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COMPLETE EXCHANGE OF PHOSPHATIDYLCHOLINE FROM INTACT ERYTHROCYTES AFTER PROTEIN CROSSLINKING

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Treatment of human erythrocytes with tetrathionate or diamide resulted in extensive crosslinking of membraneous and cytoskeletal proteins. Such treatment was followed by an incubation with phosphatidylcholine specific exchange protein to investigate the rate and extent of exchange of phosphatidylcholine between the erythrocytes and ^{14}C-labeled phosphatidylcholine containing microsomal membranes or vesicles. Exchange profiles showed that the exchange of phosphatidylcholine is facilitated in treated cells when compared to control erythrocytes and, more importantly, that all of the phosphatidylcholine is exchangeable after protein crosslinking whereas in control cells only the phosphatidylcholine pool located in the outer layer of the membrane is exchangeable. These observations demonstrate that crosslinking of cytoskeletal and membraneous proteins enhances the rate of transbilayer movement of phosphatidylcholine considerably.

The erythrocyte 'envelope' contains two separate structures: a membrane present at the outside containing (phospho)lipids and proteins and underneath this membrane a cytoskeleton composed of filamentous proteins, in particular spectrin. Both layers are thought to be interconnected by ankyrin, a protein which connects the membrane protein band 3 with spectrin, and by a direct interaction of phospholipids present in the internal membrane layer with spectrin (for a review, see Refs. 1, 2). Although detailed knowledge about the nature of the latter interaction is scarce [3,4], it is obvious that structure and function of the membrane are, at least partly, controlled by the cytoskeleton. One characteristic parameter of the erythrocyte membrane seems, in particular, dependent on the cytoskeleton structure: the specific and asymmetric distribution of phospholipids over the two membrane layers. Phosphatidylethanolamine and phosphatidylserine are, respectively, for 80 and 100% present in the inner layer of normal erythrocytes [5]. It has been argued that this specific localization is upset and more aminophospholipids can be detected in the outer layer when the cytoskeleton is modified by: (1) oxidative crosslinking of spectrin with agents like diamide or tetrathionate [4,6], (2) ATP depletion which affects phosphorylation and therefore the structure of spectrin [7,9], (3) intracellular defects as found in sickle cell anaemia, in which severely distorted skeleton structures occur [10,11]. Concurrent with the possible loss in specific phospholipid distribution, an enhancement of lipid transbilayer mobility seems to occur. Mohandas et al. [12] reported an increase in translocation rates of exogeneously added free fatty acids and lysophosphatidylcholine following crosslinking of cytoskeletal proteins. The present report extends this observation to the intact phosphatidylcholine present in the erythrocyte membrane. We have

applied a phosphatidylcholine specific exchange protein to demonstrate that all of the phosphatidylcholine in erythrocytes, modified by crosslinking in their cytoskeleton structure, is exchangeable which strongly suggests that transbilayer movement of this phospholipid is greatly enhanced in these modified cells.

Human erythrocytes collected in standard acid/citrate/dextrose buffer were washed three times with a buffer containing 90 mM KCl, 45 mM NaCl, 44 mM sucrose and 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.4 (referred to as buffer throughout). In order to modify the erythrocyte membrane proteins, cells (hematocrit, 12%) were incubated in the buffer as above but at pH 8.0 for 90 min at 37°C under gentle rotation in

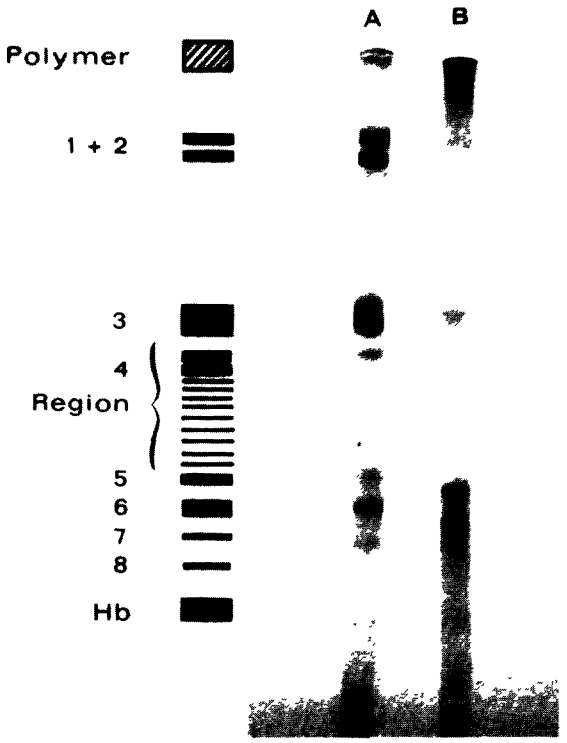

Fig 1 Membrane protein profile of control and tetrathionate treated erythrocytes Cells were treated with tetrathionate (20 mM) in buffer at a hematocrit of 12% during 90 min at 37°C After centrifugation (5 min, 2500×*g*), cells were washed twice with buffer and converted into ghosts following the procedure of Dodge et al [13] The erythrocyte membranes were analyzed for their protein composition following the procedure of Fairbanks et al [14] The gels contained 5% acrylamide, 0 3% bisacrylamide and 0 4% sodium dodecyl sulfate Total length was 11 5 cm Ghosts, 120 nmol of phosphate, were dissolved in 10% sodium dodecyl sulfate, 10mM EDTA, 100 mM Tris and 50% sucrose No disulfide reducing agent was added Electrophoresis was completed in 5 h The figure shows the protein profile of control (a) and tetrathionate-treated (b) cell membranes Proteins are numbered following Fairbanks et al [14]

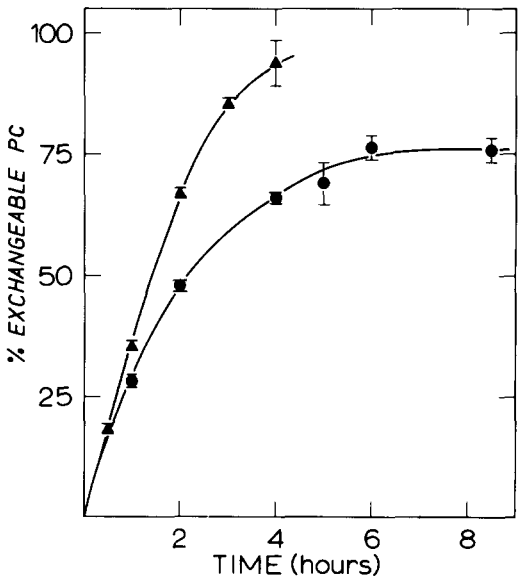

Fig 2 Exchangeability of phosphatidylcholine in normal and tetrathionate-treated erythrocytes Control and tetrathionate treated red cells (100 μl packed cells, 115 nmol phosphatidylcholine) were incubated with microsomal membranes (145 nmol phosphatidylcholine) and 25 μg phosphatidylcholine specific exchange protein in buffer supplemented with 11 mM glucose The final hematocrit value was 33 1/3% and the incubation was carried out at 37°C, with gentle rotation At the time points indicated, aliquots were taken from the mixture, diluted with 6-fold volume of the same buffer and centrifuged at 2500×*g* for 5 min Erythrocytes were isolated this way and washed two times more with buffer to remove residual microsomal membranes The erythrocytes were extracted [16] and lipid extracts were analyzed for total phosphorus content [17], total radioactivity and specific radioactivity of individual phospholipids after two-dimensional thin-layer chromatographic separation of phospholipids [18] Similarly, specific radioactivity of the phosphatidylcholine in the donor microsomal membranes was determined Calculations of the extent of exchange between microsomes and erythrocytes were carried out as described before [15] The data are corrected for the small amount of microsomal material (less than 2% of the total erythrocyte phospholipid) which is not removed by the washing procedures Data for the control experiment (●) and the tetrathionate-treated cells (▲) are expressed as % (±S D $n=2$) of the total amount of phosphatidylcholine present in the erythrocyte membrane

the presence of tetrathionate (20 mM) or diamide (5 mM) [4]. Thereafter, cells were collected by centrifugation and washed twice with buffer. During this and subsequent incubations, hemolysis did not exceed 3%. The result of such treatment is illustrated in Fig. 1. Most of the membrane proteins are crosslinked to such an extent that they do not penetrate into the gel which is used for electrophoresis.

Erythrocytes treated with tetrathionate were studied in detail with respect to the transbilayer mobility of phosphatidylcholine in the membrane. Cells modified as described above are suspended in buffer supplemented with 11 mM glucose. Purified phosphatidylcholine exchange protein from bovine liver was applied to study the exchangeability of phosphatidylcholine in the red cell membranes, essentially as described by Van Meer et al. [15]. Rat liver microsomal membranes, containing radioactively labeled phosphatidylcholine, were used as donor system in the experiments. The protein stimulated exchange of phosphatidylcholine was followed by measuring the increase in radioactive label in the erythrocyte; the results are shown in Fig. 2.

Two differences between control and tetrathionate-treated cells are evident. In agreement with previous results [5], up to 76% of the PC can be exchanged in the native cell, representing the PC pool in the outer monolayer. In treated cells, however, all of the PC appears to be available for exchange. Exchange of phosphatidylcholine is furthermore facilitated after crosslinking of proteins when compared to control cells. Similar data are obtained after diamide treatment. It is shown in the experiment depicted in Fig. 3 that also phosphatidylcholine vesicles can be used as donor system, be it that exchange is somewhat slower when compared to the microsomes. The control experiments carried out throughout all incubations show that the accumulation of radioactive phosphatidylcholine is due only to an exchange process. This is clearly indicated in Fig. 3 where data are presented which could be obtained by the inclusion of a non-exchangeable marker ([^{3}H]glyceroltrioleate) in the donor vesicles. The contamination is low and not increased after diamide and tetrathionate treatment. The conclusions which can be drawn from these data are that in modified cells

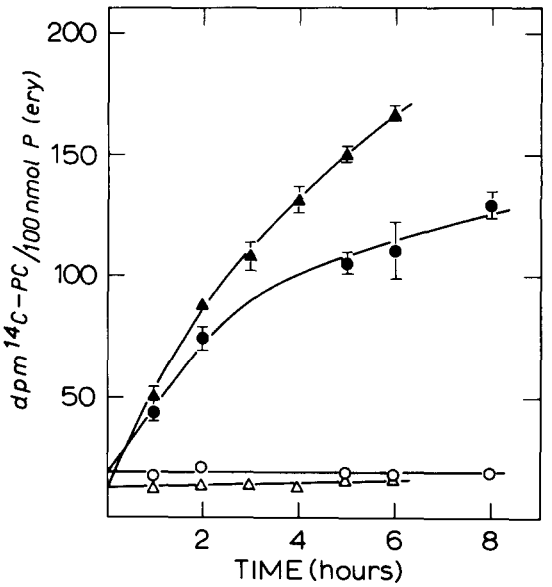

Fig. 3 Exchange of phosphatidylcholine between vesicles and tetrathionate-treated human erythrocytes Control and tetrathionate-treated erythrocytes (100 μl packed cells, 155 nmol phosphatidylcholine) were incubated with unilamellar vesicles (236 nmol of phosphatidylcholine) The vesicles were prepared by ultrasonication as described before [19] and contained equimolar amounts of cholesterol and phospholipid (97 mol% egg phosphatidylcholine + 3% phosphatidic acid) In addition egg[*Me*-^{14}C]phosphatidylcholine and [^{3}H]glyceroltrioleate were present at a concentration of 0 0034 μCi and 0 1 μCi per mol total phospholipid, respectively Buffer system, erythrocyte and exchange protein concentrations, incubation conditions and analyses were as described in Fig 2 [^{3}H]glyceroltrioleate was applied as non-exchangeable marker The amount of ^{3}H present in the erythrocytes after incubation and subsequent washings was determined and the values obtained were used to calculate the amounts of phosphatidylcholine originating from the vesicles and present as contamination in the erythrocyte pellet These data, expressed as specific activities are presented for tetrathionate (△) and control cells (○) The actual amounts of phosphatidylcholine which are exchanged are determined by measuring the ^{14}C accumulated in the tetrathionate-treated (▲) and control erythrocytes (●)

all of the phosphatidylcholine is available for exchange at a higher rate than in control cells. The interpretation of the biphasic exchange profiles obtained with control erythrocytes is that the phosphatidylcholine molecules present in the outer layer are readily exchangeable and that transbilayer movement of phosphatidylcholine is slow ($t_{1/2} = 10.8$ h, Van Meer, G., unpublished data), and rate limiting in the exchange process. Consequently, the abnormal exchange profiles obtained

for modified cells can be explained two ways; either all of the phosphatidylcholine is present in the outer layer, or the rate of transbilayer movement of phosphatidylcholine is enhanced and no longer limits the exchangeability of all of this lipid. The first explanation is highly unlikely. Most of the experiments of Haest et al., in which cells were modified as described above, provided no evidence that the treatment changed the distribution of phosphatidylcholine over the two layers of the membrane (reviewed in Ref. 20). It is furthermore unlikely because all treatments result in a shift of aminophospholipids outward, and a severe delipidation of the inner membrane would result if also all of the phosphatidylcholine should accumulate in the outer layer. It is difficult to envisage that such an unequal distribution of phospholipids between two membrane layers should still result in a stable impermeable membrane. It is more likely therefore, that the crosslinking of proteins results in an increase in the transbilayer movement of membrane phospholipids or of the phosphatidylcholine. Earlier studies of Mohandas et al. [12] indeed provide evidence to substantiate this. It has been shown that the translocation rates of added free fatty acids and lysophosphatidylcholine are enhanced after diamide treatment of the erythrocytes. Based on a variety of experimental results (summarized in Ref. 20) and considering the observation that spectrin does interact preferentially with negatively-charged phospholipids [3], Haest et al. [4,20] suggested that in the erythrocyte membrane phospholipid asymmetry is stabilized by an interaction between aminophospholipids and the cytoskeleton. The present data strongly suggest that this interaction also restricts the transbilayer mobility of the phosphatidylcholine and it may be speculated that the same may be true for the other phospholipids as well. The validity of this hypothesis will be tested by using various modified as well as pathologic erythrocytes.

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