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Release of protein 4.1-rich vesicles from diamide-treated erythrocytes under hydrostatic pressure

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The effect of cross-linking of membrane proteins on vesiculation under high pressure (2.0 kbar) of human erythrocytes was examined. To get the large molecular weight aggregates characterized by cross-linking of cytoskeletal proteins with integral ones, the erythrocytes were pretreated with diamide under pressure (1.0 kbar) where no vesiculation occurs. Vesicles released at 2.0 kbar from such erythrocytes contained protein 4.1 as major membrane protein. Upon reduction of cross-linking by dithiothreitol prior to vesiculation, the released vesicles contained membrane proteins similar to intact cells. On the other hand, in the erythrocyte pretreated with diamide at atmospheric pressure, no such large molecular weight aggregate was observed and the membrane protein composition of the vesicles released from the cells at 2.0 kbar was also similar to that of intact cells. These results suggest that the membrane protein composition of released vesicles is much affected by the properties of cross-linking of membrane proteins in erythrocytes.

Introduction

Vesiculation of human erythrocytes occurs under various conditions; ATP depletion [1], heating to 49–51°C [2], calcium loading [3] and incubation with dimyristoylphosphatidylcholine [4] and amphiphiles [5]. Membrane vesicles produced by these methods contain band 3 as a major membrane protein. In these vesicles, the content of spectrin is poor. Recently, we have reported that membrane vesicles are released from the erythrocytes subjected to high pressures above 1.4 kbar [6]. The pressure-induced vesicles contain membrane proteins such as spectrin, ankyrin, band 3, protein 4.1, protein 4.2 and actin. On the other hand, membrane phospholipid compositions of all these vesicles except for the dimyristoylphosphatidylcholine-induced ones are similar to those of intact erythrocyte membranes [1–6].

From a structural point of view, it is of interest to examine the membrane protein composition of the vesicles released from the erythrocytes in which membrane proteins are cross-linked. Diamide, SH oxidant, is widely used to cross-link cytoskeletal proteins in erythrocytes [7]. In the present work, we describe the properties of the membrane protein composition of vesicles released at 2.0 kbar from diamide-treated erythrocytes.

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Abbreviations: diamide, diazinedicarboxylic acid bis(*N*,*N*′-dimethylamide); PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; I-vesicles, vesicles from intact cells; D-vesicles, vesicles from cells pretreated with 0.5 mM diamide at 1.0 kbar.

Materials and Methods

Materials

Diazinedicarboxylic acid bis(*N*,*N*′-dimethylamide) (diamide) was purchased from Sigma. Phenylmethanesulfonyl fluoride (PMSF) was from Nacalai Tesque. Dithiothreitol (DTT) was from Wako Chemicals. All other chemicals were of reagent grade.

Cross-linking of membrane proteins by diamide

Human blood was purchased from the Fukuoka Red Cross Blood Center. The blood was centrifuged at $750 \times g$ for 10 min at 4°C and the plasma and buffy coat were carefully removed. The erythrocytes were washed three times in 10 mM Tris, 150 mM NaCl (Tris buffer, pH 7.4). To cross-link membrane proteins, intact erythrocytes suspended at 10% hematocrit in Tris buffer (pH 7.4) were incubated with diamide (0.5 or 5 mM) for 30 min at 37°C under atmospheric pressure or 1.0 kbar. The diamide-treated erythrocytes were washed three times in Tris buffer (pH 7.4). To reduce cross-linking of membrane proteins, the diamide-treated ery-

throcytes suspended at 10% hematocrit in Tris buffer (pH 7.4) were incubated with 10 mM DTT for 30 min at 37°C and atmospheric pressure. The erythrocytes were washed three times with Tris buffer (pH 7.4) to remove the excess of DTT.

Analysis of membrane phospholipids and proteins in pressure-induced vesicles

To prepare the vesicles which are used for the analysis of phospholipids, the erythrocytes suspended at 10% hematocrit in Tris buffer (pH 8.0) were incubated for 30 min at 37°C and 2.0 kbar. After decompression, the suspension was centrifuged to remove hemoglobin-rich cells at $750 \times g$ for 5 min at 35°C and then to remove large particles at $3000 \times g$ for 30 min at 4°C. The supernatant was filtered using a white plane Millipore filter of pore size 3 μm. The filtrate was centrifuged at $41\,000 \times g$ for 30 min at 4°C. The pellets were used for the analysis of membrane phospholipids. For the analysis of phospholipids, lipids were extracted from intact erythrocytes and vesicles according to the method of Rose and Oklander [8]. Phospholipids were separated by two-dimensional thin-layer chromatography [9]. Phosphorus of each spot was determined as previously described [6]. For the analysis of membrane proteins in vesicles, vesicles were prepared similarly by using Tris buffer (pH 8.0) or 0.1 M sodium phosphate buffer (pH 8.0) containing 0.1 mM PMSF. Erythrocyte ghosts were prepared according to Dodge et al. [10]. One-dimensional SDS-PAGE of membrane proteins was performed according to the method of Laemmli [11]. Two-dimensional SDS-PAGE was performed as described previously [12].

Results

Cross-linking of membrane proteins by diamide

Cross-linking of membrane proteins in diamide-treated erythrocytes was analyzed using two-dimensional SDS-PAGE. In the erythrocyte treated with 0.5 mM diamide at atmospheric pressure, spectrin oligomers and minor band 3 dimers are observed [12]. With increasing diamide concentration, e.g., at 5 mM diamide, band 3 dimers in addition to spectrin oligomers increased (Fig. 1B) [7]. On the other hand, when the erythrocytes were treated with 0.5 mM diamide at 1.0 kbar, where no vesiculation occurs [6], the electrophoretic pattern was significantly different from that of the membrane treated with diamide at atmospheric pressure, i.e., the large molecular weight aggregates were formed by cross-linking of cytoskeletal proteins with integral proteins, presumably via linking proteins

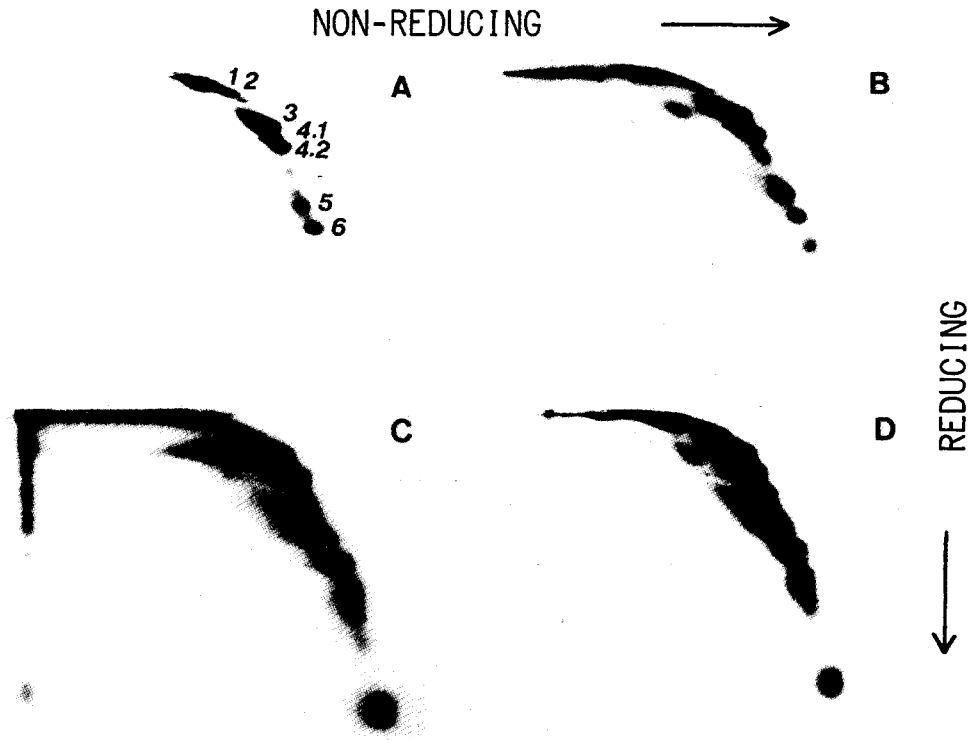

Fig. 1. Two-dimensional SDS-PAGE of membrane proteins in diamide-treated erythrocyte membranes. Ghost membranes were prepared from the erythrocytes treated with diamide for 30 min at 37°C under 1 bar or 1.0 kbar [10]. Ghost membranes were electrophoresed as described previously [12]. (A) Untreated membrane; (B) membrane treated with 5 mM diamide at 1 bar; (C) membrane treated with 0.5 mM diamide at 1.0 kbar; (D) membrane treated with 0.5 mM diamide at 1.0 kbar and then followed with 10 mM DTT at 1 bar.

TABLE I

Phospholipid composition of pressure-induced vesicles

I- and D-vesicles were prepared from intact erythrocytes and cells pretreated with 0.5 mM diamide for 30 min at 37°C and 1.0 kbar, respectively. Values are expressed as mol percent of total phospholipids and as means ± S.D. of three experiments.

	Control ghosts	Pressure-induced vesicles	
		D-vesicles	I-vesicles
Phosphatidylethanolamine	29.8 ± 1.0	31.6 ± 2.7	29.5 ± 2.0
Phosphatidylcholine	30.7 ± 0.2	30.0 ± 3.7	31.3 ± 0.9
Sphingomyelin	25.5 ± 0.6	23.8 ± 5.0	26.3 ± 1.8
Phosphatidylserine	13.0 ± 1.9	13.1 ± 2.2	12.9 ± 3.2

(Fig. 1C). Such large molecular weight aggregates disappeared upon incubation of the diamide-treated erythrocytes with 10 mM DTT (Fig. 1D).

Phospholipid composition of pressure-induced vesicles

When intact erythrocytes are subjected to a pressure of 2.0 kbar, various sizes of vesicles (I-vesicles) are released to the buffer [6]. To examine the effect of cross-linking of membrane proteins on vesiculation under high pressure, the erythrocytes were pretreated with 0.5 mM diamide at 1.0 kbar. Upon compression of the diamide-modified cells, the vesicles (D-vesicles) were released. The membrane phospholipid compositions of I- and D-vesicles were similar to that of intact erythrocyte membrane (Table I).

Membrane protein composition of pressure-induced vesicles

The composition of membrane proteins in I-vesicles is similar to that of intact erythrocyte membrane (Fig. 2A and B) [6]. It is interesting to examine whether the membrane protein composition in pressure-induced vesicles is affected by cross-linking of membrane proteins in mother cells. So, in order to cross-link membrane proteins, the erythrocytes were pretreated with 0.5 mM diamide at 1.0 kbar. The membrane protein composition of the vesicles (D-vesicles) released at 2.0 kbar from such erythrocytes is shown in Fig. 2C. A band corresponding to protein 4.1 was predominantly observed. This band did not change when membrane proteins were solubilized in the absence and presence of 2-mercaptoethanol (data not shown). The clear bands between protein 4.2 and Hb are derived from hemoglobin [13]. To reduce cross-linking of membrane proteins, the diamide-pretreated erythrocytes were treated with 10 mM DTT at atmospheric pressure. The membrane protein composition of the vesicles released from these erythrocytes was similar to that of I-vesicles (Fig. 2D).

A cross-linking reaction of membrane proteins is accelerated under high pressure [12]. So, at atmospheric pressure the erythrocytes were treated with a high concentration of diamide (5 mM). Here, the cross-linking reaction of spectrin or band 3 occurred but the large molecular weight aggregate as shown in Fig. 1B was not formed. Thus, the membrane protein composition of the vesicles released from such erythrocytes was similar to that of I-vesicles (Fig. 2E).

In further experiments, it is of interest to examine the changes in the membrane protein composition of mother cells from which protein 4.1-rich vesicles are released. When the electrophoretic pattern in SDS-PAGE of membrane proteins of the released D-vesicles showed the two bands corresponding to protein 4.1a and protein 4.1b (Fig. 3C), the membrane protein

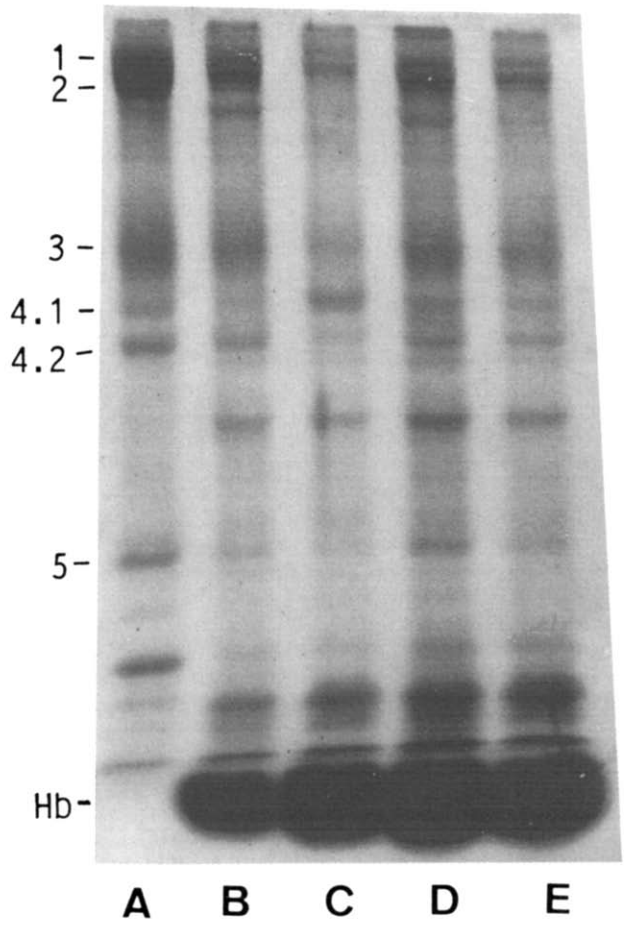

Fig. 2. SDS-PAGE of membrane proteins in released vesicles. Erythrocytes were pretreated with diamide for 30 min at 37°C under 1 bar or 1.0 kbar. An aliquot of the diamide-pretreated cells was incubated with DTT for 30 min at 37°C. To prepare the vesicles, these erythrocytes suspended in Tris buffer (pH 8.0) were subjected to a pressure of 2.0 kbar for 30 min at 37°C. Membrane proteins were solubilized in the presence of 2-mercaptoethanol. (A) Ghost membrane; (B) vesicles from intact cells (I-vesicles); (C) vesicles from cells pretreated with 0.5 mM diamide at 1.0 kbar (D-vesicles); (D) vesicles from cells pretreated with 0.5 mM diamide at 1.0 kbar and then followed with 10 mM DTT at 1 bar; (E) vesicles from cells pretreated with 5 mM diamide at 1 bar. Hb, hemoglobin.

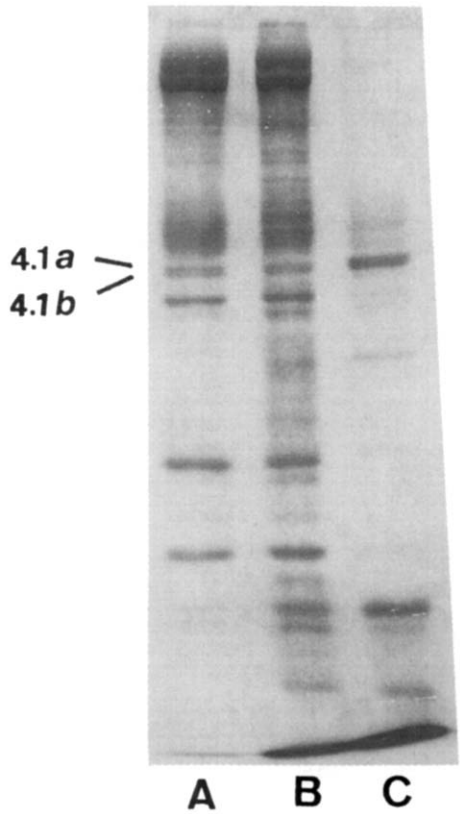

Fig. 3. SDS-PAGE of membrane proteins in D-vesicles and remanent cells. Erythrocyte suspension in Tris buffer (pH 7.4) was pretreated with 0.5 mM diamide for 30 min at 37°C and 1.0 kbar. The diamide-treated cells were suspended in 0.1 M phosphate buffer (pH 8.0) and subjected to a pressure of 2.0 kbar for 30 min at 37°C. Released vesicles (D-vesicles) were separated from remanent cells by centrifugation as described in Materials and Methods. Preparation of ghost membranes from cells was carried out according to Dodge et al. [10]. Membrane proteins were solubilized in the presence of 2-mercaptoethanol. (A) Ghost membrane; (B) remanent cell membrane; (C) D-vesicles.

composition of mother cells was apparently similar to that of intact cells (Fig. 3A and B).

Discussion

The cross-linking reaction of membrane proteins using oxidants is affected by such factors as oxidant concentration, temperature and pressure. As the SH oxidant, diamide has been extensively used to modify cytoskeletal proteins in erythrocytes [7,12–14]. At low diamide concentrations below 0.5 mM, spectrin is mainly cross-linked, whereas at higher concentrations spectrin oligomers and band 3 dimers increase [7,14]. Previously, we demonstrated that a cross-linking reaction of membrane proteins using diamide is facilitated with increasing pressure [12]. In general, chemical reactions accompanied by a negative activation volume are accelerated under high pressure [15]. Furthermore, a specific reaction occurs under high pressure. For instance, in erythrocytes treated with diamide at 1.0 kbar the large molecular weight aggregates are formed (Fig. 1C) [12]. In such aggregates, integral proteins such as band 3 are cross-linked with cytoskeletal proteins via linking proteins such as ankyrin and protein 4.1 [12]. However, no such aggregate is formed in the cells treated with diamide at atmospheric pressure (Fig. 1B). Thus, the application of high pressure to the cross-linking reaction of membrane proteins by oxidants is very useful.

In a previous paper [6], we showed that membrane vesiculation of intact erythrocytes takes place at pressures above 1.4 kbar and the amount of released I-vesicles increases with increasing pressure. Here, to compare I-vesicles with other exovesicles regarding vesicle sizes or compositions of membrane phospholipids and proteins is helpful when considering the mechanism of each vesiculation. Concerning the size of vesicles, I-vesicles are heterogeneous [6] whereas other exovesicles are relatively homogeneous [2–4]. The diameters of vesicles produced by heating [16], amphiphiles [5], ATP depletion [1] and dimyristoylphosphatidylcholine [2] are reported to be 0.42, 0.20, 0.18 and 0.15 μm, respectively. Compared with other exovesicles [1–5], I-vesicles contain a considerable amount of spectrin in addition to other membrane proteins including band 3 and also have a similar composition with respect to membrane proteins irrespective of vesicle size [6]. Hemoglobin content within I-vesicles is decreased [6]. Except for the case of dimyristoylphosphatidylcholine [4], all other vesicles including I- and D-vesicles show the composition similar to intact cells with respect to membrane phospholipids [1–3,6]. The amount of I-vesicles released is associated with the degree of hemolysis. However, membrane vesiculation induced by other methods is not associated with the hemolysis [1–4]. In erythrocytes subjected to a high pressure, parts of spectrin are detached from the membrane [13]. The hemolysis [12,13], vesiculation [6] and detachment of spectrin [13] are considerably suppressed by cross-linking of membrane proteins. Thus, the hemolysis and vesiculation under pressure seem to be associated with the destruction of the cytoskeletal structure. Similarly, in erythrocytes heated at 51°C the cytoskeletal structure is partially destroyed since the spectrin $\alpha\beta$-dimer dissociates into a monomer above 49°C [17]. Thus, vesiculation of red cells occurs. By the treatment of red cells with diamide, the vesiculation temperature is decreased due to the decrease of the heat denaturation temperature of spectrin [18]. These results suggest that the mechanism of membrane vesiculation by high pressure is very different from those by other methods.

All of the vesicles which have been reported contain band 3 as major membrane protein [1–5]. However, in D-vesicles band 3 is very much depleted. As demon-

strated in two-dimensional SDS-PAGE, band 3 is involved in large molecular weight aggregates formed among cytoskeletal proteins by diamide so that band 3 as well as other cytoskeletal proteins such as spectrin and actin also remains in mother cell membranes. Interestingly, protein 4.1 is abundant in D-vesicles. Protein 4.1 is a multifunctional binding protein [19–21]. About 25% of protein 4.1 present at about 200 000 copies per cell is responsible for the linkage of spectrin-actin complexes to the cytoplasmic domain of glycophorin C [20]. Other copies of protein 4.1 seem to be associated with phosphatidylserine [22,23] and integral proteins such as band 3 [21,24] and glycophorin A [25,26]. Thus, protein 4.1 is strongly associated with the cytoplasmic surface of the membrane so that about 80% of protein 4.1 is attached to the membrane, even when spectrin and actin are almost extracted from the membrane using a low ionic strength buffer. As with other membrane proteins, the part of protein 4.1 may be involved in large molecular weight aggregates or cross-linked products formed by the diamide treatment of erythrocyte membranes under high pressure so that it remains in mother cell membranes. The electrophoretic pattern of protein 4.1 in D-vesicles did not change in the absence and presence of 2-mercaptoethanol, suggesting that protein 4.1 which is not cross-linked with other membrane proteins but may interact with phosphatidylserine [22,23] is incorporated into D-vesicles. A similar explanation would be applicable to the membrane protein composition of vesicles released from the erythrocytes pretreated with a high concentration of diamide (5 mM) at atmospheric pressure. Thus, the membrane protein composition of the released vesicles may be affected by the properties of cross-linking of membrane proteins. In addition, the results obtained in the present work suggest that pressure-induced vesicles are not simply fragments of ruptured cell membrane such as obtained by breaking cells in a blender but may result from the perturbation of the interaction between the membrane and cytoskeletal proteins.

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