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EXPERIMENTAL ALTERATION OF PHOSPHOLIPID-PROTEIN INTERACTIONS WITHIN THE HUMAN ERYTHROCYTE MEMBRANE

DEPENDENCE ON GLYCOLYTIC METABOLISM

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SUMMARY

Phosphatidylethanolamine in freshly drawn human erythrocytes is trinitrophenylated by 2, 4, 6-trinitrobenzene sulfonic acid only slowly and to a maximum of 32 %. After different preincubation procedures at 37 °C in saline media in the absence of glucose (24 h without additive, 1–5 h with 8 mM hexanol or 1–4 h with the SH reagent, 5 mM tetrathionate) the rate of subsequent trinitrophenylation of phosphatidylethanolamine, in the absence of the additives, is greatly enhanced and the amount of phospholipid reacting increased. Glucose or inosine prevent these effects, inhibitors of glycolysis abolish this protection.

The results indicate that in fresh as well as in glycolysing incubated erythrocytes phosphatidylethanolamine in the outer layer of the membrane lipid is shielded by a protein. Conformational changes of this protein induced by metabolic starvation and perturbing agents expose the phospholipid head group to 2, 4, 6-trinitrobenzene sulfonic acid. In addition, a "flip-flop" of phosphatidylethanolamine from the inner to the outer layer may also contribute to the effects observed.

INTRODUCTION

It is generally accepted today that the matrix of the erythrocyte membrane consists of a bilayer of phospholipids, and that "intrinsic" proteins are embedded into the hydrophobic core of the bilayer [1]. Furthermore, there is considerable evidence that the arrangement of proteins and lipids in this membrane is highly asymmetric [2]. Only a minor part of the membrane protein is accessible from the outside of the cell, as demonstrated with various impermeable reagents and proteolytic enzymes [2]. Most of these proteins probably span the membrane. The major group of proteins has been shown to be localized at the cytoplasmic side of the membrane. A great part of these proteins is easily dissociated from the lipid domain, whereas the

Abbreviations: TNBS, 2,4,6-trinitrobenzene sulfonic acid; DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); PCMBS, *p*-hydroxymercuri-phenyl sulfonic acid.

remaining part is anchored strongly in the membrane by hydrophobic interaction with lipids and can only be solubilized by detergents [2]. An asymmetric distribution of the phospholipids in the erythrocyte membrane was first suggested [3, 4] on the basis of experiments in which the reactivity of the amino groups of phosphatidylethanolamine and phosphatidylserine towards non-penetrating reagents, such as trinitrobenzene sulfonic acid (TNBS) and formylmethionyl (sulphone) methyl phosphate, was tested. In contrast to cell ghosts, in the membrane of the intact erythrocyte no phosphatidylserine and only one-third of the phosphatidylethanolamine molecules could be labelled with these reagents. From these findings it was concluded that phosphatidylethanolamine and phosphatidylserine are preferentially localized at the inside surface of the membrane [3, 4]. Recently more compelling evidence for such a phospholipid asymmetry has been obtained from experiments on the hydrolytic cleavage of the erythrocyte phospholipids by phospholipases [5].

At present nothing is known about the forces responsible for the asymmetric distribution of the phospholipids. Principally, the asymmetry might already originate from the biosynthesis of the phospholipids and their subsequent incorporation into the membrane as well as from secondary exchange of phospholipids between the plasma lipoproteins and the outer layer of the membrane [6]. Preservation of the asymmetry could be due to specific lipid-protein interactions.

In the present paper this latter possibility was studied. We tried to find experimental conditions under which the interactions of lipids and proteins is likely to become disturbed. The rate of the reaction of phosphatidylethanolamine with TNBS, assumed to be measure of its "accessibility", was used as an indicator for such an alteration of lipid-protein interactions. A linkage of the observed effects to glycolytic metabolism in the erythrocyte could be demonstrated.

MATERIALS AND METHODS

Reagents. *N*-Ethylmaleimide, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), sodium iodoacetate and $Na_2S_4O_6$ were purchased from Merck AG, Darmstadt; *p*-hydroxymercuri-phenyl sulfonic acid (PCMBS) and TNBS from Sigma, St. Louis, and phloretin from Roth, Karlsruhe. Dipyridamole was a gift of the Carl Thomae GmbH, Biberach-Riss, phenopyrazone of Knoll AG, Ludwigshafen.

General incubation procedure. Erythrocytes from freshly collected, heparinized human blood were washed three times with 154 mM NaCl. One volume of the washed cells was then suspended in ten volumes of Medium A, containing (mM): KCl 90, NaCl 45, Na_2HPO_4/NaH_2PO_4 10 and sucrose 44. The pH of the suspension was adjusted at 8.0 unless otherwise indicated. Medium A was chosen because incubations had to be carried out under conditions under which cation impermeability was impaired. Media of this composition have been shown to prevent cation net movements in highly cation-permeable erythrocytes [7]. The erythrocytes were incubated under gentle agitation with various reagents usually at 37 °C in the absence or presence of glucose and metabolic inhibitors. Subsequently they were washed three times with Medium A and then exposed to TNBS.

Reaction of erythrocytes with TNBS. 1 vol. of erythrocytes was incubated at 20 °C and pH 8.0 with 10 vol. of Medium A containing 1.7 mM TNBS for 1 h unless otherwise indicated. The reaction was terminated by addition of a small volume of

1 M glycylglycine (pH 8.0) to the medium (final concentration 20 mM). The erythrocytes were isolated by centrifugation, washed and the lipids extracted according to Rose and Oklander [8]. The phospholipids were separated by thin-layer chromatography on silica plates (Merck, Darmstadt, No. 5715) using chloroform/methanol/25 % ammonia (65 : 30 : 4, v/v) for the development. The phosphatidylethanolamine that had reacted with TNBS could be directly localized because of its yellow colour. The other phospholipids were detected with molybdate spray. The trinitrophenyl phosphatidylethanolamine and phosphatidylethanolamine spots were scraped from the plates and phosphate was determined as described before [9].

RESULTS

(1) *Effect of 24 h preincubations on the trinitrophenylation of phosphatidylethanolamine*

According to results obtained by Gordesky and Marinetti [4] phosphatidylethanolamine in the membranes of freshly drawn erythrocytes reacts only slowly with TNBS at 20 °C. A maximum, obtained after about 20 h, of approx. 30 % of the total phosphatidylethanolamine could be trinitrophenylated in their experiments whereas phosphatidylserine did not react at all. These findings were confirmed in our analyses. Fig. 1 shows that the low reactivity was maintained when erythrocytes were incubated for 24 h at 37 °C in plasma or in Medium A with glucose. In the absence of glucose, however, the reaction rate increased 10-fold under otherwise identical conditions. The maximal percentage of phosphatidylethanolamine reacting with TNBS (approx. 30 %) was not altered and phosphatidylserine remained completely unreactive. Hemolysis was always less than 2 %.

The protective effect of glucose on membrane phosphatidylethanolamine amino groups indicated by these findings seems to be related to glycolytic metabolism, since inosine, which can replace glucose as a substrate of red cell energy supply [10],

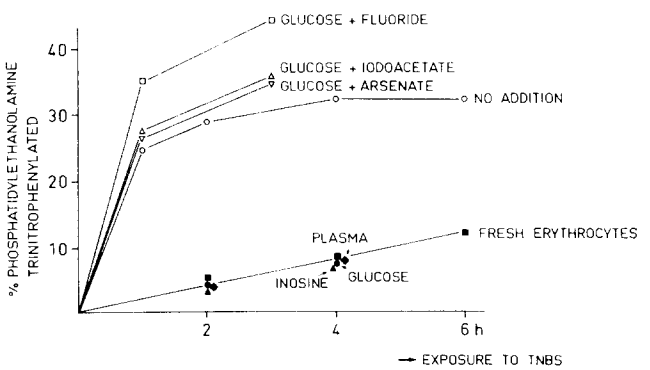

Fig. 1. Influence of metabolic substrates and inhibitors on the trinitrophenylation of phosphatidylethanolamine in human erythrocytes. ■, freshly drawn erythrocytes; ◆, erythrocytes preincubated for 24 h at 37 °C in plasma. Erythrocytes preincubated for 24 h at 37 °C in Medium A with: ○, no addition; ●, 11 mM glucose; ▲, 5 mM inosine; □, 11 mM glucose + 10 mM NaF; △, 11 mM glucose + 1 mM iodoacetate; ▽, 11 mM glucose + 20 mM arsenate. The media contained penicillin (8 mg/100 ml) and streptomycin (20 mg/100 ml). The pH was adjusted to 8.0 after addition of the cells, but declined to 7.4–7.5 during the incubation. After the incubation, the erythrocytes were washed and exposed to TNBS (see Materials and Methods).

also maintained a low rate of TNBS binding. To further substantiate the significance of metabolism, glycolysis was inhibited with fluoride, iodoacetate or arsenate [11–13]. As is evident from Fig. 1, the addition of these compounds completely abolished the protective action of glucose. In the light of such findings one may assume that glycolytic metabolism somehow protects phosphatidylethanolamine amino groups from TNBS. Lack of energy supply induces a "reorientation" in the membrane which improves the accessibility or the reactivity of phosphatidylethanolamine.

In order to elucidate the possible reasons of such a reorientation we studied the influence on TNBS binding of compounds likely to perturb hydrophobic lipid-lipid and lipid-protein interactions.

(2) *Effect of hexanol pretreatment on the trinitrophenylation of phosphatidylethanolamine*

Aliphatic alcohols are known to perturb lipid-lipid and lipid-protein interactions [14–17]. They stabilize erythrocytes against osmotic lysis, cause a disordering of the membrane lipid phase [17], and inhibit facilitated transport processes [18, 19, 26]. In our experiments we tested hexanol at a concentration of 8 mM. Cells were preincubated with hexanol at 37 °C and then washed thoroughly in order to remove the alcohol. That hexanol can actually be removed from the erythrocyte membrane by this procedure was concluded from the complete reversibility of the hexanol effect on non-electrolyte permeability (Haest, C. W. M. and Deuticke, B., unpublished results).

According to the data in Fig. 2, preincubation of erythrocytes with hexanol at 37 °C in the presence of glucose only produced a small initial enhancement of the trinitrophenylation of phosphatidylethanolamine, which did not increase on prolonged hexanol treatment. This initial effect may be interpreted as a direct consequence of the binding of hexanol to the membrane, which is a rapid process [20]. Hexanol treatment in the absence of glucose, on the other hand, or in the presence of glucose and iodoacetate, greatly enhanced the reaction rate of TNBS with phosphatidylethanolamine. Interestingly, at 20 °C, a 3-h pretreatment with hexanol resulted in a

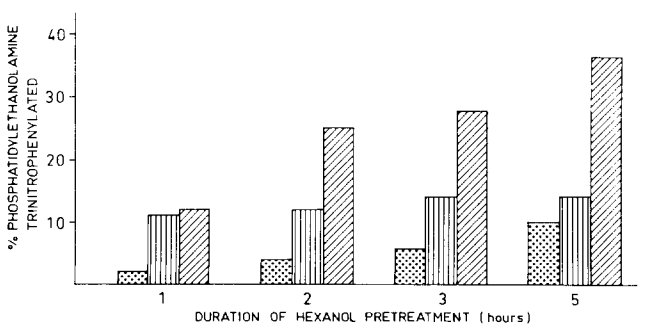

Fig. 2. Effect of hexanol on the trinitrophenylation of phosphatidylethanolamine. Erythrocytes were preincubated at 37 °C for varying times (indicated in the figure) in Medium A (pH 8.0): dots, no addition; vertical lines, 8 mM hexanol, 11 mM glucose; diagonal lines, 8 mM hexanol, no glucose. After the incubation the cells were washed four times with 20 vol. of Medium A at 37 °C and exposed for 1 h to TNBS (see Materials and Methods).

labelling of only 6.5 % of the total phosphatidylethanolamine, demonstrating a remarkable temperature dependence of the hexanol-induced perturbation.

The results described demonstrate that the effect of a hexanol pretreatment of erythrocytes at 37 °C in the absence of glucose could not be reversed by simple washings of the cells with salt solutions. However, when after such washings the cells were incubated in the presence of inosine at 37 °C for 2.5 h and subsequently exposed to TNBS for 1 h, the labelling of phosphatidylethanolamine decreased to 18.9 %, as compared to 27.8 % before the inosine treatment. In contrast, incubation with glucose had no effect (32.8 % trinitrophenylation), suggesting that after the 3-h pretreatment in the absence of metabolic substrate the erythrocytes had lost, due to degradation of ATP, the capacity to phosphorylate glucose, but were still capable of deriving metabolic energy from the phosphorylytic cleavage of inosine. It can be concluded from these findings that the effect of hexanol is slowly reversible, provided that metabolic substrate can still be utilized.

(3) *Effect of pretreatment with SH reagents on the trinitrophenylation of phosphatidylethanolamine*

In another attempt to perturb lipid-protein interactions membrane proteins were modified by preincubation of the erythrocytes with SH-specific reagents. According to the data in Table I, pretreatment of erythrocytes with DTNB had no effect on the reaction of phosphatidylethanolamine with TNBS, whereas iodoacetate stimulated the effect only slightly, although both reagents are able to react with the red cell membrane [21, 22]. In contrast, the permeable reagent *N*-ethylmaleimide as well as the essentially impermeable reagent PCMBS [23, 24] and furthermore tetrathionate strongly enhanced the rate of trinitrophenylation. The most pronounced effect was observed with tetrathionate. For this reason, and because it did not induce hemolysis, tetrathionate was used in further investigations.

(a) *Characteristics of the tetrathionate effect.* Tetrathionate had its maximal

TABLE I

EFFECT OF PREINCUBATION WITH SH REAGENTS ON THE REACTION OF PHOSPHATIDYLETHANOLAMINE WITH TNBS

Erythrocytes were preincubated with the SH reagents in Medium A (pH 8.0, 37 °C) for 3 h, then washed three times and exposed to TNBS for 1 h (see Materials and Methods).

Reagent		Phosphatidylethanolamine trinitrophenylated (%)
Control		6.8
DTNB	5 mM	6.0
Iodoacetate	1 mM	10.7
N-Ethylmaleimide	10 mM	28.4
PCMBS	0.5 mM	33.6
Tetrathionate	5 mM	42.7
Tetrathionate	20 mM	44.0
Tetrathionate after 2 h DTNB (5 mM)	5 mM	46.1

effect at 5 mM. Higher concentrations did not significantly enhance the labelling obtainable during a 1-h exposure to TNBS. After a 4-h exposure to TNBS, subsequent to a 3-h preincubation with tetrathionate, however, up to 65 % of the total phosphatidylethanolamine were found to be trinitrophenylated without any significant hemolysis (< 2 %). Whether this is the maximal amount able to react could not be investigated, since further prolongation of the incubation time led to hemolysis. Pretreatment of the erythrocytes with DTNB did not inhibit the effect of tetrathionate, indicating that the two SH reagents interact with different populations of membrane SH groups.

Although unambiguous data concerning the permeability of the erythrocyte membrane to tetrathionate are not yet available, one may assume that a divalent anion of this size at best penetrates slowly at pH 8.0 [25, 26]. The lack of inhibitory influence of tetrathionate, a potent inhibitor of the enzyme glyceraldehyde-3-phosphate dehydrogenase [27], on glycolysis in intact erythrocytes [28] provides further evidence for this assumption. Finally, tetrathionate entering the cell at a slow rate should be reduced to thiosulfate immediately by intracellular reduced glutathione (GSH) (ref. 29, p. 250). Thus it is very likely that tetrathionate only acts on the outside of the erythrocyte membrane.

In order to test whether tetrathionate on the way to its site of action uses the general pathway of anions, we studied its effect on TNBS labelling under conditions known to interfere with anion transfer. In contrast to the transfer of divalent anions, which decreases at alkaline pH values [25, 26], the effect of tetrathionate increased with pH (Fig. 3). This type of pH dependence, which is characteristic for reactions involving SH groups (ref. 29, p. 94), supports the assumption that tetrathionate reacts with such groups.

Further experiments dealt with the influence of inhibitors of anion transfer on the tetrathionate effect. As is evident from Fig. 4, the effect of tetrathionate is strongly inhibited by amphiphilic compounds like dipyridamole, phloretin and phenopyrazone.

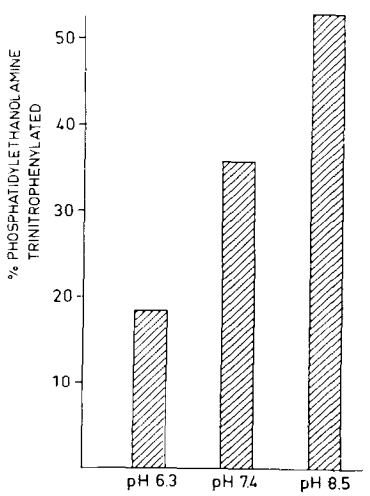

Fig. 3. pH dependence of the tetrathionate effect. Erythrocytes were preincubated for 3 h with tetrathionate at 37 °C in Medium A of varying pH. After washing of the cells they were exposed for 1 h to TNBS at pH 8.0 (see Materials and Methods).

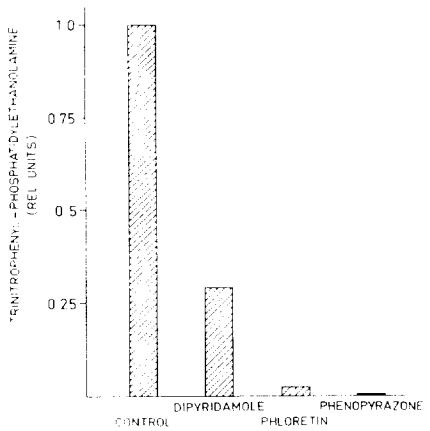

Fig. 4. Influence of inhibitors of anion transport on the tetrathionate effect. The reaction conditions were the same as described in Fig. 3, except that the pH was 8.0. The inhibitors of anion transfer were present during the incubation with tetrathionate at concentrations of $2.5 \cdot 10^{-4}$ M phloretin, $5 \cdot 10^{-5}$ M dipyridamole or 10^{-2} M phenopyrazone.

known to inhibit the penetration of phosphate, sulfate and other anions [25, 26]. From these results one might conclude that tetrathionate utilizes the anion transfer system. On the other hand, it has recently been suggested [30] that such inhibitors may merely influence in an unspecific fashion the surface potential of the erythrocyte membrane and thus block the access of anions to the membrane.

In further studies dealing with the possible binding site of tetrathionate, the proteins exposed to the outer surface of the membrane were modified by proteolytic enzymes. Treatment of erythrocytes with trypsin has been shown to cleave the sialoglycoproteins, whereas pronase also attacks the 95 000 dalton protein, which is split into a 65 000 and 35 000 dalton component [31]. Table II demonstrates a slightly stim-

TABLE II

INFLUENCE OF PREINCUBATION WITH PROTEOLYTIC ENZYMES ON THE EFFECT OF TETRATHIONATE

Erythrocytes were incubated in Medium A (pH 7.4, 37 °C) with trypsin (1 mg/ml medium, 60 min) or pronase (0.2 mg/ml medium, 30 min), washed four times with Medium A containing 1 % albumin and then exposed to TNBS for 1 h immediately or after further incubation of 3 h in presence of tetrathionate (see Materials and Methods).

Incubation conditions		Phosphatidylethanolamine trinitrophenylated (%)
Enzyme	Exposure to tetrathionate (min)	
Control	0	4.5
Trypsin	0	10.3
Pronase	0	11.9
Control	180	40.7
Trypsin	180	42.2
Pronase	180	42.3

TABLE III

TIME DEPENDENCE AND REVERSIBILITY OF THE TETRATHIONATE EFFECT

Erythrocytes were incubated in Medium A (pH 8.0, 37 °C) with 5 mM tetrathionate in the absence of glucose for the time periods indicated in the left column. After three washings with Medium A, and a further incubation in Medium A (pH 8.0, 37 °C) with or without cysteine, as shown in the right column, the washed cells were exposed to TNBS for 1 h (see Materials and Methods).

	Incubation time (h)		Phosphatidylethanolamine trinitrophenylated (%)
	With tetrathionate	Without tetrathionate	
(a)	1	0	11.2
(b)	2	0	25.4
(c)	3	0	40.1
(d)	4	0	45.9
(e)	1	2	31.6
(f)	1	2 (+ 5 mM cysteine)	18.0
(g)	0	2 (+ 5 mM cysteine)	12.3

ulating effect of the enzyme treatments on the reaction of TNBS with phosphatidylethanolamine. The effect of tetrathionate, however, was not reduced under these conditions.

In the experiments described so far a standard incubation time of 3 h was used. According to the data in Table III (lines a–d), the effect of tetrathionate approached a maximum during this time period. In addition, Table III demonstrates that further incubation (37 °C, 2 h) of the cells without tetrathionate, after 1 h exposure to tetrathionate, goes along with a further increase of the rate of trinitrophenylation of phosphatidylethanolamine, as compared to cells only exposed to tetrathionate for 1 h (line a and e). It is thus likely that the effect of tetrathionate can be separated into two steps: (1) Binding of tetrathionate to the membrane and (2) enhancement of TNBS binding to phosphatidylethanolamine. Table III (lines e–g) finally presents evidence that tetrathionate reacts with SH groups since its effect was

TABLE IV

TEMPERATURE DEPENDENCE OF THE TETRATHIONATE EFFECT

Erythrocytes were incubated in Medium A (pH 8.0, 37 °C) with tetrathionate in the absence of glucose for the time periods and at the temperatures indicated in the left column. After three washings with Medium A the cells were again incubated in Medium A without glucose for the time periods and at the temperatures shown in the right column. After another washing they were exposed to TNBS for 1 h (see Materials and Methods).

	Incubation conditions				Phosphatidylethanolamine trinitrophenylated (%)
	With tetrathionate		Without tetrathionate		
	h	Temperature (°C)	h	Temperature (°C)	
(a)	3	37	0		41.2
(b)	3	20	0		4.0
(c)	3	20	2	37	9.4
(d)	1	37	0		11.2
(e)	1	37	2	37	32.7
(f)	1	37	2	20	10.5

TABLE V

INFLUENCE OF METABOLIC SUBSTRATE ON THE TETRATHIONATE EFFECT

Erythrocytes were exposed to 5 mM tetrathionate for 1 h as described in Table IV, except that in some incubations glucose (11 mM), inosine (5 mM) or pyruvate (10 mM) were present. The incubation temperature was 37 °C.

Incubation		Phosphatidylethanolamine trinitrophenylated (%)
1 h with tetrathionate	2 h without tetrathionate	
No addition	No addition	29.5
Glucose	No addition	8.0
No addition	Glucose	26.3
No addition	Inosine	22.2
No addition	Inosine + pyruvate	17.0

reversible in the presence of cysteine, although the latter itself slightly enhances the labelling of phosphatidylethanolamine.

Changes of temperature considerably influence the effect of tetrathionate. As shown in Table IV, the labelling of phosphatidylethanolamine is almost abolished after lowering of the temperature during tetrathionate treatment from 37 to 20 °C. Moreover, it becomes evident from this table that both steps of the tetrathionate effect are strongly temperature dependent (compare lines a, b and c for the first step and lines d, e and f for the second one).

(b) *Influence of glycolysis on the effect of tetrathionate.* In view of the protective action of glucose against the "reorientation" in the membrane induced by an incubation of 24 h at 37 °C, and by hexanol, we also looked for relationships between glycolytic metabolism and the effect of tetrathionate. As shown in Table V, the

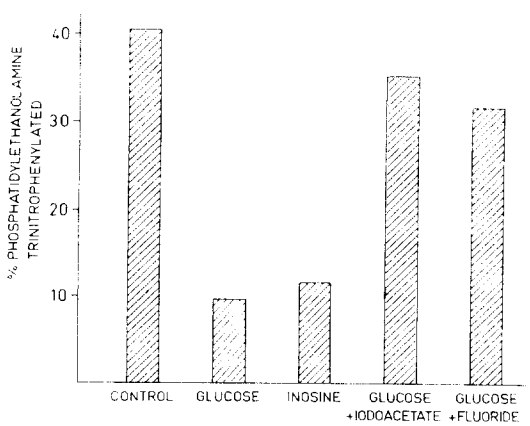

Fig. 5. Dependence of the tetrathionate effect on glycolytic metabolism. Erythrocytes were preincubated for 3 h in Medium A (pH 8.0, 37 °C) containing 5 mM tetrathionate. Concentrations of substrates and inhibitors: Glucose, 11 mM; inosine, 5 mM; sodium iodoacetate, 1 mM; NaF, 10 mM. After washing of the cells they were exposed to TNBS for 1 h (see Materials and Methods).

presence of glucose during the exposition of the cells to tetrathionate almost completely prevented the effect. Consequently, binding of tetrathionate may be regarded as a process somehow related to the metabolic integrity of the erythrocyte. This notion is further borne out by the results in Fig. 5, demonstrating that even during a 3-h preincubation with tetrathionate, glucose as well as inosine almost prevented the tetrathionate effect. The protective effect of glucose could be abolished by inhibitors of glycolysis.

On the other hand, the presence of glucose during a 2-h incubation of the cells without tetrathionate, subsequent to a 1-h tetrathionate treatment (Table V), only slightly reduced the effect of tetrathionate. Inosine, and in particular inosine plus pyruvate, under the same conditions brought about a more pronounced reduction of the effect of tetrathionate. Obviously, inosine plus pyruvate provide the best protection against TNBS binding. This observation may suggest that reduced nicotinamide adenine dinucleotide coenzymes (NADH, NADPH) are not required for the protection, since the levels of the coenzymes fall to extremely low values under these conditions [32, 33].

DISCUSSION

Phosphatidylethanolamine in freshly drawn human erythrocytes reacts slowly and only to a limited extent with an impermeable [34] amino-reactive agent such as TNBS. The results presented in this paper provide new information concerning the structural basis of this phenomenon. As could be demonstrated, incubation of erythrocytes without glucose, either in Medium A for 24 h at 37 °C, or with hexanol for 1–5 h at 37 °C, or with tetrathionate for 1–4 h at 37 °C, strongly enhances the rate of the reaction of phosphatidylethanolamine with TNBS and also increases the percentage of labelled phosphatidylethanolamine. Under all three conditions, this effect is prevented by addition of metabolic substrates such as glucose or inosine.

In the light of the findings presented above it seems likely to us that changes in the accessibility of phosphatidylethanolamine are involved. This concept is further supported by preliminary results demonstrating that under all those conditions, which lead to an increase in the rate of its reaction with TNBS, phosphatidylethanolamine is also attacked more rapidly and to a higher extent by phospholipase A_2 from *Naja Naja*.

As a preliminary interpretation we propose that the increase in accessibility of phosphatidylethanolamine results from changes of its arrangement in the membrane secondary to a perturbation of lipid-protein interactions normally "stabilized" by glycolytic metabolism. A perturbation of lipid-lipid interactions can probably be excluded since neither tetrathionate and *N*-ethylmaleimide, nor alcohols should have an effect on such interactions lasting after removal of the perturbant. A requirement of metabolic energy for the maintenance of a normal phospholipid arrangement has quite recently also been described for chicken and toad erythrocytes [35].

Although different molecular events may be involved, it seems possible that perturbation of the same protein is responsible for the effect observed under our various experimental conditions. Prolonged incubation of erythrocytes without glucose at 37 C in Medium A leads to energetic starvation. This may result in the spontaneous oxidation of membrane protein-SH groups [36] or the cleavage of phosphoproteins [37–39] which may be required for the maintenance of membrane stability.

As concerns tetrathionate, several authors have presented evidence that it reacts with SH groups of enzymes [27, 40, 41]. After initial formation of a sulfenylthiosulfate, disulfide bridges are formed. Such a two-step mechanism would be in line with our observations on the action of tetrathionate on the red cell membrane (cf. Table III): After binding of tetrathionate to a membrane protein as a sulfenylthiosulfate, the slow formation of disulfide bridges might alter the conformation of this protein, thereby release phosphatidylethanolamine from its normal interaction, and render it more accessible towards TNBS.

In contrast, it is not very likely that hexanol directly induces oxidation of SH groups, although an indirect enhancement of spontaneous oxidation processes cannot be excluded. On the other hand, the pretreatment of erythrocytes with hexanol may induce a slowly reversible disturbance of the same lipid-protein interaction affected by tetrathionate or by preincubation at 37 °C for 24 h.

The mechanism by which glycolytic metabolism prevents the protein perturbation induced by tetrathionate or hexanol remains to be elucidated. One has to consider either the energy-dependent maintenance of SH groups in the reduced state or the formation of phosphoproteins as the most likely pathways of energy input into membrane proteins. The data given in Table V may be interpreted in terms of the latter possibility.

Concerning the nature of the protein involved it seems relevant to consider the other known effects of tetrathionate on the erythrocyte membrane, namely inhibition of the facilitated diffusion of glucose [42] and enhancement of cation permeability [43]. Interference with the glucose transfer system can be excluded as explanation on the basis of the observation of Bloch [42] that tetrathionate also binds to this carrier system at 25 °C and in the presence of glucose. Under these conditions we did not find any effect of tetrathionate. The most likely candidate for the interaction with phosphatidylethanolamine would be one of the proteins which span the erythrocyte membrane [2] since, on the one hand, tetrathionate may be assumed to act on the outer surface of the erythrocyte only, whereas, on the other hand, the protection against tetrathionate, which depends on metabolic energy supply, should take place predominantly at the inner-membrane surface. The persistence of the effect of tetrathionate after treatment with pronase, which attacks a large fraction of membrane-spanning proteins [31], may indicate that the reagent interacts with SH groups in the vicinity of the hydrophobic segment of the membrane-spanning proteins.

After exposure of erythrocytes to tetrathionate, up to 65 % of phosphatidylethanolamine can react with TNBS under suitable conditions. This amount is much higher than the 20–30 % of phosphatidylethanolamine supposed to be in the outer half of the lipid bilayer [4, 5]. The following explanations for this apparent inconsistency have to be considered: (1) Part of the phosphatidylethanolamine in the outer half of the bilayer is normally screened by protein and becomes accessible to TNBS only after tetrathionate; (2) TNBS penetrates to the inner leaf of the bilayer after tetrathionate; (3) Phosphatidylethanolamine moves from the inner to the outer layer by a "flip-flop" mechanism as a consequence of tetrathionate-induced conformational changes of a protein which normally keeps phosphatidylethanolamine to the inner layer.

The first possibility seems rather unlikely since it would require that more than 50 % of the phospholipids are present in the outer layer of the red cell membrane.

This argument is based on experiments with phospholipases [5], showing that in the intact erythrocyte a maximum of 50% of the phospholipids, theoretically expected to be in the outer part of the lipid bilayer, is hydrolysed. These 50% however, comprise only 20% of the total phosphatidylethanolamine.

The second possibility cannot be fully excluded at the moment, although it is not very likely to account for the increased labelling in view of the following two reasons: Firstly, we could not demonstrate, by spectrophotometric techniques analogous to those employed by Tarone et al. [44], an enhanced binding at 20 °C of TNBS to hemoglobin in erythrocytes pretreated with tetrathionate. This finding, however, does not exclude the penetration of very small amounts of TNBS to the inner surface of the membrane. Secondly, phosphatidylethanolamine in tetrathionate-pretreated erythrocytes is also hydrolysed, as mentioned above, to a much higher extent by phospholipase A_2, an enzyme most certainly not penetrating to the inner surface of the lipid bilayer. In the light of this latter finding we are presently testing the third possibility, namely a tetrathionate-induced redistribution of phosphatidylethanolamine between the inner and the outer leaf of the membrane lipid bilayer, using various phospholipases as a tool to demonstrate changes in the accessibility of the different phospholipid classes in tetrathionate-treated erythrocytes.

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