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Influence of phloretin and alcohols on barrier defects in the erythrocyte membrane caused by oxidative injury and electroporation

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Oxidative damage by diamide, periodate and oxygen-derived reactive species, but also exposure to electroporation induce in the erythrocyte membrane dynamic, presumably fluctuating, defects having the properties of aqueous holes with definable radii and selectivities. These leaks, which can be quantified by measuring tracer fluxes or rates of colloid-osmotic lysis, are here shown to be inhibited by phloretin and a small number of related phenol compounds (phenolphthalein, hydroxyacetophenones, nitrophenol), while a host of other 'membrane-active' agents is not effective in this respect. I_{50} values range from about 200 μM for phloretin and phenolphthalein to about 10 mM for 4-nitrophenol. Inhibition by phloretin is reversible, not competitive and not related in its extent to the extent of leakiness. In contrast, the enhancement of transbilayer mobility of amphiphilic lipid probes, which invariably goes along with leak formation of the type described, is not affected by phloretin. Aliphatic alcohols (hexanol, butanol) have an amplifying effect on leaks induced by oxidative damage but do not affect leaks induced by electroporation. The alcohol-amplified leaks maintain the properties of aqueous holes as indicated by a low activation energy of leak fluxes. Since both, inhibition and stimulation of leak fluxes do not go along with appreciable changes of the apparent radii of the aqueous holes, changes in the dynamics (opening and closing) of the defects are proposed to underly the effects of phloretin and alkanols. The membrane lipid domain is likely to be the site of the leaks and of their modulation.

Introduction

In a number of recent studies we have demonstrated that various types of oxidative damage [1–6] but also physical modification (electroporation) [7] of the erythrocyte membrane induce a loss of its barrier properties. The induced defects share a number of features. They manifest themselves primarily as aqueous leaks for polar or ionic compounds up to a certain molecular size, which can be determined by measuring tracer or net fluxes. Colloid-osmotic lysis and changes of membrane potential ensuing from the formation of such defects can also be used to quantify the extent of leakiness.

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From the evaluation of such measurements it has been concluded that the aqueous leaks, if cylindrical, could have an apparent radius of 0.5–1.0 nm. They appear to be present at a very low number per cell (< 1), suggesting a fluctuating nature. The leaks due to reversible chemical or mild physical alteration are readily reversible [1,3], while more aggressive treatments, e.g. by O_2-derived reactive species, produce irreversible damage.

Much of the available data indicates that alteration of membrane proteins is the primary event leading to formation of these leaks. On the other hand, it is not yet clear whether the defects are eventually constituted by lipid head groups rearranging from the bilayer configuration to a membrane-spanning circular pore, by an induced mismatch [8] between modified proteins and lipids or even by transmembrane protein clusters having undergone conformational changes leading to the formation of channels. The first and the second of

these alternatives, which involve lipids, are supported by the finding of an additional manifestation of the barrier defect, namely a marked enhancement of the transbilayer mobility of amphiphilic lipid probes such as lysophospholipids or palmitoylcarnitine [6,9–12], which mimic the properties of native diacyl phospholipids up to a certain extent. Such an enhancement would only be expected if the membrane lipid domain is somehow perturbed and provides the pathway for a 'facilitated' transbilayer reorientation of the polar headgroups. Studies on electroporated artifical lipid membranes and theoretical considerations [13,14] also provide evidence that hydrophilic pores may occur in lipid bilayers under appropriate conditions.

Further insight into the nature of the barrier defects induced by chemical or physical modification might be provided by the availability of modifiers (inhibitors or enhancers) of the leak fluxes or the enhanced flip rates. This study reports on such modifiers. It will be demonstrated that phloretin and related phenol compounds reversibly inhibit leak fluxes while aliphatic alcohols markedly enhance the leak permeability. Preliminary results of this study have been reported elsewhere [15].

Materials and Methods

Materials

Human blood from healthy donors was obtained from the local blood bank. Sources of compounds used for oxidative modification of erythrocytes can be found in the references describing the details of the methods. Compounds tested for their inhibitory action on leak fluxes (Table II) were from Merck, Darmstadt; Fluka, Neu-Ulm; Roth, Karlsruhe; Sigma, Munich or Calbiochem, Munich, with the exception of 2,4,6-trihydroxyacetophenone (Aldridge) 2,4,6-trihydroxybenzophenone (K&K Chemicals). Labelled compounds were from Amersham Buchler, Braunschweig. Dextran 1 (FD 1, M_r = 800–1200), Dextran 4 (M_r 4000–6000), Dextran 8 (M_r 8000–12000) from Serva, Heidelberg.

Methods

Preparation of cells and of media for modification

Experiments were carried out on human blood stored for a maximum of 7 days in conventional storage medium containing glucose (20 mM) and adenine (25 μM). 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. Cells were suspended, at a hematocrit of 10% if not stated otherwise, in one of the following media (concentrations in mM) : Medium A: KCl (100) , NaCl (50) , NaH_2PO_4/Na_2HPO_4 (12.5); Medium B: KCl (90) , NaCl (40) , NaH_2PO_4/Na_2HPO_4 (12.5), trisodium citrate (20); Medium C: KCl (90), NaCl (45), NaH_2PO_4/Na_2HPO_4 (12.5), Dextran 4 (26).

Cell modifications

The following modifying treatments were applied:

(a) Diamide, as described in Refs. 1 and 2, by incubating the cells at pH 8 for 15–90 min in medium B with 5 or 10 mM reagent at 37°C.

(b) Periodate, as described in Ref. 3, by incubating the cells at pH 7.4 for 10–60 min in medium A with 5 mM sodium metaperiodate at 0°C.

(c) *t*-Butylhydroperoxide, as described in Ref. 4. Briefly, the cells were first suspended in medium B (Hct 5%) containing in addition sodium azide at 2.5 mM, and exposed to *t*-butylhydroperoxide (2 mM) for 15 min at 37°C. After rapid cooling to 0°C the cells were washed twice in medium B at 0 °C and than incubated (Hct 10%) in this medium in the presence of sodium azide (2.5 mM) at 37°C for various periods of time. The peroxidative reactions proceeding under these conditions [16] were interrupted by addition of butylated hydroxytoluene (100 μM) after appropriate periods of time.

(d) Iodoacetate/vanadate/ferricyanide as reported in detail in Ref. 5. To induce this type of peroxidative damage the cells were first treated with 0.2 mM iodoacetate in 11 vols. of medium A for 30 min, then washed twice in medium B at room temperature and incubated in this medium at 37°C in the presence of inosine (2 mM), sodium metavanadate (0.5 mM) and ferricyanide (5 mM) for 60 min. The peroxidative reactions were terminated by addition of butylated hydroxytoluene (100 μM) and the cells washed twice in medium B.

(e) Photodynamic treatment according to Ref. 6. Cells were suspended (Hct 10%) in medium C in the presence of aluminum chlorotetrasulfophthalocyanine (AlClSPc) (300 μg/ml). Illumination with white light was carried out under exactly the conditions described earlier [6] for 50–60 min. Subsequently the cells were washed twice in the dark in medium C.

(f) Tellurite treatment, following a procedure to be described in detail elsewhere. Briefly, erythrocytes were depleted of endogenous GSH by incubation with 0.3 mM chlorodinitrobenzene for 30 min at 37°C [17]. After washing, the cells were loaded with labelled erythritol (1 mM) in a phosphate-buffered saline containing 300 mg/ml Dextran 8 as an osmotic protectant. After 30 min loading, GSH (2 mM) and sodium tellurite (0.3 mM) were added and the suspension incubated for further 5 min. After subsequent centrifugation at 0°C (6000 × *g*, 10 min) the supernatants were removed and the efflux of erythritol from the cells measured as described below.

(g) Electroporation according to Ref. 7. Briefly, cells were suspended in 2 vols. of medium C, pH 7.4 at 0°C and subjected to a single, exponentially decaying pulse ($\tau = 40$ μs) of high voltage (5 or 6 kV cm^{-1}) by means of a discharge equipment with a spark gap as a switch. After the discharge the cells were kept strictly at 0–2°C to avoid resealing of the induced leaks.

Assessment of leak permeabilities

Leak permeabilities induced by the chemical modifications and by electroporation were quantified by measuring the rates of tracer fluxes of ions (Cl^-, choline) or nonelectrolytes (erythritol), the rates of net loss of K^+ into K^+-free media, and the rates of colloid-osmotic lysis.

(1) Tracer fluxes. Modified cells were suspended in 10 vols. of medium B or C containing appropriate concentrations of the test permeants (chloride 150 mM, erythritol 1 mM, choline 0.5 mM) and their ^{14}C-labelled analogs. After an incubation sufficient for equilibration or for uptake of measurable amounts of permeant, tracer efflux was measured, usually at 0°C (for details see Ref. 1). In the case of test permeants transported to an appreciable extent by specific carrier systems, these systems were inhibited (Cl^- by DIDS, erythritol by cytochalasin B (10 μM), choline by decamethonium (1 mM)).

Since choline is taken up very slowly by erythrocytes even at 37°C, a sufficient loading was achieved by incubating native cells over night in medium A containing in addition sucrose 40 mM, ^{14}C-labelled choline chloride (10 μM), gentamycin (20 μg/ml), Hct 40%. The tracer-loaded cells were than subjected to the chemical modifications by adding the modifying agent to the loading suspension. At the end of the desired exposure time the cells were washed and tracer efflux immediately measured in the presence of decamethonium (1 mM).

(2) Net fluxes. Rates of net K^+ loss through the leaks were studied by following the release of K^+ into K^+ free media (NaCl (150), Na_2HPO_4/NaH_2PO_4 (12.5), sucrose (40)) at 22°C (Hct 10%). The blockage of specific K^+ pathways (Na/K-ATPase, KCl cotransport, Ca^{2+}-activated K^+ channel) did not affect the results to any extent. K^+ was determined by flame photometry (IL 543), with an internal Li standard. Data are expressed as % loss of total intercellular K^+.

(3) Rates of colloid-osmotic lysis. Formation of membrane leaks permeable to small cations (and anions) results in colloid-osmotic lysis due to the slow uptake of water and salt driven by the, now unbalanced, osmotic drag of intracellular impermeant solutes (hemoglobin etc.) The rate of lysis is a function of the reflection coefficient of the membrane for the external electrolyte and thus a measure of the number and properties of the underlying 'defect pores' [2].

Modified cells were suspended in 20 vols. of phosphate-buffered (5 mM) isotonic NaCl and incubated at 0°C. Fractional hemolyses were determined after suitable time intervals by measuring hemoglobin contents in the supernatants and the total suspension [2].

(4) Apparent size of induced holes. Mean apparent radii of induced holes were derived, according to the principles outlined in Refs. 2 and 6, from the molecular radii (r_{SE}) of nonelectrolytes capable to protect 50% of the cells from colloid-osmotic lysis for a period of 24 h at a concentration of 40 mosmol/*l*.

Assessment of flip rates

The transbilayer movement of exogenous inserted palmitoyllysophosphatidylcholine was measured as described in detail elsewhere [9,12]. Briefly, the probe was inserted into the outer membrane layer of modified and control erythrocytes and the extent of its reorientation to the inner layer quantified by following the loss of its extractability by albumin with time.

Results

Inhibition of leaks induced by chemical membrane modification

Erythrocytes treated for 90 min with the mild SH oxidant, diamide, loose labelled erythritol (at 0°C) with a halftime of about 28 min (Fig. 1a) in contrast to native cells which are essentially impermeable to this nonelectrolyte at 0°C, particularly in the presence of cytochalasin B which blocks erythritol movements via the glucose transporter [18].

These induced leak fluxes can be inhibited by the diphenol compound phloretin at concentrations in the submillimolar range (Fig. 1a) *. 50% inhibition require about 200–250 μM. In spite of the limited water solubility of phloretin, the data for the high concentrations are meaningful, since in red cell suspensions most of the added compound is bound to intracellular binding sites, in particular hemoglobin [19]. Under our experimental conditions (Hct 5%) only 35% of the added phloretin were present in the aqueous phase, as determined by spectrophotometry (data not shown). Thus, the final aqueous concentrations, even after addition of nominally 800 μM phloretin, never exceeded 250 μM, the limiting concentration soluble at 0°C. Inhibition did neither reach 100% nor a demonstrable saturation at this level. The extent of inhibition was only slightly dependent on the hematocrit during measurement (1–10%). This would seem to indicate that at high concen-

* Concentrations given for phloretin and all other agents added refer to the amount added per volume of cell suspension and do not take into consideration any changes of these 'initial' concentrations due to binding to the membrane or other cell constituents.

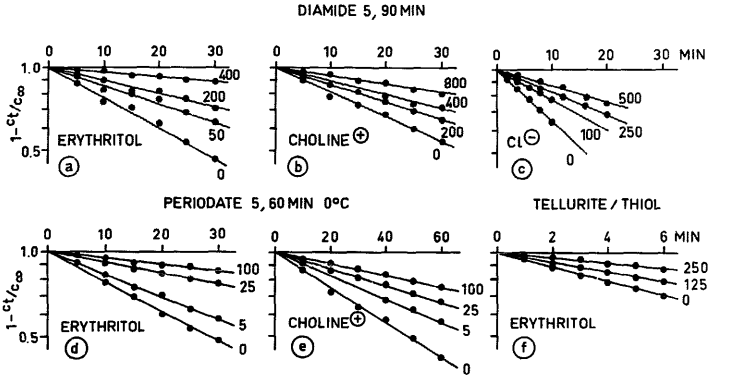

Fig. 1. Influence of phloretin on leak fluxes, at 0°C, of labelled test solutes in human erythrocytes. Leaks produced by oxidative membrane modifications. For details of experimental procedures see Methods. Numbers at the log-linear regression lines for efflux kinetics refer to concentrations of phloretin (μM). c_t and c_∞: extracellular test solute at time t and after equilibration.

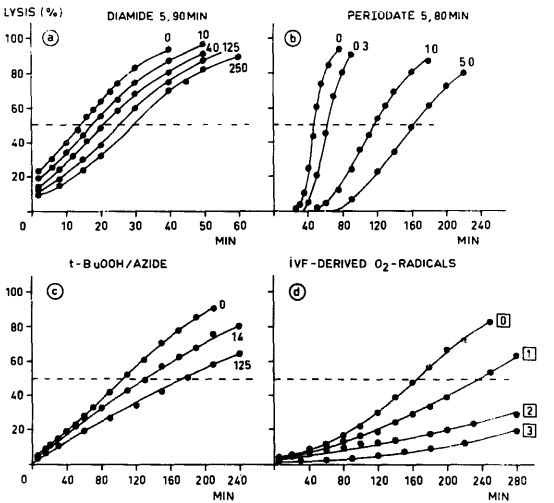

Fig. 2. Inhibition, by phloretin and other phenol compounds, of the colloid-osmotic lysis of cells made leaky to ions by various types of oxidative modification. Time courses of lysis measured as described in the Methods. Panels a–c: phloretin at the concentrations given at the curves. t-BuOOH = t-butylhydroperoxide. Panel d: Cells treated with iodoacetate/vanadate/ferricyanide (IVF) as described in Methods. 0: no additive, 1: phenolphthalein 230 μM, 2: phloretin 250 μM, 3: 4-nitrophenol 10 mM.

trations of phloretin its membrane binding is not greatly affected by its simultaneous binding to hemoglobin.

The inhibitory effect of phloretin is not restricted to nonelectrolyte leak permeability, but also demonstrable for ion leaks, e.g. to choline (Fig. 1b), K^+ (see below) or Cl^- (Fig. 1c). Even salt leaks indicated by colloid-osmotic hemolysis can be suppressed by phloretin (Fig. 2a). Moreover, the inhibitory effect of phloretin is not restricted to leaks induced by diamide. Membrane defects induced by other types of oxidative treament are also suppressed by phloretin (Figs. 1d–f, Figs. 2b–d). It should be emphasized that the formation of the leaks and the measurement of the leak fluxes in the presence or absence of the inhibitor occurred consecutively. The inhibition has therefore nothing to do with effects on leak *formation*.

Concentrations required for reducing the leak fluxes to 50% of the values observed in the absence of phloretin are compiled in Table I. As evident, these concentrations vary between 200 and 250 μM for most modifications and test permeants. As an exception, for which we have hitherto no explanation, barrier defects induced by periodate are more sensitive to inhibition by phloretin. This is surprising since at 0°C periodate produces damage by reversible oxidation of SH groups like diamide [3].

TABLE I

Inhibition, by phloretin, of various types of induced leak permeabilities in the erythrocyte membrane

Conditions of membrane modifications as described in the Methods.

	I_{50} (μM)
Diamide	
Erythritol	200
Cl^-	250
Colloid-osm. lysis	250
Periodate	
Erythritol	25
Choline	1.0
K^+	2.0
Colloid-osm. lysis	0.7
t-Butylhydroperoxide	
Erythritol	250
Colloid-osm. lysis	250
Iodoacetate/vanadate/ferricyanide	
Erythritol	250
Tellurite + thiol	
Erythritol	250
Photo-oxidation	
Erythritol	250
Electroporation	
Erythritol	> 250

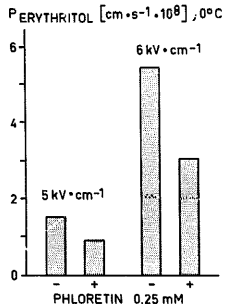

Fig. 3. Inhibitory effect of phloretin on erythritol leaks induced by electroporation. Mean values from three experiments.

Inhibition of leaks induced by electroporation

Besides chemical modification, electroporation, i.e. a physical alteration, induces leaks in the erythrocyte membrane that are stable at 0°C. These leaks, which we have studied in some detail [7,20], can also be inhibited by phloretin (Fig. 3).

Effects of other membrane-active agents

Phloretin is known to bind to the erythrocyte membrane and to inhibit numerous transport processes (see Discussion), but also to expand and modify the lipid bilayer domain [19] and to induce shape changes of erythrocytes [21]. Moreover, phloretin diminishes the interfacial dipole potential of artificial and natural lipid bilayers due to its high dipole moment [22–24]. In view of these established effects of phloretin it seemed promising to check whether other compounds with such properties would also inhibit the leak permeability. As shown in Table II, only a few compounds inhibit leak permeability like phloretin. Most of these have a high dipole moment [22] and are known or may be assumed to lower membrane dipole potentials. In contrast, leak permeability was not affected by a considerable number of drugs usually termed 'membrane-active'. The list comprises drugs known for their effects on numerous transport proteins (e.g., chlorpromazine, phenylbutazone, niflumic acid, salicylate, cytochalasin B) (see Ref. 25 for detailed references). Many of them have been shown to expand the erythrocyte membrane and thereby to decrease osmotic fragility [26] and to induce shape changes like phloretin [21]. It is therefore unlikely that such effects are the ones underlying the inhibition of leak permeabilites. Interestingly, Zn^{2+}, which has been reported to inhibit various types of induced leakiness in tumor cells but also in erythrocytes [27], is also ineffective.

TABLE II

Compounds tested for inhibitory effects on leak permeability induced by various membrane modifications

Each entry based on at least two independent measurements. Leak-forming procedure (see Methods): (a) Diamide, (b) Periodate, (c) *t*-Butylhydroperoxide, (d) photooxidation, (e) tellurite/thiol, (f) iodoacetate/vanadate/ferricyanide, (g) electroporation.

	Leak tested by measuring	
	$P_{erythritol}$	Colloid-osmotic lysis
Effective (IC_{50} (mM))		
Phloretin (≤ 0.25)	a–d, g	a–f
4-Methoxy-phloretin (0.15)	a, b	
Phenolphthalein (0.15)	a, b	b, f
4-Nitrophenol (10)	a, d	a, f
2,4-Dihydroxyacetophenone (≥ 2.0)	a	f
2,5-Dihydroxyacetophenone (≥ 2.0)	a	b
2,4,6-Trihydroxyacetophenone (≥ 1.0)	a	b, f
2,4,5-Trihydroxybutyrophenone (1.0)	a	a
2,4,6-Trihydroxybenzophenone (0.6)	a	
Ineffective (up to (mM))		
Phlorizin (5)	a	
Chlorpromazine (0.1)	a	
Phenylbutazone (5)	a	
Furosemide (2)	a	
Niflumate (1)	a	
Hexestrol (0.03)	a	
2-Nitrophenol (10)	a	
2,4-Dinitrophenol (10)	a	
4-Nitrobenzoate (10)	a	a
Phenol (19)	a	
Salicylate (10)	a, b	
Salicylamide (10)	a, d	
Dipyridamole (0.05)	a, b	
Cytochalasin B (0.02)	a	
Quinidine (1)	a, b	
Zn^{2+} (2)	a, b	b
DIDS (2)	a	

TABLE III

Lack of influence of extracellular pH on the inhibitory effect of phloretin on diamide-induced leak permeability to chloride ions

Cells treated with diamide (5 mM, 45 min, pH 8, 37°C). Chloride leak permeability (P) measured in presence of DIDS (100 μM) at 0°C. Phloretin 100 μM. Rate constants normalized to controls without phloretin.

pH_e	$\frac{P(+\text{Phlor})}{P(-\text{Phlor})}$	$\frac{[\text{HPhlor}]}{[\text{Phlor}^-]}$
6.75	0.70	3.54
7.27	0.67	1.0
7.98	0.73	0.25

Characteristics of the inhibition by phloretin

(1) Reversibility. Inhibition by phloretin requires the presence of the agent. Washing the cells with albumin-containing saline after 20 min exposure to 250 μM phloretin almost completely abolished the inhibitory effect of phloretin, on colloid-osmotic lysis induced by diamide (5 mM, 90 min) (data not shown). Saline media did not suffice for this purpose. This finding indicates the reversibility of the inhibition and shows that phloretin is not a promotor of an irreversible 'resealing' of the induced leak, but a true 'inhibitor' of leak fluxes.

(2) Competition. The question of a competitive interference of phloretin with the passage of solutes through the induced leak was addressed by determining the extent of inhibition at increasing concentrations of the test permeant. The extent of inhibition of leak flux of erythritol by 250 μM phloretin proved to be independent of the probe concentration between 0.2 and 20 mM (data not shown).

(3) pH dependence. Phloretin is a weak acid with a pK', in the aqueous phase, of 7.3 [19]. It may therefore be asked whether it causes inhibition in the charged (A^-) or the uncharged (AH) form. According to the data shown in Table III, the inhibition of the diamide-induced leak flux of Cl^- at 0°C is not affected by varying the external pH between 6.75 and 7.98, although the ratio AH/A^- in the aqueous phase decreases from 3.5 to 0.25 over this range. While this might at first sight seem to indicate that both, ionized and non-ionized phloretin, are inhibitory, an alternative explanation should also be considered: The pK' of weak acids, such as fatty acids or pentadecylhydroxycoumarin, is known to be shifted to the alkaline by about 2 pH units upon insertion of the compounds into membrane interfaces [28]. If this is also true for phloretin, the inhibitor would be in its non-ionized form over the whole extracellular pH range studied *. In this context it should also be emphasized, that carboxylate analogs of the inhibitors (e.g. 4-nitrobenzoate pK'4.8) were ineffective (Table II).

(4) Relationship between the extent of leakiness and its inhibition by phloretin. In order to understand the mechanism of phloretin inhibition, it would also be helpful to know whether the extent of inhibition depends on the extent of initial leakiness. The fractional inhibition by a given concentration of phloretin proved to be independent of the extent of the originally induced leak permeability for a number of modifications (data not shown).

(5) Influence of phloretin on the apparent size of induced 'holes'. Apparent sizes can be assigned to the holes induced by oxidative modification under the assumption of a cylindrical hole shape [2,4]. In contrast to our former mode of evaluation [4] the size, or interpolated size, of nonelectrolytes capable of protect-

* After completion of this work it has been reported (Bechinger and Seelig [45]) that the pK' of phloretin is in fact about 9.0 when the agent is bound to phospholipid membranes.

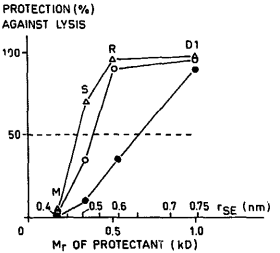

Fig. 4. Influence of phloretin and hexanol on the apparent radii of the leaks in erythrocytes treated with diamide (5 mM, 90 min, 37°C). Data derived from the extent of protection, by nonelectrolytes of varying size (40 mosmol/l), against colloid-osmotic lysis during a 24-h incubation of diamide-treated cells with: △, phloretin 250 μM, ● hexanol 25 mM, ○, controls. Apparent leak sizes derived from the Stokes-Einstein radius (r_{SE}) of nonelectrolytes protecting 50% of the cells against colloid-osmotic lysis. For further details see Methods and Ref. 5. M, mannitol; S, sucrose; R, raffinose; D1, Dextran 1.

ing 50% of the cells against colloid-osmotic lysis was taken as measure of the mean apparent hole radius. As becomes evident from Fig. 4, leaks induced by diamide (5 mM, 90 min) had a mean apparent radius of about 0.55 nm. In the presence of 250 μM phloretin this was only slightly reduced to 0.48 nm although the leak permeability was lowered to about 45% of its original value.

(6) Influence of phloretin on the enhancement of transbilayer flip rates of phospholipid analogs in leaky cells. In view of the well-established close correlation between leak formation and enhancement of the flip of phospholipid analogs it was of interest to test the effect

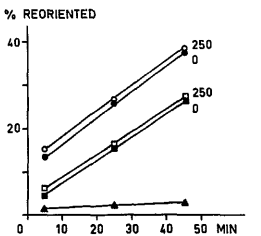

Fig. 5. Lack of effect of phloretin (250 μM) on the transbilayer reorientation of palmitoyllysophosphatidylcholine, enhanced by pretreatment with diamide 5 mM, 90 min (●, ○) or by 60 min photooxidation (□, ■). For experimental details see Methods. The membrane content of the added lysophospholipid (20 nmol/ml cells, equivalent to 0.5 mol% of the total membrane phospholipid) is well below the membrane perturbing level. Closed symbols: controls. Open symbols: phloretin. ▲, Unmodified cells.

of phloretin on the latter phenomenon. As shown in Fig. 5, the time course of transbilayer reorientation of palmitoyllysophosphatidylcholine after enhancement by diamide or photooxidative treatment is not retarded by phloretin.

Enhancement of the leaks induced by oxidative membrane modifications

As mentioned above, (see Introduction) there is some reason to believe that the leakiness induced by oxidative modification of membrane proteins stems from a secondary alteration of the membrane lipid domain. If this is true, the induced leaks should be sensitive to agents known to perturb the organization of the membrane lipid domain. Alcohols are well-known for this effect [29–33]. They enhance, for instance, the ground permeability for nonelectrolytes permeating via the lipid domain (see Ref. 29). This stimulation by alcohol can also be demonstrated for erythritol permeability at 0°C, although the effect is hard to quantify in view of the low absolute permeabilities involved. From an extrapolation of the Arrhenius plot for the native erythritol permeability to 0°C, however, and measured data for erythritol permeability in the presence of hexanol at 0°C (Fig. 7A) one can obtain an estimate of the hexanol-induced additional flux Δk. As shown in Fig. 6A, this Δk is very low. In contrast, if hexanol is added, at 0°C, to suspensions of cells made leaky by pretreatment with diamide, one obtains a large Δk, the leakiness is considerably augmented (Fig. 6A).

A more quantitative comparison between alcohol effects on the native and the oxidatively enhanced permeability is obviated by a conceptual difficulty: Quite different conclusions concerning the extent of flux enhancement by hexanol will result depending on whether the data are expressed, as above, in terms of an additive increase $\Delta k = k - k_0$ (Fig. 6A), or as a multiplicative amplification k/k_0 (Fig. 6B). The former would for instance seem appropriate in the case of aqueous pores formed *in parallel* to the lipid pathway of passive ground permeability. The concept of a multiplicative amplification, on the other hand, would be appropriate to describe the consequences of a fluidization or an increase of polarity of the lipid bilayer which have been proposed to underly the effects of alcohols on nonelectrolyte or ion permeabilities [29,32,33].

Expressed in multiplicative terms (Fig. 6B), the effect of hexanol on the diamide-induced leak permeability is less pronounced than its effect on the native permeability. Expressed in additive terms (Fig. 6A) it is much larger in diamide-treated than in native cells. This opposite behaviour underlines the relevance of the conceptual problem addressed above.

Mechanistically, the enhancement of leakiness by hexanol observed in oxidatively modified cells might, in

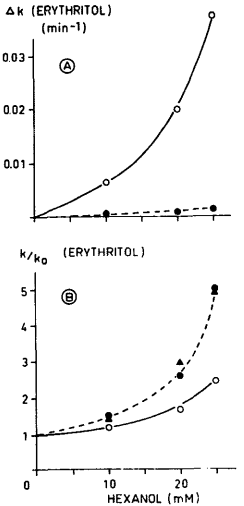

Fig. 6. Stimulation, by hexanol, of erythritol fluxes in native cells and in cells made leaky by pretreatment with diamide (5 mM, 90 min, 37°C). Fluxes measured at 0°C (●, ○) and 35°C (▲). ------, Native cells; ———, diamide-treated cells. Panel A: Changes described under the assumption of an additional, parallel leak ($\Delta k = k - k_0$). Panel B: Changes described under the assumption of a multiplicative amplification of an existing pathway (k/k_0). For details see text. Mean values from three experiments.

principle, be based on two different processes. On the one hand, it could arise from an influence on the apparent size or number of the aqueous defects produced by the primary oxidative modification of membrane proteins. On the other hand, it could result from an amplification, by a preceding protein modification, of the above-mentioned fluidizing effects of alcohols on the lipid bilayer. These two mechanisms should be discernible by the activation energies of the leak fluxes. They should be low in the former case, since aqueous leaks are involved, but high in the latter case, in view of the high activation energy of the alcohol-enhanced erythritol fluxes in native erythrocytes, shown in Fig. 7A.

Experiments designed to solve this problem (Fig. 7B) clearly demonstrated that the low temperature coefficient of erythritol fluxes in diamide-treated cells ($Q_{10} \approx 1.7$, $E_a \approx 30$ kJ/mol) remains low after flux enhancement by hexanol ($Q_{10} \approx 1$, $E_a \approx 0$), and is thus very different from the temperature coefficient of erythritol fluxes in cells only exposed to hexanol ($Q_{10} = 6.1$, $E_a = 100$ kJ/mol), which is not different from that of erythritol fluxes in native control cells (see Fig. 6A). This finding would seem to justify the conclusion that hexanol either enlarges the aqueous pores produced by oxidative treatment or facilitates their formation.

To distinguish between these two alternatives, the size of the aqueous 'holes' was determined in the presence of hexanol. As shown in Fig. 4, the apparent radius of the diamide induced hole is increased by a factor of about 1.2 (from 0.55 to 0.65), when the leak permeability is increased about 2.5-fold by 25 mM hexanol. This increase in radius cannot account for the increase of permeability (see Discussion).

A stimulation by hexanol is also observed in cells made leaky by periodate, *t*-butylhydroperoxide or iodoacetate/vanadate/ferricyanide (Table IV). From the data it becomes evident, that about the same effectivity of hexanol is observed for various modifying treatments, essentially independent of the magnitude of the induced leak permeability. This finding is also in line with the observation (data not shown) that increasing leakiness induced by a modifier does not alter the relative enhancement of this leak permeability by hexanol. As an exception, periodate treatment renders the cells more sensitive to hexanol than all the other modifications.

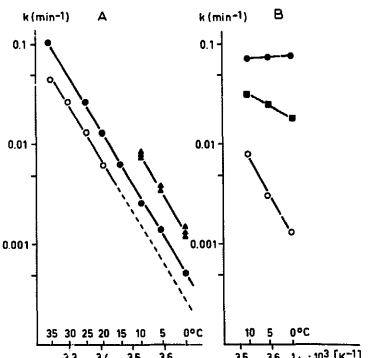

Fig. 7. Panel A: Arrhenius plots for erythritol fluxes in native cells in the absence (open symbols) and presence (closed symbols) of hexanol: ●, 20 mM; ▲ 25 mM. From the slopes, activation energies between 96 (control) and 112 kJ/mol (hexanol) can be obtained. Panel B: Arrhenius plots for erythritol fluxes: ○, in native cells in the presence of hexanol 25 mM; ■, in diamide-pretreated cells and ●, in diamide-pretreated cells exposed to hexanol 25 mM. Data for a representative experiment out of a series of three with coinciding results.

TABLE IV

Stimulation, by hexanol (25 mM), of leakiness induced by oxidative chemical membrane modification

Modification as described in the Methods. k, leak flux or rate of lysis (0°C). IVF, iodoacetate/vanadate/ferricyanide (see Methods).

	Leak defined by	k ($\times 10^2$) (min^{-1})	$\frac{k(+Hex)}{k(-Hex)}$
Periodate 5 mM, 60 min	$k_{erythritol}$	1.68	4.8
Periodate 5 mM, 40 min	k_{lysis}	1.39	4.6
t-Butylhydroperoxide, 40 min	k_{lysis}	3.63	2.2
IVF, 60 min	k_{lysis}	3.77	2.8
Photooxidized, 50 min	$k_{erythritol}$	1.90	1.9
Diamide 5 mM, 90 min	$k_{erythritol}$	2.16	2.5
Diamide 5 mM, 90 min	k_{lysis}	3.73	2.4

Besides hexanol, other alcohols (e.g. butanol) also enhance leakiness at concentrations at which they have no detectable effects in unmodified cells at 0°C (data not shown). Moreover, preliminary results indicate that detergents and local anesthetics have similar stimulating effects.

The concept of an effect of hexanol on the pre-existing aqueous leaks is also supported by our finding (Table V) that phloretin exerts its inhibitory action to the same extent in the presence of hexanol as in its absence. The effect may even be more pronounced, if anything. In contrast, hexanol stimulation of the native membrane permeability, i.e., its effect on fluidity or polarity, is not counteracted by phloretin (Deuticke, B., unpublished results).

Lack of stimulating effects of alcohols on leak permeability induced by electroporation

As shown above, phloretin inhibits leak fluxes induced by oxidative modification as well as by electroporation. Surprisingly, this parallelism does not hold for stimulation by alcohols. The rate of leak flux k of erythritol (0°C) induced by a field pulse of 5 kV cm^{-1} ($\tau = 40$ μs) was even slightly inhibited by alkanols.

TABLE V

Extent of inhibition, by phloretin, of the leak fluxes induced by oxidative membrane modification or by combination of oxidative modification and addition of hexanol

Flux rates (k) of erythritol at 0°C. For details of oxidative treatments see Methods.

Oxidant	Phloretin (μM)	$\frac{k(+Phlor)}{k(-Phlor)}$	
		no hexanol	hexanol 25 mM
Diamide 5 mM, 90 min, 37°C	200	0.50	0.14
Periodate 5 mM, 60 min, 0°C	12.5	0.45	0.33
t-Butylhydroperoxide 2.5 mM	250	0.54	0.34

$k(+alkanol)/k(-alkanol)$ was 0.90 ± 0.28 ($n = 8$) for 25 mM hexanol and 0.81 ± 0.25 ($n = 6$) for 200 mM butanol.

Discussion

Inhibition by phloretin

Phloretin (3-(4'-hydroxyphenyl)-2,4,6-trihydroxypropiophenone) has a long history as potent inhibitor of protein-mediated, facilitated and active, transport processes in the erythrocyte membrane. Transports affected comprise nonelectrolytes (monosaccharides, glycerol, urea and other amides) organic ions (amino acids, monocarboxylates, choline) as well as inorganic anions and cations (for references see 25, 29, 34, and 35). Phloretin may thus be regarded as a rather unspecific inhibitor in spite of its high effectivity.

Kinetic details, as far as they are known, seem to vary among the different transport processes. Inhibitor constants (K_i, I_{50} values) for most transport inhibitions fall into a range of concentrations (< 2–3 μM) coinciding with the dissociation constant ($K_d = 1.5$ μM [19]) for high affinity binding of phloretin to about $2.5 \cdot 10^6$ sites per cell on erythrocyte membrane proteins. In addition, about $5.5 \cdot 10^7$ low-affinity binding sites ($K_d \approx 50$ μM) for phloretin have been demonstrated in the erythrocyte membrane [19]. Since this type of sites is also present in extracted membrane lipids and in artificial vesicular [36] and planar [23,37] lipid membranes ($K_d = 8$ μM), its location in the lipid bilayer domain is generally taken for granted. At the saturating level of $5.5 \cdot 10^7$ phloretin molecules per cell the molar ratio lipid/phloretin is about 4:1.

The major known consequence of phloretin binding to lipid bilayers, besides a moderate increase of membrane 'fluidity' indicated by slight increases of nonelectrolyte permeability [38,39], consists in a lowering of the endogenous dipole potential (membrane interior positive) at each membrane interface [22,23]. The molecular origin of these potentials has not been finally clarified [41]. They may be as large as 300–400 mV in artifical membranes, but are probably smaller in the erythrocyte membrane [22,24,40]. Due to their sign, these potentials increase the energy barrier of the membrane interior for cations, while lowering that for anions [41]. The concept of a diminution of these dipole potentials by phloretin and related compounds with high dipole moments [22] is based on the finding [22,23] that the permeability to cationic species (hydrophobic cations, inorganic cation/ionophore complexes) is increased by such compounds, while that for comparable anionic species is lowered.

The results reported in this work establish a new facet of phloretin's diverse effects on membranes. Although fairly high concentrations of the agent are required to inhibit leak fluxes, the effect can be re-

garded as specific inasmuch as it is not shared by numerous other membrane active agents (Table II). Since many of these other agents act like phloretin in expanding the erythrocyte membrane and decreasing the cell's osmotic fragility [26], the inhibition of leak permeability is unlikely to result from a mere insertion into the membrane of drug molecules.

In view of the lack of effect of compounds like furosemide, dipyridamole and DIDS it is also not very likely that a modified transport protein, e.g band 3, acts as a leak. Moreover, since Zn^{2+} ions do not inhibit the leak fluxes induced by these covalent membrane modifications, our leaks are probably different from leaks induced by toxins, low doses of complement and certain detergents, reported by Bashford et al. [27] to be sensitive to inhibition by Zn^{2+}. This view is also supported to some extent by the observation (Deuticke, B., unpublished results) that membrane leakiness induced by high concentrations of the polyene antibiotic amphotericin B is not inhibited by phloretin.

The high concentrations of phloretin required for inhibition of leak fluxes suggest a mechanism of action involving the membrane lipid domain. The concentration I_{50} required for 50% inhibition (250 μM in most cases) is even higher than the K_d for phloretin binding to the erythrocyte membrane lipid domain [19]. It can be estimated that the I_{50} corresponds to a molar ratio of about 1 phloretin per 5 lipid molecules. Assuming equidistribution of phloretin between both sides of the bilayer, this corresponds to a surface concentration of 1 phloretin per 4 nm^2 in each layer. The reason for the much lower I_{50} values for the inhibition of the leakiness induced by periodate is not clear and subject of work in progress.

Besides phloretin, a number of related compounds also inhibit the leak fluxes, though mostly at much higher concentrations. This difference is probably due to their lower partition coefficients. Since most of these compounds have a high dipole moment like phloretin [22], a causal involvement of this feature in the inhibitory effect of phloretin has to be considered. The lack of effect of 4-nitrobenzoate and 2-nitrophenol, as opposed to 4-nitrophenol, is also in line with such a hypothesis. The lack of effect of salicylamide, which has been shown to affect dipole potentials in artifical bilayers [42] may only be due to a quantitative problem, since this compound is much less effective as a modifier of dipole potentials than phloretin [42]. On the other hand, the pronounced inhibitory effect of phenolphthalein is not readily reconcilable with an involvement of the dipole potential. While direct data for the dipole moment of this molecule are not available, it could be shown (Deuticke, B., unpublished results) that it lacks the stimulating effect on the permeability of hydrophobic cations, which would be expected for a compound lowering the membrane dipole potential.

The decrease of dipole potential going along with the inhibition of leak flux by 250 μM phloretin would be about 65–75 mV. This can be predicted, on the basis of theoretical considerations [33,43] from the extent of stimulation (13–20-fold) of the flux of methyltriphenylphosphonium, a hydrophobic cation, across the erythrocyte membrane in the presence of 250 μM phloretin (Deuticke, B., unpublished results).

An interesting conclusion can be drawn from our finding of a lack of effect of phloretin on the enhancement of lipid transbilayer reorientation in chemically modified leaky cells (Fig. 5). We have previously proposed that the leaks, which can be envisaged as dynamic structures alternating between open and closed states, might also serve as flip sites [20]. This unifying hypothesis is hard to maintain in view of the insensitivity of the enhanced flip rates to phloretin, unless one assumes that the inhibitor affects solute transport across the open pore by a simple steric blockage. We tend to prefer the concept that flip enhancement and leakiness do not involve the same 'sites'. Material to be published elsewhere also supports the view that the altered state of the membrane allowing for flip enhancement in chemically modified cells may *precede* the formation of the dynamic aqueous leaks, and that phloretin affects the transition between these two states. This interpretation is also in line with the finding (Fig. 4) that a 50% inhibition of leak flux by phloretin only goes along with a decrease of the apparent radius of the holes by 13%. Such a decrease by itself could only induce a 25% inhibition of the leak flux. A 50% inhibition would require a lowering of the apparent radius to 0.38 nm. This decrease should shift the protection curve in Fig. 4 to much lower radii of protective nonelectrolytes.

Stimulation by alcohols

The stimulating effect of alcohols on the fluxes through the aqueous leaks in oxidatively modified cells shall first be considered under the aspect of the required membrane levels of the alcohols. Such levels can be obtained using the fairly well established partition coefficients (P_m) compiled in Ref. 26. Assuming membrane lipid concentrations of 450 mmol phospholipid and 350 mmol cholesterol/kg membrane [44] one obtains a molar ratio hexanol/total lipid of 1:6 to 1:2.5, required for stimulating leak permeability 2–4-fold.

Marked changes of the membrane lipid composition are thus involved in the stimulation. These changes are equivalent to those required for maximal protection of the cells against osmotic lysis due to 'membrane expansion' [26], or for increasing the 'fluidity' of microsomal membranes about 1.5–2-fold [30]. Moreover, insertion of alcohols at these concentrations has been claimed to increase the 'polarity' of the bilayer core [32,33].

In principle, an enlargement of the apparent pore radii or an increase of pore numbers (or frequency of opening) might be responsible for the effects of the alcohols. In order to distinguish between the two mechanisms, one can estimate the increase in radius that would account for the 2.5-fold increase of leak permeability produced by 25 mM hexanol (Fig. 4). Assuming an apparent radius of the original oxidatively induced 'holc' of 0.55 nm (Fig. 4) one would have to postulate an increase to 0.85 nm. Such an increase would, however, have to go along with an increase in the limiting size of test solutes passing through the leaks to values exceeding the radius of D1 (0.75 nm). Since this was not observed (Fig.4) it seems more likely that, besides a minor increase of the apparent pore size, the 'opening frequencies' or 'open times' of the pores are increased by the alcohols as a consequence of altered physical properties of the lipid domain. Changes in polarity, but also of the motional or order parameters of the membrane lipids might equally well be involved in this effect.

A feature that does not fit into this concept is the surprising observation that leaks produced by electroporation are not enhanced by hexanol (see above). From this difference in sensitivity of chemically and electrically induced defects one must either conclude that the above explanation for the effect of alcohols is wrong (if a common structure of the two types of defects is taken for granted) or that the two types of defects in fact differ in important structural properties.

Regardless of this ambiguity and in conclusion, the new results presented here support the dynamic nature of the membrane leaks induced by oxidative membrane modification and open the possibility to modulate these dynamics. Moreover, they support the involvement of membrane lipids in leak formation and suggest a chain of events leading to leak formation, in which the enhancement of transbilayer mobility of amphiphiles precedes the formation of aqueous defects. Phloretin and related compounds constitute a new class of agents capable of suppressing leaks caused by various types of membrane injury. It will be interesting to test the beneficial effect of this aglycon of a plant glycoside (phlorizin) in other membrane systems.

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Bechinger and Seelig ((1991) Biochemistry 30, 3923–3928) have very recently published data indicating that phloretin at the membrane concentrations to be expected in our study (up to 20 mol%) not only alters the orientation of the headgroup of choline phospholipids in liposome membranes but also replaces water in the headgroup region, thereby interfering with the structural organization of this domain. The hydrocarbons were found to be, at best, slightly disordered by the agent. It is tempting to speculate that the headgroup perturbation affects the dynamic fluctuations of the phospholipid configuration proposed to produce the membrane leaks and is thus responsible for the inhibition demonstrated in our study.

References

1 Deuticke, B., Poser, B., Luetkemeier, P. and Haest, C.W.M. (1983) Biochim. Biophys. Acta 731, 196–210.
2 Deuticke, B., Luetkemeier, P. and Sistemich, M. (1984) Biochim. Biophys. Acta 775, 150–160.
3 Heller, K.B., Poser, B., Haest, C.W.M. and Deuticke, B. (1984) Biochim. Biophys. Acta 777, 107–116.
4 Deuticke, B., Heller, K.B. and Haest, C.W.M. (1987) Biochim. Biophys. Acta 899, 113–124.
5 Heller, K.B., Jahn, B. and Deuticke, B. (1987) Biochim. Biophys. Acta 901, 67–77.
6 Deuticke, B., Henseleit, U., Haest, C.W.M., Heller, K.B. and Dubbelman, T.M.A.R. (1989) Biochim. Biophys. Acta 982, 53–61.
7 Schwister, K. and Deuticke, B. (1985) Biochim. Biophys. Acta 816, 332–348.
8 Israelachvili, J.N. (1977) Biochim. Biophys. Acta, 469, 221–225.
9 Bergmann, W.L., Dressler, V., Haest, C.W.M. and Deuticke, B. (1984) Biochim. Biophys. Acta 769, 390–398.
10 Haest, C.W.M., Heller, K., Schwister, K., Kunze, I.G., Dressler, V. and Deuticke, B. (1983) Biomed. Biophys. Acta 42, 127–129.
11 Dressler, V., Schwister, K., Haest, C.W.M. and Deuticke, B. (1983) Biochim. Biophys. Acta 732, 304–307.
12 Classen, J., Deuticke, B. and Haest, C.W.M. (1989) J. Membr. Biol. 111, 169–179.
13 Chernomordik, L.V. and Chizmadzhev, Y.A. (1989) in Electroporation and Electrofusion in Cell Biology (Neumann, E., Sowers, A.E. and Jordan, C.A., eds.), pp. 83–95, Plenum Press, New York.
14 Weaver, J.C. and Powell, K.T. (1989) in Electroporation and Electrofusion in Cell Biology (Neumann, E., Sowers, A.E. and Jordan, C.A., eds.), pp. 111–126, Plenum Press, New York.
15 Deuticke, B. (1989) Stud. Biophys. 134, 99–104.
16 Deuticke, B., Heller, K.B. and Haest, C.W.M. (1986) Biochim. Biophys. Acta 854, 169–183.
17 Awasthi, Y.C., Garg, H.S., Dao, D.D., Partridge, C.A. and Srivastava, S.K. (1981) Blood 58, 733–738.
18 La Celle, P. and Passow, H. (1971) J. Membr. Biol. 4, 270–283.
19 Jennings, M.L. and Solomon, A.K. (1976) J. Gen. Physiol. 67, 381–397.
20 Deuticke, B. and Schwister, K. (1989) in Electroporation and Electrofusion in Cell Biology (Neumann, E., Sowers, A.E. and Jordan, C.A., eds.), pp. 127–148, Plenum Press, New York.
21 Deuticke, B. (1968) Biochim. Biophys. Acta 163, 494–500.
22 Andersen, O.S., Finkelstein, A., Katz, I. and Cass, A. (1976) J. Gen. Physiol. 67, 749–771.

23 Melnik, E., Latorre, R., Hall, J.E. and Tosteson, D.C. (1977) J. Gen. Physiol. 69, 243–257.
24 Macey, R.I. and Orme, F.W. (1980) in Membrane Transport in Erythrocytes (Lassen, U.V., Ussing, H.H. and Wieth, J.O., eds.), pp. 498–509, Munksgaard, Copenhagen,
25 Deuticke, B., Grebe, R. and Haest, C.W.M. (1990) in Blood Cell Biochemistry (Harris, J.R., ed), pp. 475–529, Plenum Press, New York, London.
26 Seeman, P. (1972) Pharmacol. Rev. 24, 583–655.
27 Bashford, C.L., Alder, G.M., Graham, J.M., Menestrina, G. and Pasternak, C.A. (1988) J. Membr. Biol. 103, 79–94.
28 Tocanne, J.-F. and Teissié, J. (1990) Biochim. Biophys. Acta 1031, 111–142.
29 Deuticke, B. (1977) Rev. Physiol. Biochem. Pharmacol. 78, 1–97.
30 Zavoico, G.B. and Kutchai, H. (1980) Biochim. Biophys. Acta 600, 263–269.
31 Chi, L.-M., Wu, W.-g, Sung, K.-L. and Chien, S. (1990) Biochim. Biophys. Acta 1027, 163–171.
32 Orme, F.W., Moronne, M.M. and Macey, R.I. (1988) J. Membr. Biol. 104, 57–68.
33 Reyes, J. and Latorre, R. (1979) Biophys. J. 28, 259–279.
34 Rosenberg, R. (1981) Biochim. Biophys. Acta 649, 262–268.
35 Devés, R. and Krupka, R.M. (1990) Biochim. Biophys. Acta 1030, 32–40.
36 Verkman, A.S. and Solomon, A.K. (1980) J. Gen. Physiol. 75, 673–692.
37 Awiszus, R. and Stark, G. (1988) Eur. Biophys. J. 15, 321–328.
38 Owen, J.D., Steggall, M. and Eyring, E.M. (1974) J. Membr. Biol. 19, 79–92.
39 Poznansky, M., Tong, S., White, P.C., Milgram, J.M. and Solomon, A.K. (1976) J. Gen. Physiol. 67, 45–66.
40 Moronne, M.M. and Macey, R.I. (1991) Biophys. J. 59, 642a.
41 Flewelling, R.F. and Hubbell, W.L. (1986) Biophys. J. 49, 541–552.
42 McLaughlin, S. (1977) Current Topics in Membranes and Transport, Vol. 9, pp. 71–135, Academic Press, New York.
43 De Levie, R., Seelig, P.F. and Andersen, O.S. (1979) Biophys. J. 25, 295–300.
44 Broekhuyse, R.M. (1974) Clin. Chim. Acta 51, 341–343.
45 Dechinger, B. and Seelig, J. (1991) Biochemistry 30, 3923–3928.