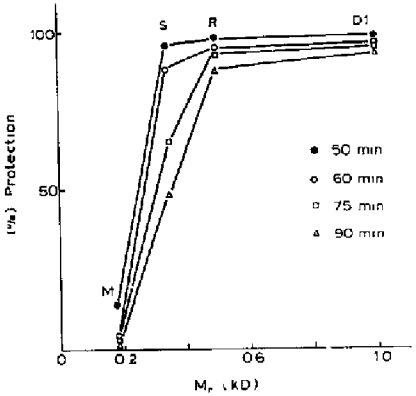

Fig. 3 Determination of apparent sizes of photodynamic membrane leaks from molecular radii (r_{SE}) of nonelectrolytes protecting the cells against colloid-osmotic lysis After illumination for the time periods given at each curve the cells were washed and incubated in saline media containing 40 mosmol/l of the nonelectrolytes for 26 h at 0 °C Subsequently, the extent of colloid-osmotic lysis was determined. 100% lysis = 0% protection M = mannitol, $r_{SE} = 0\,36$ nm, S = sucrose $r_{SE} = 0\,46$ nm, R = raffinose, $r_{SE} = 0\,57$ nm, D_1 = Dextran 1, mean $r_{SE} = 0\,75$ nm Radii adopted from Ref 51

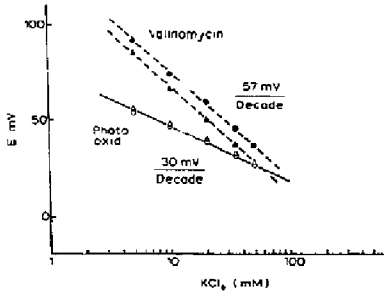

Fig 4 Membrane potentials determined for different extracellular K^+ concentrations as described in the Methods and Ref 27 Membrane potentials were derived from measurements of the steady-state pH in unbuffered suspensions of cells subjected to photodynamic treatment in the presence of AlClSPc (pH 7 4 23°C) Suspension media contained the KCl concentrations given on the abscissa, isotonicity was maintained by sucrose For further details see Methods Ratios P_K/P_{Cl} were derived from the slopes obtained Data for membrane potentials in native cells treated with valinomycin (1 μmol/l) are given to demonstrate the validity of the Method ○ 50 min △ 60 min illumination Valinomycin ● KCl/NaCl medium ▲ KCl/sucrose medium

reorients slowly to the inner leaflet with a half-time of 11 h [17,25] This slow flip in the native membrane is considerably enhanced after photodynamic membrane damage (Fig 5) The first increase becomes already detectable after 10 min illumination Cells exposed to the photosensitizer in the dark do not show flip enhancement The initial rates of transbilayer reorientation increase overproportionally with the time of illumination (Fig 6) When cells are illuminated with Photofrin II as sensitizer (20 μg/ml cell suspension, 15–40 min illumination) an even more pronounced increase of the flip rate becomes evident (data not shown) These findings clearly indicate that the membrane lipid phase is somehow perturbed by the photodynamic membrane damage

Role of lipid peroxidation

In previous studies, we have demonstrated [14,18,20] that various types of oxidative membrane damage produce an enhancement of the transbilayer reorientation of amphiphilic lipid probes In these earlier studied cases of oxidative damage, membrane phospholipids were oxidatively cleaved giving rise to typical degradation products such as aldehydes It could therefore not be excluded that the enhancement of flip rates was due to the chemical alteration of the lipid domain In the case of photodynamic treatment of erythrocyte ghosts previous studies have demonstrated formation of lipid hydroperoxides [8,29,32,33], but only little oxidative cleavage of alkyl chains

In order to investigate the role of lipid peroxidation under our experimental conditions, the cells or cell

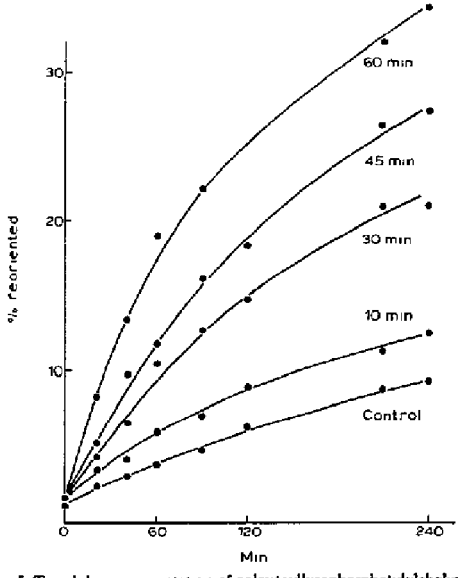

Fig. 5 Transbilayer reorientation of palmitoyllysophosphatidylcholine from the outer to the inner membrane layer of human erythrocytes after various time periods of photodynamic damage After illumination in the presence of AlClSPc (300 μg/ml) the cells were washed and the reorientation of the probe inserted into the outer layer followed by the albumin extraction procedure described in Methods

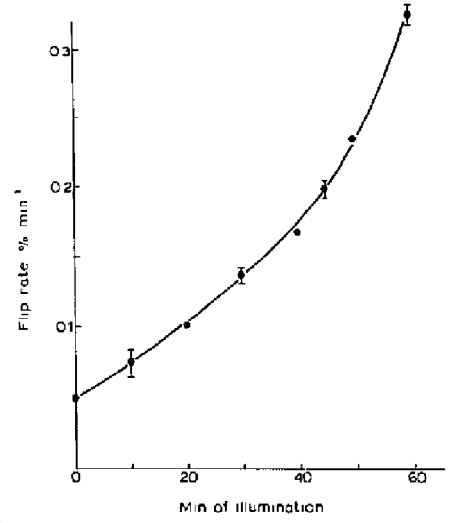

Fig 6 Overproportional increase of the flip rates of palmitoyllysophosphatidylcholine in photodynamically damaged human erythrocytes Flip rates were obtained from the initial part of uptake curves as given in Fig 5 Mean values ± S D from three experiments

suspensions, resp , were assayed for fatty acyl hydroperoxides and thiobarbituric acid reactive material after photodynamic treatment with AlClSPc Levels of thiobarbituric acid-reactive material (quantified as 'malondialdehyde' (see Methods)) did not exceed 4 nmol/ml erythrocyte after illumination times up to 90 min, to be compared with 10- or 20-fold higher values in cells subjected to treatment with *t*-butylhydroperoxide under conditions producing comparable leakiness [20]

Even more revealing, no fatty acyl hydroperoxides could be detected in the illuminated cells above the ground levels of ≤ 5 nmol/mg protein Similar results were obtained for erythrocytes illuminated in the presence of protoporphyrin IX (10 μmol l^{-1}) as described in the Methods, for periods up to 60 min This treatment induces loss of K^+ (during a subsequent dark period) with a half time of about 3 min indicating extensive leak formation

As a safeguard against artifacts hemoglobin-free ghosts were subjected to the conditions of photodynamic treatment also applied to the intact cells In the presence of 10 μmol l^{-1} protoporphyrin IX, a 60 min illumination went along with the formation of up to 250 nmol fatty acyl hydroperoxide/mg protein and 20 nmol malondialdehyde/ml packed ghosts ($n = 3$) This finding, which is qualitatively in line with recent observations of others [33], clearly indicates that photodynamic damage is greatly attenuated in erythrocyte suspensions as compared to ghost suspensions This difference most likely results from the different optical properties of the two suspensions

Illumination of ghosts in the presence of AlClSPc, under the conditions used for modifying the native cells, induced considerably less lipid hydroperoxide formation (44 nmol/mg protein after 60 min illumination, $n = 3$) than protoporphyrin IX and produced levels of malondialdehyde not exceeding 2–3 nmol/ml packed ghosts. The reasons for these different capacities of the two photosensitizers in promoting lipid peroxidation in ghosts, as opposed to a comparable extent of leak production in intact cells, are not yet clear In any case, the lipid analyses of the photodynamically treated whole cells clearly indicate that a contribution of lipid peroxidation to the leakiness and the flip enhancement occurring under our conditions is highly unlikely

On the other hand, protein damage of all kinds is well established in cells subjected to photodynamic treatment [6,34–40] The enhancement of the transbilayer mobility shown in Figs 5 and 6 is therefore likely to result from the alteration of membrane proteins leading to a secondary perturbation of the lipid domain

Phospholipid asymmetry in photodynamically treated erythrocytes

Oxidative and other membrane modifications of the

erythrocyte which lead to a flip enhancement have sometimes [19,41–44], although not generally [22,25, 42,45,46] found to be accompanied by a partial loss of the well-established [47] preferential orientation of the aminophospholipids, phosphatidylethanolamine and phosphatidylserine, to the inner half of the membrane bilayer

Illumination of erythrocytes in the presence of AlClSPc (300 μg/ml) does not enhance the extent of cleavage of phosphatidylcholine by phospholipase A_2 over the fraction of 75% present in the outer membrane layer of native erythrocytes [47] While in controls, however, this fractional cleavage cannot be completely obtained within the short exposure time to the phospholipase, cleavage occurs more rapidly after the photodynamic treatment (Fig 7B) * On the other hand, photodynamic treatment enhances not only the rate but also the extent of cleavage of phosphatidylethanolamine and phosphatidylserine to values exceeding the fractions of these phospholipids normally present in the outer layer (20% and 0%, respectively [47]) This becomes particularly clear after a 75 min illumination of the cells (Fig. 7C) Subsequent treatment with phospholipase for 5 min cleaves about 20% of the phosphatidylethanolamine and no phosphatidylserine Enzyme treatment for 10 or 15 min, however, increases phosphatidylethanolamine and phosphatidylserine cleavage beyond these values without evidence for saturation with time Since bee venom phospholipase A_2 cleaves the three glycerophospholipids at comparable rates (Ref 19 and Haest, C W M , unpublished results), we interpret these results by a combined effect, on the transbilayer distribution of the aminophospholipids, of photodynamic damage *and* phospholipase treatment, none of the two modifications by itself being capable of perturbing aminophospholipid asymmetry Photodynamic damage thus belongs to the type of membrane damage in which phospholipid asymmetry is preserved although the enhanced flip rates of our lipid probe indicate an increased passive transbilayer mobility of lipids

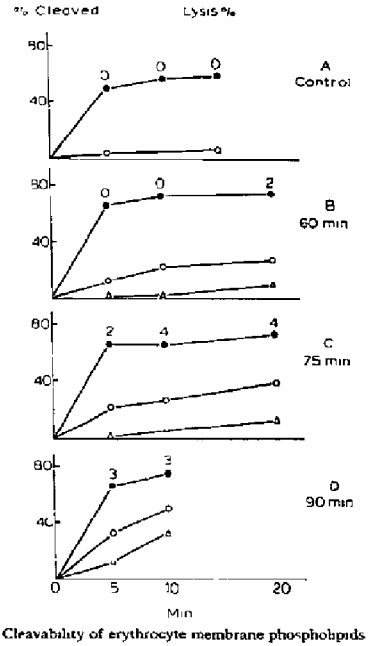

Fig 7 Cleavability of erythrocyte membrane phospholipids by exogenous phospholipase A_2 (bee venom) after photodynamic treatment of the cells Erythrocytes were illuminated in the presence of AlClSPc (300 μl/ml) for 0, 60, 75 or 90 min, washed and exposed to the enzyme for the time periods given on the abscissa. ●, phosphatidylcholine, ○, phosphatidylethanolamine, △, phosphatidylserine Cleavage in percent of each individual phospholipid class Numbers in italics lysis (%) after enzyme treatment

Concluding remarks

In this study we have investigated whether photodynamic erythrocyte membrane damage affects the simple, passive transbilayer mobility (the 'flip-flop') of membrane-intercalated amphiphilic lipids This transbilayer mobility provides, in our view, a measure of the extent of local perturbations of the lipid domain According to our results photodynamic membrane damage enhances the passive transbilayer reorientation of membrane-intercalated amphiphilic compounds in parallel to the formation of membrane leaks In order to facilitate a comparison of the data with those for other types of membrane damage we have also characterized the leaks induced by photodynamic treatments in terms of their apparent number and size

We have further shown here, that photodynamic leak formation in native erythrocytes does not involve a significant extent of lipid peroxidation Advanced stages of photo-generated leakiness (to glucose 6-phosphate) in resealed ghosts have been assigned to lipid peroxidation [6], which in fact occurs in ghosts after photodynamic treatment [29,32,33,34,37] as also confirmed by our results These findings in ghosts can obviously not be generalized Photodynamic perturbation of the erythrocyte membrane function, as indicated by leakiness and flip enhancement, does not require covalent modification of membrane lipids

* It might be tempting to assign this apparent enhancement of the *rate* of cleavage of phosphatidylcholine after photodynamic treatment to a formation of phospholipid hydroperoxides which are more sensitive to phospholipase A_2 than native phospholipid [30] In view of the lack of hydroperoxide formation this explanation has to be discarded.

Photodynamic treatment produces well-established alterations of erythrocyte membrane proteins Most prominently, skeletal proteins (spectrin, actin, band 4 1) are cross-linked [6,34–36] This goes along with a loss of cell deformability [36] Intrinsic membrane proteins such as band 3 or the glycophorins are cross-linked to a lesser extent [34,35,37,40] but photodynamic alterations of their side chains [38] as well as inhibition of transport processes [37,39] and other functional properties [39,40] have been demonstrated

According to our results membrane leakiness can be assigned to protein damage (see also Refs 6 and 9) There is earlier evidence that it may not be the cross-linking of skeletal proteins but oxidation of intrinsic proteins that causes leak formation [9,48,49]

In former studies on various types of membrane damage induced by selective modifications of membrane protein SH-groups [10,11,17,50] or less selective oxidative damage by O_2-derived radicals [13,14] we have advocated the concept that dynamic fluctuating barrier defects rather than defined pores are the sites acting as membranes leaks Moreover, a very consistent parallelism between the formation of leaks and of 'flip sites' seems to indicate that an induced 'mismatch' at lipid/protein interfaces may be the important event in chemically and physically induced losses of membrane barrier properties The processes leading to such a mismatch remain to be elucidated In some instances cross-linking of membrane skeletal proteins may be causally involved [10,11,17,45,49] In other cases the issue is less clear [13,14,20] Since photodynamic leak formation seems to involve the oxidation of intrinsic rather than the cross-linking of extrinsic, skeletal membrane proteins [9], the parallelism between leak formation and flip enhancement now established for this type of damage raises the question whether the flip enhancement might also be the consequence of oxidative alteration of *intrinsic* membrane proteins Studies to clarify this issue are under way

Using protectants against colloid-osmotic lysis we have also assigned sizes to the leaks According to our results complete hemolysis within about 160 min arises from less than 1 'hole' per cell not exceeding in its apparent radius 0 75 nm, the mean radius of Dextran 1 Lesser extents of photodynamic damage seem to produce even smaller leaks Our radii are smaller than those reported by Deziel and Girotti [7] who characterized (in ghosts) the leaks that allow to- the efflux ($t_{1/2}$ about 150 min) of glucose 6-phosphate They claimed pores with radii of $1\,1 < r < 4\,2$ nm

The apparent number of induced leaks per cell (< 1) proved to be surprisingly low This finding as well as the simultaneous occurrence of an enhanced transbilayer mobility of phospholipid analogues are in line with the concept of randomly occurring, fluctuating defects Interestingly, the photodynamically enhanced transbilayer mobility is not accompanied by a detectable loss of phospholipid asymmetry In this respect photodynamic damage may be compared with membrane alterations by periodate [45], gramicidin D [26] or heat treatment [22,46], but differs from treatment with diamide [18] and noncovalent perturbations, e g , by bacterial toxins or amphotericin B [42], which induce a loss of phospholipid asymmetry

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References

1 Pooler, J P and Valenzeno, D P (1981) Med Phys 8, 614–628
2 Blum, H F (1964) Photodynamic Action and Diseases Caused by Light, Hafner New York
3 Schothorst A A , Van Steveninck, J , Went L N and Suurmond, D (1970) Clin Chim Acta 28, 41–49
4 Pooler, J P (1985) Biochim Biophys Acta 812, 193–198
5 Pooler, J P (1985) Biochim Biophys Acta 812, 199–205
6 Deziel, M R and Girotti, A W (1980) J Biol Chem 255, 8192–8198
7 Deziel, M R and Girotti, A (1982) Int J Biochem 14, 263–266
8 Schothorst, N A and Van Steveninck, J (1972) Clin Chim Acta 39, 161–170
9 Dubbelman, T M A R , Haasnoot, C and Van Steveninck, J (1980) Biochim Biophys Acta 601, 220–227
10 Deuticke, B , Poser, B , Lütkemeier, P and Haest, C W M (1983) Biochim Biophys Acta 731, 196–210
11 Deuticke B Lutkemeier, P and Sistemich, M (1984) Biochim, Biophys Acta 775, 150–160
12 Van der Zee, J , Dubbelman, T M A R and Van Steveninck, J (1985) Biochim Biophys Acta 818, 38–44
13 Deuticke, B , Heller, K B and Haest, C W M (1986) Biochim Biophys Acta 854 169–183
14 Heller, K B , Jahn, B and Deuticke, B (1987) Biochim Biophys Acta 901, 67–77
15 Girotti, A W and Thomas, J P (1984) J Biol Chem 259 1744–1752
16 Schwister, K and Deuticke, B (1985) Biochim Biophys Acta 816, 332–348
17 Bergmann, W L , Dressler V , Haest, C W M and Deuticke, B (1984) Biochim Biophys Acta 769, 390–398
18 Haest, C W M , Heller, K , Schwister, K , Kunze, I G Dressler, V and Deuticke, B (1983) Biomed Biophys Acta 42, 127–129
19 Dressler, V , Schwister, K , Haest, C W M and Deuticke B (1983) Biochim Biophys Acta 732, 304–307
20 Deuticke, B , Heller, K B and Haest, C W M (1987) Biochim Biophys Acta 899, 113–124
21 Bartmann, V (1987) M D Thesis, RWTH Aachen
22 Claszen, J (1989) M D -Thesis, RWTH Aachen.
23 Devaux, Ph F (1988) FEBS Lett 234, 8–12
24 Mohandas, N , Wyatt, J , Mel, S F , Rossi, M E and Shohet, St B (1982) J Biol Chem 257 6537–6543
25 Bergmann, W L Dressler, V Haest, C W M and Deuticke, B (1984) Biochim Biophys Acta 772, 328–336

26 Claszen, J., Haest, C W M Tournois H and Deuticke B (1987) Biochemistry 26, 6604–6612
27 Macey, R I, Adorante, J S and Orme, F W (1978) Biochim Biophys. Acta 512, 284–295
28 Wieth J O (1979) in Membrane Transport in Erythrocytes Relation between Function and Molecular Structure (Lassen, U V, Ussing, H H and Wieth, J O, eds), pp 512–519, Munksgaard, Copenhagen
29 Thomas, J P and Girotti A W (1988) Biochim Biophys Acta 962, 297–307
30 Van Kuijk F J G M, Sevanian A Handelman G J and Dratz, E.A (1987) Trends Biochem Sci 12 31–34
31 Stocks, J and Dormandy T L (1971) Br J Haematol 20 95–111
32 Girotti, A W, Thomas J P and Jordan J E (1985) Arch Biochem Biophys 236, 238–251
33 Girotti, A W, Bachowski G J and Jordan, J E (1987) Lipids 22 401–408
34 Girotti A W (1978) J Biol Chem 253, 7186–7193
35 Dubbelman, T M A R, De Goey, A F P M and Van Steveninck J (1978) Photochem Photobiol 28, 197–204
36 Dubbelman, T M A R, De Bruijne A W and Van Steveninck J (1977) Biochem Biophys Res Commun 77, 811–817
37 Dubbelman, T M A R, De Goey, A F P M, Christiaanse, A and Van Steveninck, J (1981) Biochim Biophys. Acta 649 310–316
38 Dubbelman, T M A R. De Goey A F P M and Van Steveninck J (1978) Biochim. Biophys Acta 511, 141–151
39 Schothorst A A Van Steveninck J Went L N and Suurmond D (1971) Clin Chim Acta 33 207–213
40 De Goey A F P M Ververgaert P H J Th and Van Steveninck, J (1975) Clin Chim Acta 62 287 292
41 Haest C W M Plasa, G Kamp D and Deuticke B (1978) Biochim Biophys Acta 509 137–155
42 Schneider E., Haest, C W M Plasa, G and Deuticke, B (1986) Biochim Biophys Acta 855 325–336
43 Jain, S K (1984) J Biol Chem 259, 3391–3394
44 Jain, S K (1985) J Clin Invest 76, 281–286
45 Haest, C W M Erusalimsky J Dressler V and Deuticke B (1983) Biomed Biochim Acta 42, 17–21
46 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
47 Verkleij, A J, Zwaal R F A Roelofsen B Comfurius P Kastelijn D and Van Deenen L L M (1973) Biochim Biophys Acta 323, 178–193
48 Van Steveninck J Boegheim J P J and Dubbelman T M A R (1985) Biochim Biophys Acta 821 1–7
49 Pooler J P and Girotti A W (1986) Photochem Photobiol 44 495–499
50 Heiler K B Poser B Haest C W M and Deuticke B (1984) Biochim Biophys Acta 777 107–116
51 Scherer R and Gerhardt Ph (1971) J Bacteriol 107 718–735