Biochimica et Biophysica Acta 857 (1986) 127–130
Elsevier

BBA 70304

BBA Report

Does diamide treatment of intact human erythrocytes cause a loss of phospholipid asymmetry?

P.F.H. Franck *, J.A.F. Op den Kamp, B. Roelofsen ** and L.L.M. van Deenen

Department of Biochemistry, State University of Utrecht, Padualaan 8, NL-3584 CH Utrecht (The Netherlands)

(Received December 24th, 1985)

Key words: Phospholipid asymmetry; Phospholipase A_2; Fluorescamine; Diamide treatment; (Erythrocyte membrane)

Diamide-treated human erythrocytes have been compared with native red cells as to the accessibility of their amino phospholipids to both phospholipase A_2 hydrolysis and fluorescamine labeling. In agreement with observations by others (Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32), treatment of intact human erythrocytes with diamide resulted in considerably enhanced degradation of amino phospholipids upon subsequent incubation of the cells with bee venom phospholipase A_2. The hydrolysis of phosphatidylethanolamine (PE) in control cells reached a plateau value at 5% after 10 min. In diamide-treated cells, on the other hand, PE hydrolysis did not level off. Contrastingly, dose-response curves recorded for the labeling of PE with the very fast reacting NH_2-group-specific reagent, fluorescamine, showed identical results for both native and diamide-treated erythrocytes. In each of these two cases, a plateau was reached after approx. 15% of the PE had been labeled. These results strongly suggest that the enhanced phospholipase-A_2-induced hydrolysis of amino phospholipids in diamide-treated erythrocytes may reflect a destabilization of the lipid bilayer, rather than an *in situ* loss of phospholipid asymmetry.

The asymmetry in transverse distribution of phospholipids in the human erythrocyte membrane has been well established [1–3]. The two choline-containing phospholipids (sphingomyelin (SM) and phosphatidylcholine (PC)) dominate the outer monolayer, whereas about 80% of the phosphatidylethanolamine (PE) and all of the phosphatidylserine (PS) reside in the inner leaflet of this membrane. One of the intriguing questions that was raised regarding this highly pronounced asymmetry is how this situation is maintained during the lifetime of the erythrocyte.

Haest and co-workers [4,5] have shown that treatment of intact human erythrocytes with either diamide or tetrathionate causes drastic changes in the accessibility of the aminophospholipids for exogenous probes, most specifically phospholipase A_2. For instance, exposure of either diamide- or tetrathionate-treated cells to bee venom phospholipase A_2 resulted in the non-lytic degradation of not less than 50% of the PE and 30% of the PS, whereas in native erythrocytes only 5% of the PE and none of the PS can be digested by this enzyme [4].

Fig. 1 shows that, in agreement with the earlier studies by Haest and co-workers [5,6] we also observed enhanced degradations of amino phos-

* Present address: Laboratory of Clinical Chemistry, Academic Hospital, State University of Leiden, Rijnsburgerweg 10, NL-2333 AA Leiden, The Netherlands.
** To whom correspondence should be addressed.

Abbreviations: SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

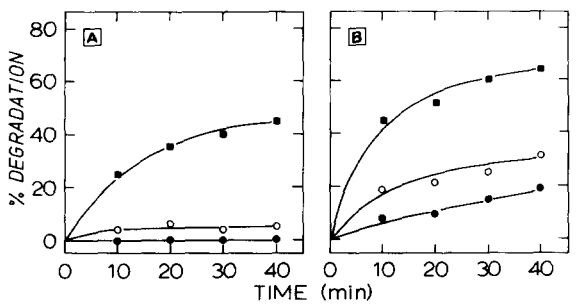

Fig. 1. Non-lytic degradation of phospholipids during incubation of control (A) and diamide-treated (B) erythrocytes with phospholipase A_2. Intact cells were incubated at 37°C as a 10% suspension in the following buffer: 90 mM KCl, 45 mM NaCl, 44 mM sucrose and 10 mM Na_2HPO_4/NaH_2PO_4 (pH 8.0), also containing 5 mM diamide (Calbiochem, San Diego, CA), essentially following the procedure described in Ref. 6. After 40 min of incubation, cells were collected by centrifugation (5 min, 2500 × *g*) and washed twice with the above buffer (pH 7.4) and not containing diamide. Control and diamide-treated erythrocytes were washed once with a buffer consisting of 100 mM KCl, 50 mM NaCl, 10.0 mM $CaCl_2$, 0.25 mM $MgCl_2$, 44 mM sucrose and 10 mM glycylglycine (pH 7.4). (In the context of this buffer, it should be noted that similar results are obtained using 0.25 mM $CaCl_2$, although phospholipid hydrolysis proceeds more slowly.) A 10% suspension of cells in the latter buffer was incubated at 37°C with bee venom phospholipase A_2 (Sigma Chemical Co., St. Louis, MO; 1 IU per ml suspension). Samples (1.5 ml) were taken at the time-points indicated, the cells collected by centrifugation and further action of the phospholipase was terminated by suspending them in 0.5 ml of the phospholipase A_2 incubation buffer, containing 100 mM EDTA. The cells were subsequently lysed by adding at least 20 vol. of a 10 mM EDTA solution, saturated at 4°C with CO_2. After 1 h at 4°C, ghost membranes were collected by centrifugation for 30 min at 2500 × *g* and their lipids were extracted according to Rose and Oklander [19]. The extent of hydrolysis of PC (■), PE (○) and PS (●) was determined after fractionation of the lipids by two-dimensional thin-layer chromatography according to Broekhuyse [20] and phosphate analysis by the method of Rouser et. al. [21]. Haemolysis of the cells, determined by measuring the release of haemoglobin at 418 nm [22] was always less than 3%.

pholipids when diamide-treated intact human erythrocytes are subsequently incubated with bee venom phospholipase A_2. Under our experimental conditions, this enzyme caused considerable hydrolysis of both PE and PS in diamide-treated erythrocytes, reaching values of 30 and 20%, respectively, after 40 min of incubation. It is obvious from Fig. 1B that, at this time point, at which cell lysis is still less than 3%, the degradation of both amino phospholipids has not yet leveled off. Unfortunately, however, lysis increased rapidly when the diamide-treated cells were exposed to the action of the phospholipase A_2 for longer time periods. Treatment of control erythrocytes with the bee venom phospholipase A_2 resulted, under identical conditions, in the hydrolysis of no more than 5% of the PE, which level of degradation was already reached after 10 min of incubation (Fig. 1A). As could be expected [1,2], hydrolysis of PS did not occur in the non-treated cells. Similar results were obtained when phospholipase A_2 concentrations were used that were either 5-times lower or 2-times higher than the one applied in the experiment depicted in Fig. 1.

The most obvious and unequivocal interpretation from the above results seems to be that treatment of intact human erythrocytes with either diamide or tetrathionate results in a considerable loss of phospholipid asymmetry in their membranes [5–7]. Since both these chemical reagents cause an extensive crosslinking of spectrin [5], one of the major components of the membrane skeleton that underlies the cytoplasmic half of the bilayer [3], it was concluded that this protein plays a key role in maintaining the asymmetric phospholipid distribution in the red cell membrane. This view was supported by the observation of enhanced phospholipase-A_2-induced hydrolyses of amino phospholipids in sickled erythrocytes [8], in which the interaction between the membrane skeleton and the lipid bilayer may be lost in those areas of the membrane that are in spicular form [9,10].

The exposure of considerable fractions of the PS in the outer membrane leaflet of the intact erythrocyte should be easily detectable by the prothrombinase assay, which is a specific and sensitive method to probe the presence of PS in a lipid surface [10,15]. However, the response of this system towards either diamide-treated normal human red cells (Zwaal, R.F.A. and Bevers, E.M., personal communication) or deoxygenated (sickled) reversibly sicklable cells [10] is absolutely negative. These observations raised our suspicion against the in situ occurrence of an altered phospholipid distribution, not only in case of the sickled erythrocyte [10], but also in that of the diamide-treated cell.

In this context it is relevant to note that abnormalities in the structure of the membrane skeleton or its interaction with the membrane bilayer – either chemically induced or naturally occurring – invariably result in enhanced transbilayer movements of *glycero*-phospholipids [6,11–14]. Hence, the question may be raised whether the treatment with phospholipase A_2 as such could induce transbilayer rearrangements of substrates in those destabilized membranes. In other words, does the increased hydrolysis of PE and the degradation of part of the PS – as observed by treatment of those cells with phospholipase A_2 during incubations that take at least 60 min – indeed indicate a (partial) loss of phospholipid asymmetry in their membranes, or is it rather an artefact caused by transverse reorientations of *glycero*-phospholipids induced by the action of this enzyme?

To explore this problem further, we studied the localization of PE in native and diamide-treated human red cells, using the NH_2-group-specific reagent fluorescamine as the probe of choice. Compared to phospholipase A_2 treatments of intact erythrocytes, which require prolonged incubations, the reaction between fluorescamine and its target molecules in membranes proceeds very rapidly, i.e., within 1 s [16,17]. This feature of fluorescamine, combined with its rapid aqueous hydrolysis which destroys any excess within 1 min [16–18], makes this reagent extremely suitable for taking a 'snap shot' of the actual localization of PE in those membranes in which *glycero*-phospholipids experience enhanced transbilayer dynamics.

The addition of increasing amounts of fluorescamine to suspensions of erythrocytes resulted in labeling patterns of PE which were similar for both control and diamide-treated cells (Fig. 2). In both cases, the labeling reached a maximum at about 15% of all PE present. This value is in reasonable agreement with the 20% of this phospholipid that can be degraded when intact native erythrocytes are exposed to phospholipase A_2 in the presence of sphingomyelinase C [1,2]. Furthermore, it was observed that the phospholipase A_2 is unable to degrade an additional fraction of the PE in control erythrocytes, previously exposed to a saturating concentration of fluorescamine. This

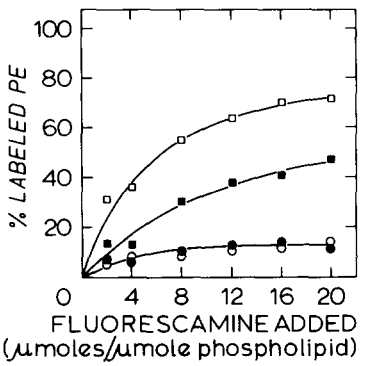

Fig. 2. Dose-response curves for the labeling of phosphatidylethanolamine by fluorescamine. Suspensions (3 ml) of control (●), diamide-treated (○) and sonicated native (■) erythrocytes, as well as sonicated lipid vesicles (□), in a buffer composed of 100 mM KCl, 50 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM $NaHCO_3$ and 20 mM Tricine (pH 8.0) (buffer A), each suspension corresponding to 167 μM of total phospholipid, were added to each of a series of tubes containing a sufficient amount of a stock solution of fluorescamine (Sigma Chemical Co., St. Louis, MO; 114 mM in DMSO/acetone, 1:2.5, v/v) to give final ratios of 2–20 μmol fluorescamine per 1 μmol phospholipid. All mixtures were immediately vortexed for 30 s exactly. The reaction was stopped by adding 7.5 ml of a buffer (B) consisting of 100 mM KCl, 50 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 6 mM glycylglycine and 20 mM Tricine (pH 8.0). Intact and sonicated erythrocytes were collected by centrifugation and lysed in 350 μl of 20 mM glycylglycine and their lipids were extracted and analysed as mentioned in the legend to Fig. 1. Lipids from the sonicated vesicle suspension were recovered using the procedure of Bligh and Dyer [23]. Sonication of erythrocytes (■), as a 50% suspension in buffer A, was performed using a Branson sonifier at 70 W for 2 min. The released haemoglobin was removed by centrifugation at 100000 × *g* for 30 min and two subsequent washes with buffer A. Small unilamellar vesicles of total erythrocyte lipids (□) were prepared by sonication of 5 mg of extracted lipids in 10 ml buffer A, using the same sonifier at 70 W for 15 min. Sonication was performed under a nitrogen atmosphere, while the temperature was kept below 20°C by cooling in ice. Larger lipid aggregates and metal particles released from the sonifier probe were removed by centrifugation for 60 min at 100000 × *g*.

observation indicates that both phospholipase A_2 and fluorescamine react with the same (exterior) pool of PE in the normal erythrocyte. That, under our experimental conditions, the limited extent of labeling of PE in the intact cells could be ascribed to a low reactivity of this phospholipid towards fluorescamine can be excluded, because extensive labeling of PE was observed when the reagent had been added to either erythrocytes disrupted by

sonication or vesicles prepared from total red cell lipids (Fig. 2). These results do not seem to leave much room for any conclusion other than that treatment of normal erythrocytes with diamide does not give rise to a spontaneous translocation of appreciable amounts of PE from the inner to the outer membrane leaflet. Based on the above-mentioned observations by Zwaal and Bevers, the same conclusion seems to hold for PS.

In conclusion, oxidative cross-linking of membrane proteins – most specifically spectrin – by treatment of intact human erythrocytes with diamide does not necessarily result in an immediate loss of phospholipid asymmetry. However, such a structural modification of the membrane skeleton causes a destabilization of the lipid bilayer, as expressed by accelerated flip-flop of endogenous PC molecules [11], which may enable particular reagents – such as phospholipases – to induce transbilayer reorientations of *glycero*-phospholipids. This implies that, in contrast to the native normal erythrocyte on which phospholipases successfully can be used to determine transbilayer phospholipid distributions [1,2], these probes may easily give rise to erroneous results when applied to destabilized membrane bilayers. Finally, it seems worth noting that these implications may not be restricted to those erythrocytes in which the stability of the lipid bilayer has been impaired by chemical means, but may also have consequences for naturally occurring red cells in which enhanced transbilayer phospholipid movements have been demonstrated, as, for instance, the deoxygenated reversibly sicklable cell [8,10,13].

The authors thank Dr. P. van der Schaft for helpful discussions. The present investigations were carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial support from The Netherlands Organization for Advancement of Pure Research (Z.W.O.).

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