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EFFECTS OF PHOSPHOLIPASE A_2 TREATMENT OF HUMAN ERYTHROCYTE MEMBRANES ON THE RATES OF SPECTRIN-ACTIN DISSOCIATION

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This work examines the extent to which alterations in the composition of the phospholipid bilayer of the erythrocyte membrane influences the stability of the association of the 'cytoskeletal network' to the rest of the membrane. Rates of spectrin-actin dissociation at low ionic strength were used as a measure of the stability, and composition of the phospholipid bilayer was altered by the action of the enzyme phospholipase A_2. Hydrolysis of all of the phosphatidylcholine of the outer leaflet of the bilayer had no effect on dissociation rates, whether or not the hydrolysis products were extracted with albumin. Hydrolysis of inner leaflet phospholipids increased the rates by up to 2-fold if the hydrolysis products were not extracted; for $\leq$50% hydrolysis, the rates were unaffected if the hydrolysis products were extracted. The moderate magnitudes of the increases in dissociation rates indicate that interactions between the 'cytoskeletal network' and the phospholipid bilayer are not a decisive factor in maintaining the stability of the membrane, at least under low ionic strength conditions.

The basic molecular organization of the erythrocyte membrane is well understood. The membrane phospholipids are arranged in a bilayer with the several phospholipids asymmetrically distributed between the inner and outer leaflets [2]. The bilayer is penetrated by various 'intrinsic' proteins, chiefly the glycoproteins and the anion transport protein, band 3 [3]. The inner surface of the bilayer is lined by a 'cytoskeletal network' of proteins, composed principally of spectrin and actin. This protein network is linked to the remainder of the membrane through another protein, ankyrin (band 2.1), which in turn is associated with band 3 [4]. The binding of the cytoskeletal network to ankyrin is presumably largely through electrostatic forces, since it depends strongly on ionic strength [5].

This report examines the extent to which the binding of the cytoskeletal network to the rest of the membrane is sensitive to the structure and composition of the phospholipid bilayer. Such sensitivity could arise, for example, through an influence of bilayer characteristics on the conformation of band 3, which might modulate this protein's interactions with ankyrin. Bilayer characteristics could also influence direct interactions, such as those between spectrin and phospholipids proposed by Haest et al. [6].

The rate of dissociation of spectrin and actin from the membrane at low ionic strength was used as a measure of the stability of the association of the cytoskeletal network with the rest of the mem-

Abbreviations and terminology: Membrane proteins are referred to according to Steck (Ref. 1). Phospholipids: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine. Phosphate-buffered saline, 0.15 M NaCl/5 mM sodium phosphate, pH 7.4; NaCl/Na_2EDTA buffer, 0.15 M NaCl/4 mM Na_2EDTA, pH 7.4. PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

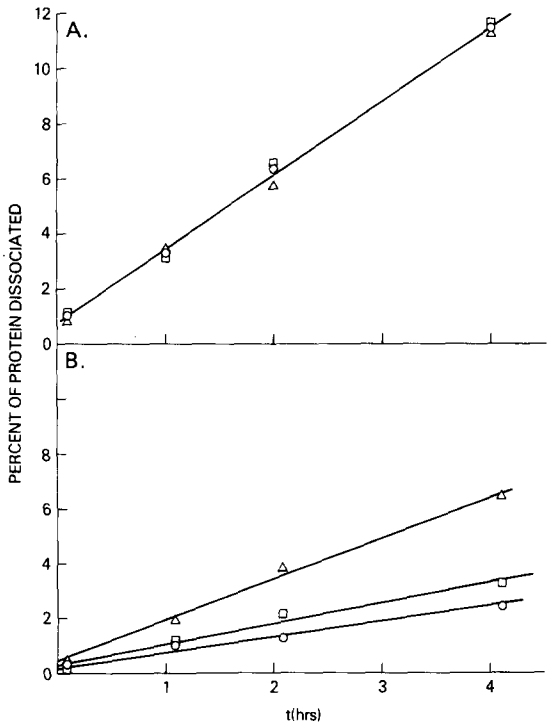

Fig. 1. Time course of spectrin-actin dissociation from erythrocyte membranes at 23–25°C in 1.4 mM sodium phosphate solution, pH = 8.0. ○, Control membranes, not exposed to phospholipase A_2; △, membranes exposed to phospholipase A_2; □, membranes exposed to phospholipase A_2 subsequently extracted with albumin.

Fig. 1A shows results from erythrocyte membranes in which the outer leaflet, comprising 67% of the total membrane PC, was hydrolyzed by exposure of intact erythrocytes to phospholipase A_2 *Naja naja*. Erythrocytes from 1-day-old human blood were washed three times with 10–15 vol. of phosphate-buffered saline. Treatment with enzyme essentially followed that by Zwaal et al. [2], using enzyme directly as obtained from Sigma and incubating the cells with enzyme at an activity of 50 units/ml (supplier's assay) for 1 h at 37°C. Albumin was the 'essentially fatty acid free' bovine product of Sigma, also used directly as obtained; extractions were with 10 vol. of 0.5% albumin in phosphate-buffered saline at 23–25°C for 0.5 h. Cells were washed three times with phosphate-buffered saline following enzyme treatment or albumin extraction. The three cell samples were subject to identical wash, incubation, etc. procedures except for the presence or absence of enzyme or albumin. Cells were converted into 'leaky' ghosts by hemolyis and washing in cold 5 mM sodium phosphate (pH 8). The free movement of spectrin-actin from such ghosts was established in preliminary experiments which showed that measured dissociation rates were unaffected by subsequent ultrasonic fragmentation of the ghosts (model W-350, Heat Systems Ultrasonics, Plainview, NY, setting 7, microtip, 5 s). The dissociation rate measurement was initated by adding 1 ml of the suspension of sedimented ghosts (typically containing about 4 mg of membrane protein) to 9 ml of a 1 mM sodium phosphate (pH 8) solution (which also contained 0.3 mM NaN_3, 0.2 mM Na_2EDTA and 0.002% PMSF). Aliquots of the suspension were withdrawn at appropriate intervals and centrifuged for 30 min at 25000 rev./min at 5°C using the Ti60 rotor of the Beckman L5-65 ultracentrifuge. Supernatants were removed and analyzed for protein on the same day, using the Coomassie blue method [9]. The total amount of cell protein in the suspension was determined by diluting an aliquot with an equal volume of 4% SDS/0.05 M Na_2CO_3 and assaying by the method of Lowry et al. [10]. The percentage of cell protein dissociated was computed on the basis of an observed 40% greater sensitivity of spectrin in the Coomassie blue method than in the method of Lowry. Proteins in the sediment and supernatant were analyzed by polyacrylamide gel electrophoresis, (7.5% SDS gels) using standard techniques [11,12]. The only proteins found in the supernatants were spectrin and actin, in about the same ratios as in the original ghosts. The patterns for the sediments were similar to those for the original ghosts (except for decreased spectrin and actin). Phospholipids were extracted from the ghosts by the isopropanol-$CHCl_3$ method [13], separated by thin-layer chromatography (Applied Science silica gel GF 'Prekotes'; $CHCl_3/CH_3OH$/conc. ammonia soln./H_2O (90:40:4:4, v/v). Phospholipid spots were visualized [14], scraped from the plates and assayed [15]. Lysophosphatidylcholine was only detected in ghosts from cells which had been treated with enzyme without albumin extraction.

Fig. 1B is for membranes in which approx. 15% of the PC and 50% of the PE and PS were hydrolyzed by exposure of the inner surface to pancreatic phospholipase A_2 (used as obtained from Sigma). Source and washing of cells was as described for Fig. 1A. The restriction of the enzyme action to the inner surface was based on its Ca^{2+} requirements, following its entrapment into resealed ghosts. The procedure of Zwaal et al. [2] was used, but with some modifications: cells were hemolyzed in cold 5 mM sodium phosphate (pH 7.4), and washed twice with this buffer followed by addition of phospholipase A_2 in cold NaCl/Na_2 EDTA buffer (here at a concentration of 9 units/ml by supplier's assay), resealing of ghosts in the NaCl/Na_2 EDTA buffer at 37°C for 1 h, removal of the enzyme by washing the resealed ghosts with the same buffer, carrying out the enzymatic reaction by incubating with Ca^{2+}-containing buffer [7] at 23–25°C for 30 min, and removal of Ca^{2+} by washing with NaCl/Na_2 EDTA buffer. The maintenance of the integrity of the membrane during the enzymatic reaction was verified by β-NADH assay [8]. The resealed ghosts were broken open by subjecting them to three freeze (solid CO_2/acetone)-thaw (25°C water bath) cycles; β-NADH assay indicated that 90–120% of the ghosts became unsealed by this procedure. As previously, subsequent ultrasonic fragmentation did not affect measured spectrin-actin dissociation rates. Ghosts were stored at −20°C overnight, and albumin extraction and dissociation measurements done the following day. Other procedures were as described for Fig. 1A, except that individual phospholipids were estimated from the diameters of the thin-layer chromatography spots (estimated accuracy ± 15%). SDS-polyacrylamide gel electrophoresis patterns were similar for control and enzyme-treated membranes, but differed from those of fresh ghosts in the occurrence of a 2–3-fold increase in peak height at the position of band 2.1. This increase took place while the freshly prepared ghosts were incubated at 37°C in NaCl/Na_2 EDTA buffer for resealing, and was not eliminated by the addition of 0.002% PMSF.

brane. The composition of the phospholipid bilayer was altered by the action of the enzyme phospholipase A_2, which hydrolyzes phospholipids to their lyso derivatives and free fatty acid. This enzyme reacts with all erythrocyte membrane phospholipids, except sphingomyelin, and its action can be restricted to a particular leaflet of the bilayer [2]. Since the hydrolysis products ordinarily remain in the membrane, but are extractable by albumin [7], it is possible to examine, in each leaflet, the effects of substituting hydrolysis products for phospholipids and of reducing the total lipid content.

Fig. 1 shows a comparison of the effects of enzymatic hydrolysis of the phospholipids of the outer (Fig. 1A) and inner (Fig. 1B) leaflets of the bilayer. The outer leaflet phospholipids include about equal amounts of PC and sphingomyelin and possibly small amounts of PE [2]. As shown in Fig. 1A, hydrolysis of all the PC of this leaflet has no effect on the spectrin-actin dissociation rates, whether or not the hydrolysis products are extracted from the membrane. Similar results were obtained in two other experiments.

In contrast Fig. 1B shows that hydrolysis of about half the inner leaflet phospholipid (which consists of PC, PE and PS [2]) results in approximately a 70% increase in the protein dissociation rate in the absence of albumin extraction, but that no such increase occurs with such extraction. (The differences between the absolute dissociation rates in Figs. 1A and 1B are not significant; absolute rates were found to vary widely, probably because of uncontrolled differences in the preparation of ghosts.)

Results of varying the extent of hydrolysis of the inner leaflet phospholipids are shown in Table I. The rate of dissociation of spectrin-actin increases with increasing extent of hydrolysis if albumin extraction is not carried out. The effects of albumin extraction are complex, with this extraction preventing the increase in dissociation rate at moderate extents of hydrolysis, but not having this effect when all inner leaflet phospholipid is hydrolyzed. Qualitatively similar results were obtained in two other experiments.

Thus by this criterion the binding of the cytoskeletal proteins to the rest of the membrane is unaffected by substituting the hydrolysis products of PC for this phospholipid in the outer leaflet of the bilayer, or by reducing the total lipid content of this leaflet by approximately half. The dissociation rates of the cytoskeletal proteins are also unaffected when the total lipid content of the inner leaflet is reduced by up to 50%, but are significantly increased when either all lipid is removed from this leaflet or the phospholipids are substituted for by their hydrolysis products.

Speculation on the reasons for the increases in the rates of dissociation of the cytoskeletal proteins following hydrolysis of inner leaflet phospholipids is obviously not presently warranted, but

TABLE I

RATES OF SPECTRIN DISSOCIATION AT VARIOUS EXTENTS OF HYDROLYSIS OF INNER LEAFLET PHOSPHOLIPIDS

Procedures were as described for Fig. 1B. The fraction of inner leaflet phospholipids remaining was computed on the basis that 33% of the PC and all of the PS are in the inner surface. Dissociation rates were computed from least squares regression lines of data similar to that of Fig. 1, and 95% confidence limits for the slopes were computed as described [16].

Enzyme concn. (units/ml)	Dissociation rate (% h^{-1} of total membrane protein ± 95% confidence limit)		% of original inner bilayer surface phospholipids remaining		
	No albumin treatment	Extracted with albumin	PC	PE	PS
0	0.43 ± 0.08	0.36 ± 0.05	100	100	100
1	0.61 ± 0.08	0.42 ± 0.04	80	80	60
10	1.11 ± 0.13	0.44 ± 0.06	50	50	0
50	0.89 ± 0.11	0.86 ± 0.10	0	0	0

two points can be made. First, the fact that (at up to 50% hydrolysis) the increased rates only occur if the hydrolysis products remain in the membrane, suggests that it is the presence per se of these products in the membrane which is responsible and not the removal of phospholipid. This argues against the increased dissociation rates being a result of disruption of direct spectrin-phospholipid interactions. However, it is also possible that extraction of the hydrolysis products results in a reorganization of the membrane phospholipids, conceivably including transbilayer migration. Second, and more important, is the fact that whatever the underlying causes, the increases in protein dissociation rates are not large; the greatest increase found here is about equal to that occurring on lowering the ionic strength from the 1.4 mM used here to 0.9 mM (unpublished observations). Since bilayer alterations as drastic as removal of all inner leaflet lipid do not have a more major influence, it appears that interactions between the bilayer and the cytoskeletal network do not play a decisive role in maintaining membrane stability. These conclusions of course apply only to the low ionic strength conditions of the measurements reported on here. Evaluation of bilayer-cytoskeletal interactions under physiological, higher ionic strength, conditions must await the development of methods for studying such interactions which do not involve dissociation of the cytoskeletal proteins.

References

1 Steck, T.L. (1972) J. Mol. Biol. 66, 295–305
2 Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and Van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83–96
3 Marchesi, V.T. and Furthmayr, H. (1976) Annu. Rev. Biochem. 45, 667–698
4 Branton, D., Cohen, C.M. and Tyler, J. (1981) Cell 24, 24–32
5 Bennett, V. and Branton, D. (1977) J. Biol. Chem. 252, 2753–2763
6 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32
7 Deuticke, B., Grunze, M., Forst, B. and Luetkemeier, P. (1981) J. Membrane Biol. 59, 45–55
8 Steck, T.L. (1974) Methods Membrane Biol. 2, 245–281
9 Bradford, M. (1976) Anal. Biochem. 72, 248–254
10 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
11 Laemmli, U.K. (1970) Nature 227, 680–685
12 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2616
13 Rose, H.G. and Oklander, M. (1965) J. Lipid Res. 6, 128–131
14 Vaskovsky, V.E. and Kostetsky, E.Y. (1968) J. Lipid Res. 9, 396
15 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
16 Natrella, M.G. (1963) Experimental Statistics, pp. 5–19, NBS Handbook 91, U.S. Government Printing Office, Washington, DC