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Involvement of ATP-dependent aminophospholipid translocation in maintaining phospholipid asymmetry in diamide-treated human erythrocytes

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Crosslinking of membrane skeletal proteins such as spectrin by oxidation of their SH-groups can be provoked by treatment of intact erythrocytes with diamide. Shortly after exposure of human erythrocytes to diamide and despite the transverse destabilization of the lipid bilayer that was observed in these cells (Franck, P.F.H., Op den Kamp, J.A.F., Roelofsen, B. and Van Deenen, L.L.M. (1986) Biochim. Biophys. Acta 857, 127–130), no abnormalities could be detected regarding the asymmetric distribution of the phospholipids when probed by either the prothrombinase assay or brief exposure of the cells to a modified phospholipase A_2 with enhanced membrane penetrating capacity. This asymmetry appeared to undergo dramatic changes however, when the ATP content of the cytosol had decreased to less than 10% of its original level during prolonged incubation of the treated cells. These observations indicate that the initial maintenance of phospholipid asymmetry in diamide-treated erythrocytes can be solely ascribed to the action of the ATP-dependent aminophospholipid translocase. This view is supported by experiments involving radiolabeled phospholipids of which trace amounts had been inserted into the outer membrane leaflet of diamide-treated red cells and which still showed a preferential translocation of both aminophospholipids in favour of the inner monolayer, be it that the efficiency of the translocase was found to be impaired when compared to control cells.

Introduction

One of the most intriguing aspects of the well-established asymmetric transbilayer distribution of phospholipids in the (human) red cell membrane is the absolute confinement of phosphatidylserine (PS) to the cytoplasmic leaflet [1,2]. From the numerous studies that have been performed during the last decade to elucidate the mechanism(s) responsible for the maintenance of this asymmetry, the following phenomena seem to be of importance: (i) interactions between membrane skeletal proteins and the aminophospholipids phosphatidylethanolamine (PE) and PS [3–5] and/or (ii) an ATP-dependent translocation of both aminophospholipids from the outer towards the inner monolayer [6,7]. Whether this asymmetry is virtually maintained by either these two mechanisms in concert with each other or just only one of them, appears to be subject to some debate [8,9].

The membrane skeleton largely determines the typical discoid shape and flexibility of the erythrocyte and has also been shown to exert a stabilizing effect on the structure of the plasma membrane of other cell types [10]. Treatment of intact erythrocytes with diamide, under appropriate conditions, has been claimed to cause a rather selective crosslinking of the spectrin molecules in the membrane skeleton by oxidative conversion of their sulfhydryl groups into disulfide bridges [11]. Furthermore, it was observed that diamide-treated erythrocytes exhibited an enhanced accessibility of both aminophospholipids to exogenous phospholipase A_2, suggesting that this treatment has caused a loss of

Abbeviations: RBC, red blood cell; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; ns-LTP, non-specific lipid transfer protein; PC-TP, PC-specific transfer protein. NBD-PS, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylserine; Pal-116-AMPA, N^{ϵ}-palmitoyl-Lys-116 ε-amidinated phospholipase A_2.

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phospholipid asymmetry in their membranes [3]. Since a great variety of entirely independent studies have shown that membrane skeletal proteins, such as spectrin and band 4.1, preferentially interact with PE and – more specifically – with PS [12–20], the most obvious conclusion was that those interactions play an important (if not an exclusive) role in the maintenance of phospholipid asymmetry in the erythrocyte membrane. More recently, however, it has been shown that treatment of intact human red cells with diamide causes a considerable destabilization of the lipid bilayer, as expressed by an enhanced transbilayer mobility of the glycerophospholipids, rather than an in situ alteration in their asymmetric distribution [21]. The latter observation implied that the red cell membrane should be equipped with at least one other system to maintain its (normal) phospholipid asymmetry. This system was supposed to be a very efficient one as it is apparently able to maintain phospholipid asymmetry in diamide treated erythrocytes, despite the marked enhancement in transverse dynamics of glycerophospholipids in the membrane of those cells.

The discovery of an ATP-dependent aminophospholipid-specific translocase in the human erythrocyte membrane [6] brought a possible solution to the problem that was raised by the above studies. This translocase, which has been shown to be present also in the plasma membrane of cell types other than the erythrocyte [22–24], requires hydrolyzable ATP as a source of energy. Furthermore, the high degree of specificity that the system appears to exhibit regarding the chemical structure of the phospholipids which can be accepted for translocation [24], as well as the fact that its activity is (partially) inhibited by SH-group reagents such as *N*-ethylmaleimide and diamide [25–28], strongly suggest that the translocase is a protein. From studies involving diamide as inhibitor of translocase activity [26–28] it did not become clear whether this inhibition had any consequences for the asymmetric distribution of the endogenous phospholipids in the membrane of cells treated with this reagent. Moreover, a serious consequence of such an inhibitory effect of diamide seems to be that the ATP-dependent aminophospholipid translocase could not be held responsible for maintaining phospholipid asymmetry in diamide-treated erythrocytes.

The present studies were undertaken to elucidate this apparent contradiction. Using previously described techniques [7,29], we first studied the inward translocation of radiolabeled species of three long-chain diacylglycerophospholipids inserted into the outer membrane leaflet of intact diamide-treated human red cells. Transbilayer reorientations of endogenous glycerophospholipids, that may eventually occur in diamide-treated erythrocytes as a consequence of ATP-depletion, were detected by brief exposure of the intact cells to Pal-116-AMPA (N^{ϵ}-palmitoyl-Lys-116 ε-amidinated phospholipase A_2), a chemically modified pig pancreatic phospholipase A_2 with superior membrane-penetrating properties [30,31], as well as by using the prothrombinase assay which is known as a very gentle, highly sensitive and specific system to determine the presence of PS in a membrane surface [32]. PC-specific transfer protein (PC-TP) mediated exchangeability of PC in intact cells was determined as a marker for enhanced transbilayer movements of this phospholipid in ATP-containing as well as ATP-depleted diamide-treated erythrocytes.

Materials and Methods

Chemicals. Egg phosphatidylcholine, egg phosphatidic acid, bee venom phospholipase A_2 and diamide (diazinedicarboxylic acid bisdimethylamide) were obtained from Sigma, St. Louis. Cholesterol was purchased from Merck, Darmstadt, F.R.G. 1,2-[^{14}C]-Dioleoylphosphatidylcholine, 1,2-dioleoylphosphatidyl-[^{14}C]serine and 1,2-dioleoylphosphatidyl[^{14}C]ethanolamine were from Amersham International, U.K. Pal-116-AMPA (N^{ϵ}-palmitoyl-Lys-116 ε-amidinated phospholipase A_2, a chemically modified phospholipase A_2 containing a palmitic acid residue at position 116) was synthesized according to the procedure described by Van der Wiele et al. [31] and coagulation factors Xa, Va and prothrombin were isolated and purified as described by Bevers et al. [32]. Chromogenic substrate *H*-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroaniline (S2238) was from Kabi Diagnostica (Stockholm, Sweden). A constant light signal ATP determination kit was from Boehringer, Mannheim. All other chemicals were of analytical grade.

Erythrocytes. After obtaining informed consent, blood from healthy volunteers was collected into acid/citrate/dextrose buffer and used immediately. Erythrocytes were collected by centrifugation (1500 × *g*, 5 min) and washed three times in buffer A (90 mM KCl, 45 mM NaCl, 10 mM glucose, 44 mM sucrose, 30 mM Hepes (pH 7.4), also containing 10^5 IU/l penicillin and 10^5 μg/l streptomycin). The buffy coat was removed after each step.

Diamide treatment of intact erythrocytes. Red cells were resuspended at 10% hematocrit in buffer A, also containing 5 mM diamide. After adjusting the pH to 8.0 the suspension was incubated at 37°C for 40 min. Subsequently, cells were washed twice in buffer A (pH 7.4).

ATP depletion. Erythrocytes were deprived of their ATP by a 22 h incubation period at 37°C in buffer A, not containing glucose.

ATP determination. Diamide-treated and control cells were incubated at 1% hematocrit in buffer A with or without glucose. At timed intervals samples were taken,

diluted 40–80-times in water and kept on ice. The ATP content of the cells was determined by transferring 50 μl of these lysates to 50 μl of a luciferin/luciferase mixture; immediately followed by measuring the light signal in a CA 2000 Tricarb scintillation counter (Packard), using a single photon counting protocol. ATP contents were calculated by comparing the measured light signals with those generated by an ATP calibration solution. Concentrations of ATP as low as $5 \cdot 10^{-9}$ M could easily be detected this way.

Phospholipid donor systems. For monitoring the transbilayer mobility of different phospholipids, vesicles were prepared from equimolar amounts of phospholipid (egg PC and egg PA in the molar ratio of 10 to 1) and cholesterol, with trace amounts of radioactive dioleoyl PC, -PE and -PS. After removal of the solvents under nitrogen, the lipids were dispersed in the following buffer: 280 mM sucrose, 10 mM NaCl, 20 mM glucose, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4) referred to as 'sucrose buffer' throughout, and sonicated (Branson sonifier, microtip, 65 W, 10 min) under nitrogen, while cooled on ice. The suspension was centrifuged at 19000 × *g* for 10 min. The final phospholipid concentration in the vesicle suspension was 0.24 mM, the total radioactivity was 0.16 μCi/ml.

For the PC exchange experiments, [*methyl*-^{14}C]PC-labeled rat liver microsomes, isolated from Wistar rats following the procedure of Kamp and Wirtz [33] were used as the PC donor system. Before use, the microsomes were sonicated for 2 min under nitrogen, using a Branson sonifier (65 W); the mixture was cooled on ice. The suspension was centrifuged for 10 min at 10000 × *g* and used in the exchange assay immediately thereafter.

Lipid transfer proteins. A non-specific lipid transfer protein (ns-LTP) was purified from bovine liver essentially as described in Ref. 29. The protein was dialyzed extensively against sucrose buffer before use. The final protein concentration was 1 mg/ml.

A PC-specific transfer protein (PC-TP) was purified from bovine liver according to Kamp and Wirtz [33]. After overnight dialysis against 300 volumes of buffer A, the volume of the protein solution was reduced by covering the dialysis bag with polyethylene glycol flakes (Aquacide III, Calbiochem) until the desired protein concentration was reached (2–3 μM).

Incorporation of radiolabeled phospholipids and determination of their transbilayer reorientation. Diamide-treated as well as control erythrocytes were incubated at 30% hematocrit for 20 min at 37°C in the presence of donor vesicles and ns-LTP, essentially following the procedure as described in Ref. 7. The molar ratio of erythrocyte phospholipid versus vesicle phospholipid was 27 to 1; 0.6 ml of the ns-LTP solution was added per 1 ml of packed cells. After incorporation of the radiolabeled probe molecules the intact cells were washed twice, resuspended at 5% hematocrit in buffer A and reincubated at 37°C. At timed intervals, samples were taken, the cells pelleted (5 min, 1500 × *g*), resuspended in buffer B (90 M KCl, 45 mM NaCl, 22 mM sucrose, 10 mM glucose, 10 mM $CaCl_2$, 0.25 mM $MgCl_2$ and 30 mM Hepes (pH 7.4)) at 5% hematocrit and incubated for either 5 min with Pal-116-AMPA (60 IU; 125 μg) per 400 μl packed cells) or 60 min with bee venom phospholipase A_2 (70 IU per 400 μl packed cells). Enzyme action was terminated by the addition of 100 μmol EDTA per 400 μl packed cells. Cells were collected by centrifugation (5 min, 1500 × *g*). Lipids were extracted according to Rose and Oklander [34] and phospholipid classes were separated by two-dimensional TLC (silica coated plates from Merck, Darmstadt) using the developing solvents according to Broekhuyse [35]. The specific radioactivity of each phospholipid class was determined as described before [29].

Determination of the transbilayer distribution of endogenous glycerophospholipids. The localization of endogenous phospholipids in diamide-treated red cells was determined by treatment of the intact cells with Pal-116-AMPA or bee venom phospholipase A_2, as described above. The presence of PS in the outer membrane leaflet of these cells was further tested by applying the prothrombinase assay, an assay system involving purified coagulation factors Xa, Va, prothrombin and Ca^{2+}. Full details of this method were published by Bevers et al. [32]. ATP depleted and fresh red cells were treated with diamide and subsequently incubated at 37°C (1.5% hematocrit) in buffer A with or without glucose. Samples were taken at timed intervals and prothrombinase activity was determined using the following coagulation factor concentrations: 3 nM factor Xa, 6 nM factor Va, 4 μM prothrombin and 5.6 mM $CaCl_2$ in isotonic buffer. The final phospholipid concentration was 4.5 μM. All data were corrected for cell lysis as described before [29]. For some samples, prothrombinase activity was also determined in a 750 × *g* (10 min) supernatant of the cell suspension, in order to determine the activity contributed by microvesicles that had been tied off from diamide-treated and/or ATP-depleted cells during prolonged incubations.

Exchangeability of PC in intact red cells. Diamide-treated and control cells, both fresh and ATP-depleted, were incubated at 10% hematocrit in the presence of ^{14}C-labeled microsomes and PC-TP (2–3 μM) at 37°C. The ratio of red cell PC to microsomal PC was 1 to 3. At timed intervals, samples were taken and cells were isolated by centrifugation. After two additional washes to remove microsomal membranes that were still present, lipids were extracted and the specific radioactivity of the PC was determined. The extent of PC exchange was calculated as described by Van Meer and Op den Kamp [36].

Red cell lysis. In all experiments, erythrocyte lysis was determined after the various treatments by measur-

ing the release of hemoglobin from the cells at 418 nm in a 1500 × *g* supernatant (5 min) and comparing this to a completely lysed cell sample. Cell lysis never exceeded 8%.

Results

Transbilayer reorientation of (radiolabeled) phospholipids

The transbilayer migration of exogenously introduced radiolabeled glycerophospholipids from the outer towards the inner membrane leaflet was monitored in diamide-treated as well as in control human erythrocytes (Fig. 1). Because of the limited availability of Pal-116-AMPA, control cells had to be treated mainly with bee venom phospholipase A_2. However, some of the control samples that had been incubated for 21 h (Fig. 1, panel A) were also treated with Pal-116-AMPA to ascertain that under those experimental conditions both phospholipases gave essentially similar results. In diamide-treated cells, the aminophospholipids were hydrolyzed by PAL-116-AMPA to an appreciably higher extent than in control cells by bee venom phospholipase PLA_2. This difference was particularly pronounced during the first couple of hours after the introduction of the probe molecules. Clearly, 5–10 hours after insertion of the radiolabeled PS into the membrane of the diamide-treated erythrocytes, not less than 90–95% of it escaped from the hydrolytic action of this modified PLA_2 (Fig. 1, panel B). These experiments demonstrate that, though its efficiency may have been impaired, particularly for PE, the aminophospholipid translocase is still functionally active, at least during the first hours after treatment of the cells with diamide. Furthermore, it is of interest to note that after prolonged incubation of these cells for up to 20 to 25 h, the fraction of the radiolabeled PS that could be degraded by Pal-116-AMPA appeared to have increased (Fig. 1, panel B). This indicates that during such long incubations and in contrast to what is observed in control cells (Fig. 1, panel A), part of the PS that had been previously translocated into the inner membrane leaflet of the diamide-treated cell appeared again in the outer monolayer.

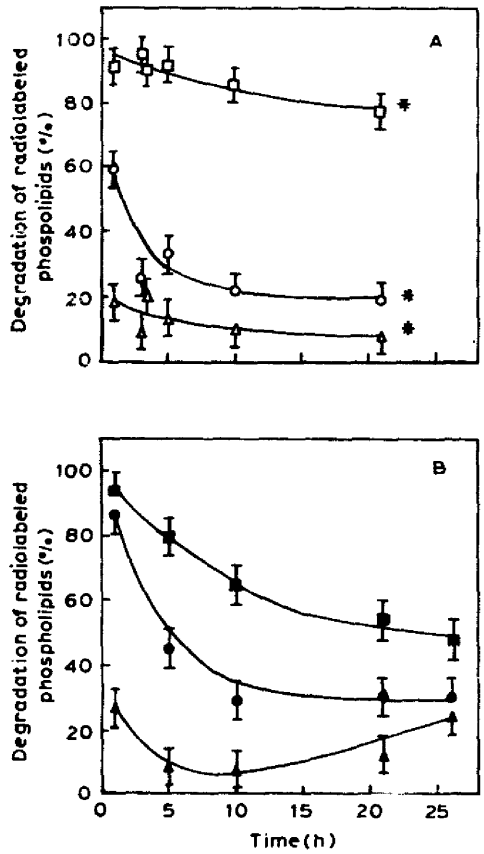

Fig. 1. Translocation of radiolabeled glycerophospholipids in the membrane of control (panel A) and diamide-treated (panel B) human erythrocytes. Ns-LTP mediated insertion of the probe molecules was started at zero time (see Materials and Methods) and the cells were subsequently incubated at 37°C. At the indicated time points, intact cells were treated with phospholipase A_2 from bee venom (panel A) or Pal-116-AMPA (panel B). The percentages of radiolabeled PC (□, ■), PE (○, ●) and PS (△, ▲) that could be degraded this way were assumed to be still present in the outer membrane leaflet. Results shown are the means of eight independent experiments; bars indicate the corresponding S.D. values. (*, including results obtained by treatment of cells with Pal-116-AMPA).

The relatively fast transbilayer equilibration of the radiolabeled PC in diamide-treated erythrocytes (Fig. 1, panel B) is in full agreement with earlier observations by Franck et al. [37], showing a considerable acceleration of PC flip-flop in the membrane of such cells. In contrast to the situation in normal erythrocytes (where the ultimate distribution of the radiolabeled PC closely resembled that of the endogenous PC, i.e., 76% in the outer monolayer (Fig. 1, panel A)), the PC that had been introduced into the diamide-treated cells reached an essentially equal distribution over both halves of the bilayer after more than 25 h (Fig. 1, panel B). The latter indicates that the enhanced hydrolysis of PS and PE observed in these cells cannot be ascribed to cell lysis, as in such a case also the hydrolysis of PC would have increased rather than decreased, relative to the 76% degradation of PC in control cells.

The ultimate distribution of the three radiolabeled phospholipids (Fig. 1) indeed closely resembled the transbilayer distribution of the corresponding endogenous phospholipids, both in case of control as well as of diamide-treated erythrocytes. When the diamide treatment was immediately followed by a brief (5 min) exposure to Pal-116-AMPA, virtually no PS could be hydrolysed whilst PE and PC were degraded by 15% and 70%, respectively (results not shown). Similar results were obtained with control erythrocytes. However, when the treatment of the cells with Pal-116-AMPA was

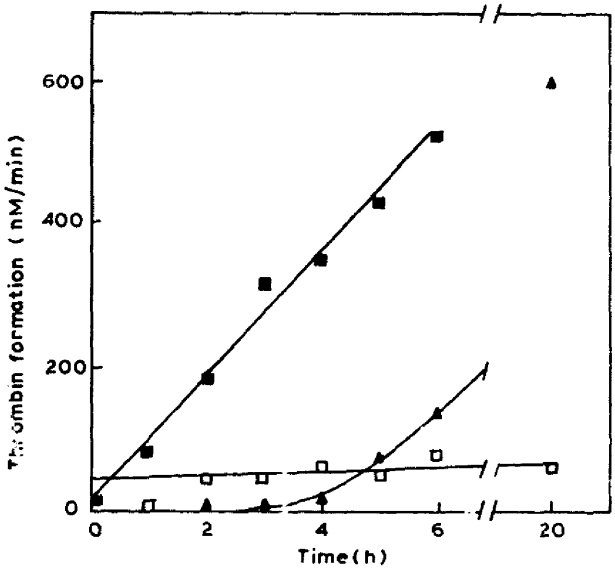

Fig. 2. Prothrombinase activity of diamide-treated fresh (▲) and ATP-depleted (■) human erythrocytes as a function of the time of their incubation at 37 °C. Cells were treated with diamide and subsequently incubated as described under Materials and Methods. Prothrombinase activity of the cells was determined at the time points indicated and all data were corrected for cell lysis. The figure represents the results, including those obtained with ATP-depleted cells that had not been exposed to diamide (□), of a typical experiment.

performed 20 to 25 h after their exposure to diamide, the endogenous phospholipids were degraded under still non-lytic conditions to the following extents: 10% of the PS, 30% of the PE and only 50% of the PC (not shown).

Procoagulant activity of diamide-treated red cells

The prothrombinase assay was used to ascertain beyond doubt that the Pal-116-AMPA mediated hydrolysis of PS that could be observed in erythrocytes some 20 h after their treatment with diamide, indeed represented an alteration in the asymmetric distribution of this phospholipid rather than an artifact induced by the action of this enzyme. In agreement with previous observations [29], neither fresh (not shown) nor ATP-depleted normal human erythrocytes showed any appreciable response in this assay (Fig. 2). Even after incubation of the ATP-depleted cells for up to 24 h at 37 °C, the activity was as low as 59 nM thrombin formed per min, which could be ascribed to microvesicles that had been pinched off from the cells during such long-term incubations. Similarly, procoagulant activity was negligible up to 4 h after treatment of fresh erythrocytes with diamide, but increased slowly during prolonged incubation of these cells at 37 °C (Fig. 2). Contrastingly, procoagulant activity was already observed within 1 h after treatment of ATP-depleted red cells with diamide, prothrombinase activity increasing linearly with time (Fig. 2). No more than only 12% of the total activity that was observed in these samples after 6 h could be ascribed to the contribution of micro-vesicles that had been tied off from the cells during this incubation. Reversing the treatment of the cells, i.e., treatment with diamide followed by ATP-depletion during a 20 h starvation, had a similar effect as to the procoagulant activity of the cells. In a typical experiment, such cells induced the formation of approx. 600 nM thrombin per min under the conditions described in the legend to figure 2, approx. 30% of it originating from membrane vesicles. When compared to the activity of a preparation of cells disrupted by sonication, it could be estimated that the optimal response of the diamide-treated ATP-depleted cells corresponded to the presence of 10–15% of the PS in the outer membrane leaflet of these cells. This correlates very well with the above data regarding the Pal-116-AMPA mediated hydrolysis of both the radiolabeled as well as the endogenous PS in diamide-treated ATP-depleted erythrocytes (Fig. 1).

ATP levels in control and diamide-treated erythrocytes

Cytosolic ATP levels were monitored during incubation of control as well as diamide-treated erythrocytes in buffers with and without glucose (Fig. 3). Clearly, the presence of glucose in the incubation buffer is not sufficient to stabilize the ATP content of diamide-treated cells. In fact, the decline in ATP concentration in diamide-treated cells proceeds about as fast in a glucose containing buffer as it does in the absence of this energy source (Fig. 3). This might be explained by the fact that diamide treatment of the cells also provokes an inhibi-

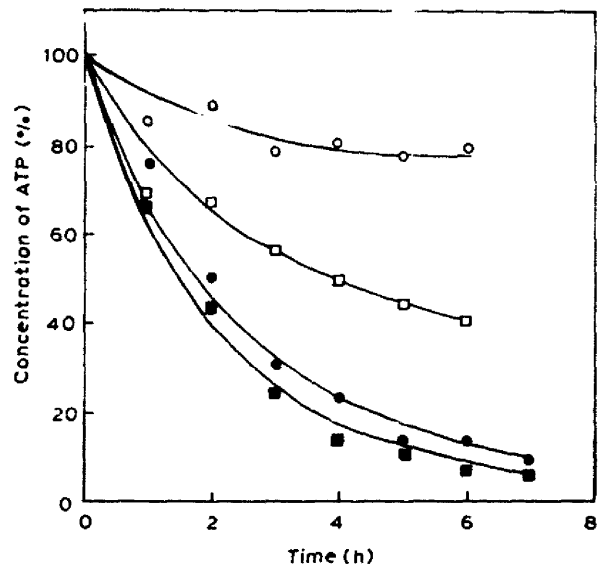

Fig. 3. ATP-content of control (open symbols) and diamide-treated (closed symbols) erythrocytes, as a function of the time of incubation at 37 °C in a buffer with (○, ●) or without (□, ■) glucose. Diamide treatment was completed at zero time. Cellular ATP concentrations are expressed as percentages of those determined in the corresponding cell samples at zero time.

tion of the glucose transporter [38], making it impossible for those molecules to enter the cell and to regenerate ATP. It is also of interest to note that the net ATP consumption in diamide-treated erythrocytes is considerably higher than that in control cells during incubation in a glucose free buffer. The ATP concentration in diamide-treated red cells is reduced to 10% of its original level within 5 to 7 h of incubation at 37°C (Fig. 3). This time period correlates very well with the onset of procoagulant activity in diamide-treated fresh erythrocytes (Fig. 2).

PC-TP mediated exchangeability of PC in intact erythrocytes

The PC-TP mediated exchangeability of the endogenous PC in intact erythrocytes for radiolabeled rat liver microsomal PC was determined to get some insight in the effects that ATP depletion, whether or not combined with diamide treatment, of the cells might have on the transbilayer mobility (flip-flop) of their PC. In full agreement with earlier observations [37], treatment of (fresh) red cells with diamide resulted in an exchangeability of the entire PC complement of the membrane, which, under the present experimental conditions, was completed within 6 h of incubation, such in contrast to the situation in control erythrocytes in which only the outer monolayer pool appeared to participate in this exchange process (Fig. 4, panel A).

Depleting the cell of its ATP caused a considerable increase in the rate at which the outer monolayer pool of PC could be exchanged but, on the other hand, appeared not to affect the relatively slow flip-flop of PC in the membrane as, in the time scale of the experiment,

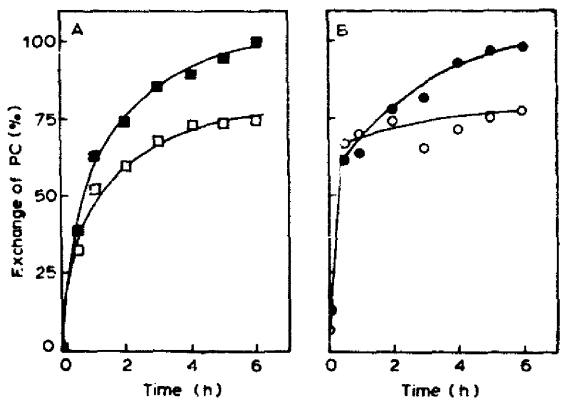

Fig. 4. Exchangeability of PC in control (□, ○) and diamide-treated (■, ●) fresh (panel A) and ATP-depleted (panel B) human erythrocytes. After completion of the diamide treatment at zero time, the cell suspensions were supplemented with PC-TP and [^{14}C]PC containing rat liver microsomes. The extent of exchange, expressed as percent of the total PC present in the erythrocyte, was calculated from the specific radioactivity of the red cell PC as determined at the time points indicated. For further details see Materials and Methods. Data are presented as the mean from five entirely independent experiments.

the exchange remained confined to the PC in the outer monolayer (Fig. 4, panel B). Similarly, as in the case of fresh erythrocytes, diamide treatment of ATP-depleted cells caused a markedly enhanced transbilayer movement of PC, as reflected by the complete exchangeability of this phospholipid (Fig. 4, panel B).

Discussion

An ATP-dependent aminophospholipid-specific translocation system is believed to play an important role in the maintenance of phospholipid asymmetry in the plasma membrane of human erythrocytes [6,7,25–28] as well as other cell types [22–24]. Particularly in situations where the membrane skeleton is no longer able to exert its stabilizing effect on the lipid bilayer, as for instance in deoxygenated sickled erythrocytes [39], it appeared that this translocase manages to maintain an essentially normal phospholipid asymmetry despite the disturbances in the bilayer/membrane skeleton interaction that occur in those cells [29].

Disturbances of these interactions can be achieved also by treatment of intact erythrocytes with diamide [3,11]. This treatment, however, has been reported not only to enhance the accessibility of both aminophospholipids towards exogenously added phospholipases A_2, presumably caused by a considerable destabilization of the lipid bilayer [21], but also to result in a marked inhibition of the aminophospholipid translocase activity [26–28]. The present studies were therefore undertaken to answer the question of how phospholipid asymmetry is maintained in diamide-treated red cells as this treatment drastically perturbs both mechanisms that are responsible for its maintenance and, moreover, also induces an acceleration in the transbilayer movements of lipid molecules [37].

Using an earlier developed technique [7], we studied the inward translocation of exogenously inserted (radiolabeled) dioleoyl-species of three glycerophospholipids in diamide-treated red cells and observed that – at least during the first hours after this treatment – the activity of the translocase was not yet completely inhibited (Fig. 1, panel B). When compared to control cells (Fig. 1, panel A), however, the net rate of translocation of the aminophospholipids toward the inner membrane leaflet appeared to be decreased in diamide-treated cells. In principle, this impairment could have been caused by a 'dilution' effect in the outer monolayer pools, because relatively vast amounts of both aminophospholipids might have been migrated towards the outer leaflet as a consequence of a disruption in their interaction with the membrane skeleton. However, previous studies involving the use of fluorescamine [21], as well as the present study in which both Pal-116-AMPA and the prothrombinase assay have been used as two entirely independent methods, all clearly demonstrated that diamide

treatment of fresh red cells does not result in such changes in the transbilayer orientation of endogenous aminophospholipids. Alternatively, and in agreement with observations by Daleke and Huestis [28] as well as by Connor and Schroit [26,27], the most obvious conclusion seems to be that exposure of intact erythrocytes to diamide caused an inhibition of the translocase which is ascribed to oxidation of its SH-group(s). It should be noted, however, that the extent to which the translocase appeared to be inhibited by diamide treatment is much more pronounced in the study by Connor and Schroit [27] than it is in ours (Fig. 1). Connor and Schroit reported a translocation of no more than 16% of the NBD-PS within one hour after insertion of this PS-analogue in the outer membrane leaflet; the corresponding value for control cells being 81%. We observed in diamide-treated cells, and in spite of a retarded inward translocation, a distribution of at least 90% of the PS in favour of the inner monolayer, which situation is reached after 10 h (Fig. 1B). This is essentially identical to the ultimate distribution of those probe molecules in control erythrocytes (Fig. 1A). Whether this difference could be indicative of NBD-PS being a less favourable target molecule for the translocase than the naturally occurring dioleoyl-species, cannot be answered at present. The lower specificity of the translocase for dioleoyl-PE as compared to that for dioleoyl-PS, already observed in the native erythrocyte (Fig. 1A), is further reduced in the diamide-treated cell, resulting in a less pronounced asymmetry in the transbilayer distribution of this aminophospholipid (Fig. 1B). This situation appeared to be maintained up to 26 h after insertion of the probe molecules into the diamide-treated cells.

The ultimate transbilayer distribution of the radiolabeled PE in the diamide-treated erythrocyte was identical to that of the endogenous PE when probed 20–25 h after this oxidative treatment of the cells. This implies a transbilayer rearrangement of the endogenous PE, as its fraction present in the outer monolayer increased from 20% to 30%. A similar time-dependent reorientation appears to occur for PS in diamide-treated erythrocytes. After the inward translocation of approximately 90% of the PS within the first 10 h, a prolonged incubation of the cells caused part of this PS fraction to reappear in the outer monolayer (Fig. 1B). Similarly, 20–25 h after that treatment with diamide, Pal-116-AMPA degraded approximately 10% of the endogenous PS in the intact cells, whereas shortly after diamide treatment this PS was found to be completely inaccessible to this enzyme. To fully appreciate these observations, it should be remembered that: (i) among the glycerophospholipids present, PS is the most favourite substrate for Pal-116-AMPA; (ii) this modified enzyme has superior membrane-penetrating capacities which, already in the case of normal erythrocytes, allows incubation times in the order of minutes [27] for a complete hydrolysis of the glycerophospholipids present in the outer membrane leaflet of the red cell and (iii) the packing of the lipids in the outer membrane leaflet of diamide-treated erythrocytes is markedly reduced as is evident from the fact that those cells can be attacked by native pig pancreatic phospholipase A_2 [3,40], an enzyme that completely fails to do so in the case of control red cells [41]. Moreover, a considerably decreased packing of the outer monolayer lipids may also be manifest in ATP-depleted erythrocytes, as is suggested by the accelerated PC-TP mediated exchangeability of the PC pool in this membrane leaflet of ATP-depleted cells (Fig. 4, panel B). Hence, in the membrane of diamide-treated, ATP-depleted erythrocytes, all conditions will be fulfilled to enable Pal-116-AMPA to achieve a rapid hydrolysis of all of the PS that might be present in the outer membrane leaflet of such cells. As, judging from the results shown in Fig. 3, incubation of diamide-treated erythrocytes for 10 h at 37°C will cause a virtual complete depletion of their ATP, there will be no doubt that the 90% of the radiolabeled PS in those cells that escaped the hydrolytic action of this particular phospholipase A_2 (Fig. 1B), indeed represents that fraction located in the inner membrane leaflet.

A time-dependent appearance of endogenous PS in the exofacial membrane leaflet of diamide-treated erythrocytes was also noticed when the prothrombinase assay was applied. Intact red cells started to develop procoagulant activity some 4 h after their treatment with diamide (Fig. 2). This phenomenon coincided with a considerably decreased cellular ATP content which had dropped to less than 20% of its original level (Fig. 3). The maximum response in the prothrombinase assay, reached some 20 h after diamide treatment of the red cells, was estimated to correspond to the presence of 10–15% of the PS in the outer membrane leaflet. This figure correlates well with the abovementioned data regarding the hydrolysis of both the radiolabeled as well as the endogenous PS that can be achieved by exposing intact cells to Pal-116-AMPA, some 20–25 h after their treatment with diamide. The apparent discrepancy between the time points at which radiolabeled PS becomes available again in increasing amounts for Pal-AMPA hydrolysis (Fig. 1B) and the onset of procoagulant activity (Fig. 2) in diamide treated cells, might be ascribed to the very high sensitivity of the prothrombinase assay when compared to methods involving phospholipase hydrolyses.

The above observations demonstrate that an alteration in transbilayer distribution of the aminophospholipids in diamide-treated erythrocytes occurs only when this chemical modification of the membrane is accompanied by a complete loss of cellular ATP. Neither ATP-depletion [42], nor diamide treatment alone [21] causes even minor changes in phospholipid asymmetry

in the red cell membrane. It is of interest to note therefore that, even though diamide-treatment of intact cells not only affects the structural organization of the membrane skeleton, but also clearly diminishes the efficiency of the translocase, this system is still capable of maintaining the asymmetric distribution of the (endogenous) phospholipids. A similar situation has been previously observed in deoxygenated reversibly sicklabe cells [29]. As long as the sickled cell contains sufficient amounts of ATP, changes in phospholipid asymmetry will be only marginal, if indeed occurring [29], despite locally broken membrane skeleton-lipid bilayer interactions [39] and a possibly decreased efficiency of the translocase in those cells [43]. The similarity between the previous [29] and present studies also concerns the conclusion that was derived from them, namely that phospholipid asymmetry in the red cell membrane is maintained by both an ATP-dependent translocation of aminophospholipids towards the inner membrane leaflet and their interaction with the membrane skeleton.

In agreement with previous observations [37], it was again found that PC experiences markedly accelerated transbilayer movements in the membrane of diamide-treated erythrocytes as is expressed by the complete PC-TP mediated exchangeability of the inner monolayer pool of PC (Fig. 4), as well as by the accelerated transbilayer reorientation of the inserted radiolabeled PC (Fig. 1) in those cells. In marked contrast to what is observed regarding the aminophospholipids, the rate of PC flip-flop does not seem to be affected by depletion of the cells of their ATP, neither in control, nor in diamide-treated cells (Fig. 4). PC flip-flop remains fast in diamide-treated cells, even after ATP-depletion, which provides another indication that the transbilayer movement of this phospholipid is controlled by a mechanism that is different from that of the aminophospholipids.

It is also of particular interest to note that, unlike what is observed in control erythrocytes (Fig. 1A and Ref. 7), exogenously introduced radiolabeled as well as endogenous PC ultimately reaches an equal distribution within the bilayer. Obviously, this rearrangement of PC over both halves of the membrane bilayer serves to compensate for the ultimate net migration of parts of the inner monolayer pools of either aminophospholipid towards the outer leaflet. In this context, it may be worthwhile recalling that the asymmetric distribution of sphingomyelin in the (human) red cell membrane appeared to be a highly static one, as it is disturbed neither by diamide treatment of normal erythrocytes [3], nor by sickling of reversibly sicklable erythrocytes [44]. Also, sphingomyelin is the only phospholipid of which the asymmetric distribution in the plasma membrane of a proerythroblast is already identical to that in the corresponding mature erythrocyte [45]. Moreover, various independent studies [7,46,47] indicate that its transbilayer mobility must be extremely slow, even when compared to that of PC in the normal erythrocyte. Sphingomyelin does not therefore seem to be a suitable candidate to compensate for any changes in transbilayer orientation of aminophospholipids in the human red cell, and consequently this role has to, and can be, fulfilled by PC, particularly because this phospholipid experiences enhanced transbilayer dynamics in the diamide-treated cell.

It may be assumed that the translocase will not be able to discriminate the exogenous (radiolabeled) dioleoyl-PE from the endogenous PE that is present in the outer membrane leaflet. Furthermore, these probe molecules are inserted in tracer amounts only and will not therefore disturb the mass distribution of PE over both halves of the bilayer to any appreciable extent. Hence, the rate of inward translocation of the radiolabeled PE (Fig. 1) may truely reflect that of the endogenous PE. In other words, the translocase maintains a steady state by continuously translocating PE molecules from the outer towards the inner half of the bilayer [48,49]. Whether the same phenomenon also applies to PS, as suggested by others [48,49], is less clear. Surely, whatever type of probe molecule is used, the ultimate distribution of the exogenously inserted PS never reaches a 100% localization in the inner monolayer [6,7,25–27], although the naturally occurring dioleoyl-PS comes very close to it (Fig. 1A and Ref. 7). On the other hand, it can be deduced from the absolutely negative response that normal (human) erythrocytes exhibit in the prothrombinase assay (see also Refs. 29 and 39), that the outer membrane leaflet contains less than 1% of the endogenous PS. Hence, it may be argued that data which suggest the presence of PS in the exterior leaflet of normal erythrocytes, may be due to some experimental imperfection. The continuous translocation of PE from the outer towards the inner monolayer, however, already infers a continuous consumption of ATP. Because of the fact that this ATP-energized process maintains a steady-state type of situation, it is self-evident that the number of PE molecules that are translocated towards the inner monolayer has to be compensated for by an identical number of PE molecules that 'flop' back to the outer leaflet. This implies that the rate of this outward diffusion is directly related to that of the inward translocation of PE and consequently to the availability of ATP. However, the strict coupling between these processes does not necessarily imply that ATP is also required for the outward diffusion of aminophospholipids as such, as was suggested recently [9,48,49]. Although this back transport is a logical consequence of the inward translocation and therefore indirectly dependent of ATP, there is as yet no experimental evidence that excludes a passive outward diffusion of PE molecules.

One possible argument in favour of the physiological significance of the membrane skeleton in contributing

to the maintenance of phospholipid asymmetry in the red cell membrane could be that it provides a means by which the ATP consumption by the translocase can be kept within acceptable limits. Such an involvement of the membrane skeleton had been ascribed to the interaction of its major component spectrin with aminophospholipids [4,11,12], an interaction which had been demonstrated to occur also in various model systems [13–16]. However, as it was recently shown that such interactions might be (very) weak [50,51], the involvement of spectrin – and thus of the whole membrane skeleton – in maintaining phospholipid asymmetry is highly questioned. On the other hand, it may be argued that even weak interactions may provide, in concert with the translocase, an essential contribution in retaining the aminophospholipids in the inner membrane leaflet. Moreover, experimental evidence is rapidly accumulating recently that PS can also interact with another membrane skeletal protein, namely band 4.1 [17–20]. Nevertheless, it seems to be firmly established that the ATP-dependent translocase plays an important role in maintaining phospholipid asymmetry, a role which may even become essential in those cases where the structural integrity of the membrane skeleton is impaired. Some typical examples in which such defects have been demonstrated to cause a transverse destabilization of the lipid bilayer that do not result in a loss of phospholipid asymmetry are, in addition to the above mentioned diamide-treated normal and sickled [29,39] erythrocytes, hereditary pyropoikilocytes [52] and malaria infected red cells [53].

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