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Vesicle release from rat red cell ghosts and increased association of cell membrane proteins with cytoskeletons induced by cadmium

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When rat red cell ghosts were incubated with 0.1–0.5 mM $CdCl_2$ in 10 mM Tris-HCl (pH 7.4) at 37°C for 30 min, they became irregular in shape and released small vesicles. The release of vesicles was dependent on the incubation temperature and Cd^{2+} concentration. The maximum release occurred at 37°C in the presence of 0.2 mM Cd^{2+}. The protein composition of Cd^{2+}-induced vesicles was similar to that of the vesicles released from ATP-depleted red cells. Upon incubation with 0.1–0.2 mM Cd^{2+}, more than 90% of the Cd^{2+} added to the incubation buffer was recovered in ghosts and 15–20% of the ghost Cd^{2+} was located on the cytoskeletons prepared by washing ghosts with 0.5% Triton X-100 solution containing 0.1 M KCl and 10 mM Tris-HCl (pH 7.4). Moreover, the cytoskeletons prepared from Cd^{2+}-treated ghosts markedly contained cell membrane proteins, bands 2.1, 3, 4.2 and 4.5, and glycophorins. The association of bands 3 and 4.2 with cytoskeletons increased with increasing concentrations of Cd^{2+} added to the incubation buffer and saturated at 0.2 mM Cd^{2+}. The solubilization of cytoskeletal proteins, bands 1, 2 and 5, from ghosts at low ionic strength was almost completely suppressed by preincubation of ghosts with 0.1 mM Cd^{2+}. $HgCl_2$, $PbCl_2$ and $ZnCl_2$ at 0.2 mM each also produced an increased association of cell membrane proteins with cytoskeletons, whereas $CaCl_2$ and $MgCl_2$ did not.

Introduction

The red cell ghosts prepared by hypotonic lysis can exhibit some of the morphology seen in intact cells. Evidence has been accumulated that in human red cells the interactions of peripheral cytoskeletons with the bilayer membranes, which may be dynamic through band 2.1 and other connecting proteins **, play an important role in maintaining cell shape [1–4]. Through these interactions, the cytoskeletal network composed of bands 1, 2, 5 and 4.1 provides resistance and deformability to the cell membrane and prevents membrane fragmentation [3]. The ghost shape can be altered by a number of agents, such as changes in pH and temperature and various drugs and some of them cause vesicle formation [5–7].

Cadmium is a well-known industrial and environmental toxicant. Anemia has been a common finding in cadmium-administered mammals [8,9] and has been attributed to a cadmium-induced hemolysis or an iron-deficiency [9]. However, the exact mechanism responsible for cadmium-induced anemia is still unclear. Recently, we showed that administration of low doses of Cd^{2+} to rats resulted in an increase in red blood cells with higher density in blood followed by an enhanced

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** The nomenclature for red cell membrane proteins is according to Steck [1].

Abbreviations: PCMB, *p*-chloromercuribenzoic acid; SDS, sodium dodecyl sulfate; PS, phosphatidylserine; PE, phosphatidylethanolamine.

clearance of red blood cells from the circulation [10]. Subsequently, we confirmed that in in vitro experiments Cd^{2+} accelerated age-related changes of red blood cells such as density increment, shape changes, decreased deformability and shortened in vivo survival [11]. It has been shown that sulfhydryl-reactive compounds such as *p*-chloromercuribenzoic acid, *N*-ethylmaleimide and diamide are bound to red cell membranes leading to decreased deformability and shortened in vivo survival of red blood cells [12–14]. Since Cd^{2+} is a sulfhydryl-reactive metal, it is conceivable that Cd^{2+} accelerates age-related changes of red blood cells through interactions with the cell membrane. The aim of the present study is to clarify the effect of Cd^{2+} on red cell ghosts.

In this report, we show that Cd^{2+} at submillimolar concentration induces shape changes of rat red cell ghosts and a vesicle release. We also show that Cd^{2+} mediates an increased association of cell membrane proteins with cytoskeletons.

Materials and Methods

Preparation and incubation of red cell ghosts

Blood samples were obtained from male Jcl:Wistar rats (440–470 g, CLEA-Japan Co., Tokyo) using heparin as anticoagulant. Plasma and the buffy coat were removed by centrifugation and red blood cells were washed three times with 0.95% NaCl solution and hemolyzed by 1:20 dilution with 10 mM Tris-HCl (pH 7.4). The ghosts were pelleted at $22\,000 \times g$ for 20 min at 0°C and washed four times with the same buffer. The washed ghosts were suspended in 5 volumes of the same buffer containing $CdCl_2$ at the concentration indicated subsequently and incubated at 37°C for 30 min.

To estimate the binding of Cd^{2+} to ghost components, Cd^{2+}-treated ghosts were extracted with chloroform/methanol (2:1, v/v) according to the method of Folch et al. [15]. Resulting lipid fraction and residues were used for determination of Cd content.

Separation of vesicles

To collect vesicles released from Cd^{2+}-treated ghosts, the ghost suspension was cooled on ice 30 min after incubation and layered on 20% sucrose solution containing 10 mM Tris-HCl (pH 7.4) and centrifuged at $22\,000 \times g$ for 30 min. The vesicles formed were recovered on the 20% sucrose cushion, while the ghosts sedimented on the bottom of the centrifuge tubes. The vesicle fraction was carefully removed, suspended in 10 mM Tris-HCl (pH 7.4) and pelleted at $105\,000 \times g$ for 30 min.

The vesicles released from ATP-depleted red cells were obtained by the method of Lutz et al. [16].

Preparation of Triton shells

To prepare Triton shells, Cd^{2+}-treated ghosts were recovered by centrifugation at $22\,000 \times g$ for 20 min and washed once with 12 vol. of 10 mM Tris-HCl (pH 7.4). The washed ghosts were mixed with 12 vol. of 0.5% (v/v) Triton X-100 solution containing 0.1 M KCl, 10 mM Tris-HCl (pH 7.4) and 20 μg/ml phenylmethylsulfonyl fluoride (solution A) and stayed at 2°C according to the method of Bennett [17] with a slight modification. After 10 min, Triton shells were pelleted by centrifugation at $22\,000 \times g$ for 20 min, washed twice with the solution A and termed the cytoskeleton.

SDS-polyacrylamide gel electrophoresis of red cell ghosts and cytoskeletons

Electrophoreses in 7.5% polyacrylamide gels containing 0.1% SDS and in 5% polyacrylamide gels containing 0.2% SDS were performed according to the methods of Laemmli [18] and Fairbanks et al. [19] as modified by Steck and Yu [20], respectively. Gels were stained with Coomassie brilliant blue R-250 or periodic acid-Schiff reagent and scanned in a densitometer (Auto Scanner Flur-Vis, Helena Lab. Co., TX., U.S.A.). The area of each peak was integrated with MOP-10 (Kontron, Munich, F.R.G.) and the ratio of each band was calculated.

Release of spectrin and actin from ghosts at low ionic strength

The ghosts treated with either $CdCl_2$, $CaCl_2$ or $MgCl_2$ were washed once with 9 vol. of 0.6 mM Tris-HCl (pH 7.4) and incubated in 9 volumes of the same buffer at 37°C for 30 min. After incubation, the ghost suspension was centrifuged at $105\,000 \times g$ for 30 min and the resulting super-

natant was used for SDS-gel electrophoresis and the determination of protein content.

Other methods

The amount of Cd was determined using atomic absorption spectrophotometer (Hitachi Model 170-50. Hitachi Co., Tokyo). Lipid phosphorus and phospholipid composition were determined as described before [21]. The protein content was estimated by the method of Lowry et al. [22]. The protein content of samples containing Triton X-100 was measured by the method of Sugawara [23]. Microscopic photographs of ghosts and vesicles were obtained in a darkfield microscope (Optiphoto, Nikon, Tokyo). The ghost volume was determined according to the method of Johnson et al. [24]. Spectrin-depleted inside-out vesicles were prepared by the method of Bennett of Branton [25].

Results

Binding of Cd^{2+} to red cell ghosts

When ghosts were incubated with 0.1–0.5 mM Cd^{2+} at 37°C for 30 min, Cd^{2+} bound to ghosts increased almost linearly up to 0.2 mM Cd^{2+} and saturated (Fig. 1). More than 90% of the Cd^{2+} added to the incubation buffer was recovered in ghosts at 0.1–0.2 mM Cd^{2+}, while 48% at 0.5 mM Cd^{2+}. To examine a participation of ghost lipids in the Cd^{2+} binding, the amounts of Cd^{2+} in the lipid and chloroform-methanol extracted residue were determined. The amount of Cd^{2+} of the lipid fraction was less than 3% that of ghosts (Fig. 1). Residual Cd^{2+} was recovered mostly in the unextracted residue. Therefore, a major binding site for Cd^{2+} appears to be protein components of ghosts.

Shape change of ghosts induced by Cd^{2+}

When ghosts are prepared from red blood cells by lysis with 10 mM Tris-HCl buffer, most of them are spheres [26]. During 30-min incubation at 37°C in 10 mM Tris-HCl buffer, most ghosts maintained spherical shapes but a few ones became cup-shaped (Fig. 2A). As shown in Fig. 2B, incubation with 0.2 mM Cd^{2+} caused an increase in the ghosts with irregular shapes and smaller size (control; 5.79 ± 0.38 μm and 0.2 mM Cd^{2+}; 5.15

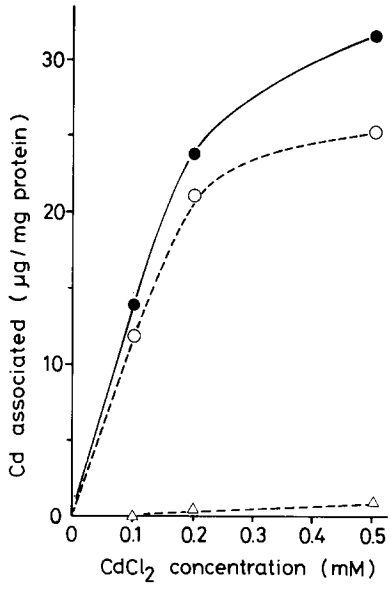

Fig. 1. Binding of Cd^{2+} to red cell ghosts. Red cell ghosts were incubated with 0.1, 0.2 and 0.5 mM $CdCl_2$ for 30 min at 37°C and the lipids were extracted from the Cd^{2+}-treated ghosts. The amounts of Cd^{2+} bound to ghosts (●), extracted lipids (△) and residues (○) were determined as described in Materials and Methods.

± 0.34 μm, $n = 100$, mean ± S.D.). Some of them appeared to be created and small vesicles were seen outside the ghosts. The volume of ghosts also was decreased to 80% that of the control at 0.2 mM Cd^{2+} (data not shown). The vesicles recovered on 20% sucrose cushion by centrifugation were rather uniform population of spheres with a diameter of less than 1 μm (Fig. 2C).

Vesicle release from ghosts induced by Cd^{2+}

The release of vesicles induced by Cd^{2+} proceeded rapidly within 30 min at 37°C and then continued at a slower rate for at least 90 min (Fig. 3A). The vesicle release was dependent on the incubation temperature and the maximum release was observed at 37°C (Fig. 3B). The release of vesicles was also dependent on the concentration of the Cd^{2+} added to the incubation buffer and reached a maximum at 0.2 mM (Fig. 3C). Above 0.2 mM the release was drastically suppressed. When the vesicles released from Cd^{2+}-treated ghosts were incubated with untreated ghosts in the presence of 0.5 mM Cd^{2+} and centrifuged on 20%

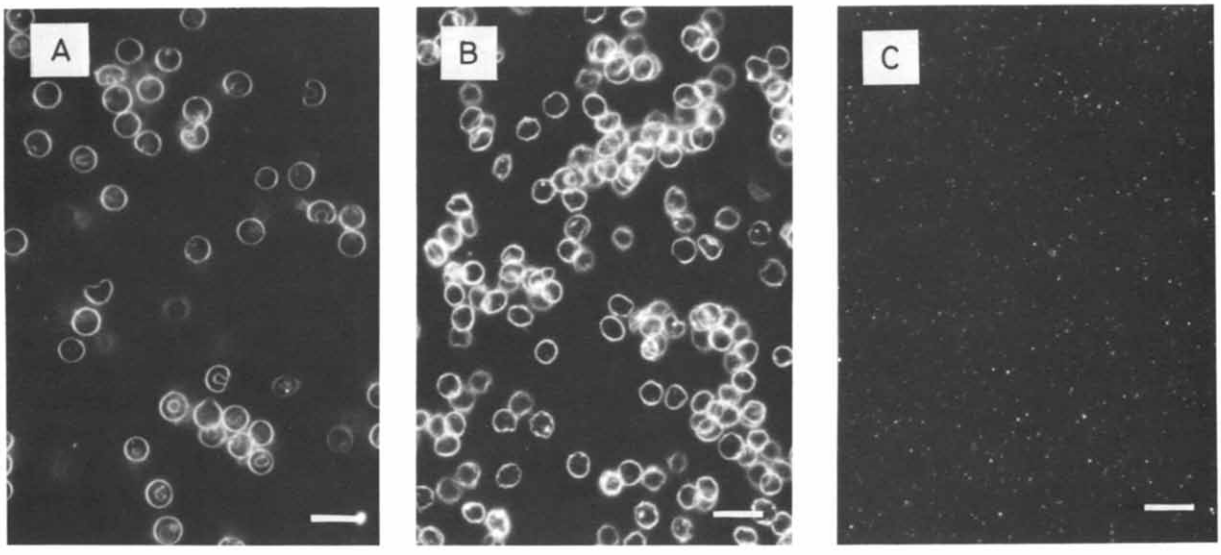

Fig. 2. Effect of Cd^{2+} on the shape of ghosts. Ghosts treated with $CdCl_2$ and the vesicles formed by Cd^{2+}-treatment were photographed in a dark-field microscopy as described in Materials and Methods. (A) Ghosts incubated without $CdCl_2$. (B) Ghosts incubated with 0.2 mM $CdCl_2$. (C) Vesicles from the ghosts incubated with 0.2 mM $CdCl_2$. Scale bar, 10 μm.

sucrose cushion, 80% of vesicles cosedimented with ghosts on the bottom of the centrifuge tube (data not shown). Therefore, the decrease in vesicle release at higher Cd^{2+} concentrations is not necessarily attributed to inhibition of vesicle formation.

Table I shows the chemical composition of ghosts and recovered vesicles. The vesicles contained less PE and PS, innerleaflet phospholipids of cell membranes, than the ghosts, whereas the vesicles released from ATP-depleted red cells

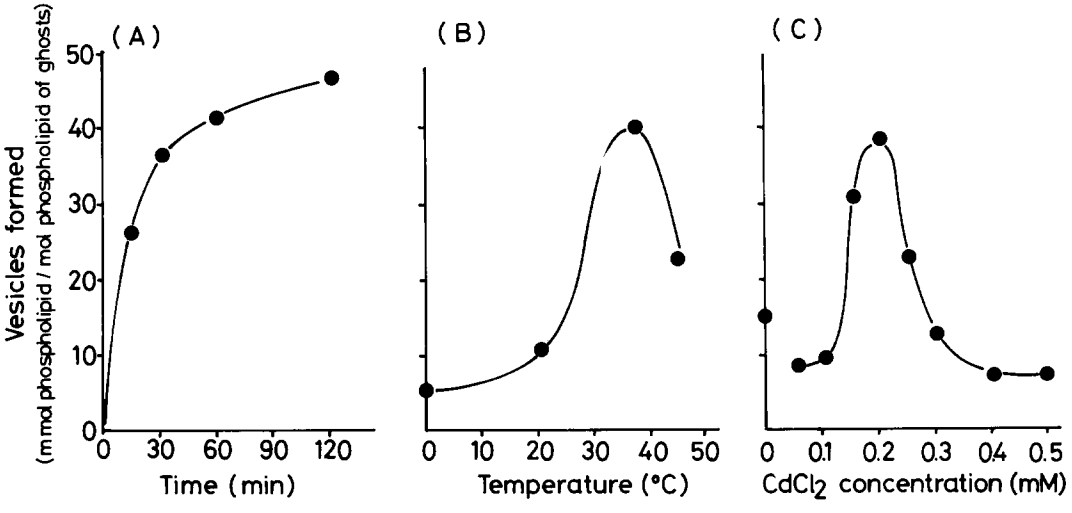

Fig. 3. Time-course and effects of temperature and Cd^{2+} concentration on the vesicle release. (A) Time-course of vesicle release. Ghosts were incubated with 0.2 mM $CdCl_2$ at 37°C and at intervals the formed vesicles were recovered and analyzed as described in Materials and Methods. (B) Effect of temperature on vesicle release. Ghosts were incubated with 0.2 mM $CdCl_2$ for 30 min at 0, 20, 37 and 45°C. (C) Effect of Cd^{2+} concentration on vesicle release. Ghosts were incubated for 30 min at 37°C with 0.1, 0.15, 0.2, 0.25, 0.3, 0.4 and 0.5 mM $CdCl_2$.

TABLE I

COMPOSITION OF RED CELL GHOSTS AND VESICLES

Values are the mean of duplicate.

	Protein/phospholipid (mg/μmol)	Phospholipid composition [a] (%)					
		PC	PE	PS	SM	PI	LPC
Ghost	1.31	44.9	24.8	12.1	11.1	4.8	2.3
Vesicle (Cd^{2+})	0.30	47.9	21.9	7.5	15.3	4.4	3.0
Vesicle (ATP depletion)	3.40 [b]	44.7	24.0	12.8	11.3	4.5	2.6

[a] PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; PI, phosphatidylinositol; LPC, lysophosphatidylcholine.

[b] The vesicles contain hemoglobin (2.12 mg/μmol phospholipid) and other soluble proteins.

showed the phospholipid composition similar to that of ghosts. The ratio of protein to phospholipid was 0.23-fold that of ghosts. The Cd^{2+}-induced vesicles showed the protein composition similar to that of vesicles from ATP-depleted red cells (Fig. 4).

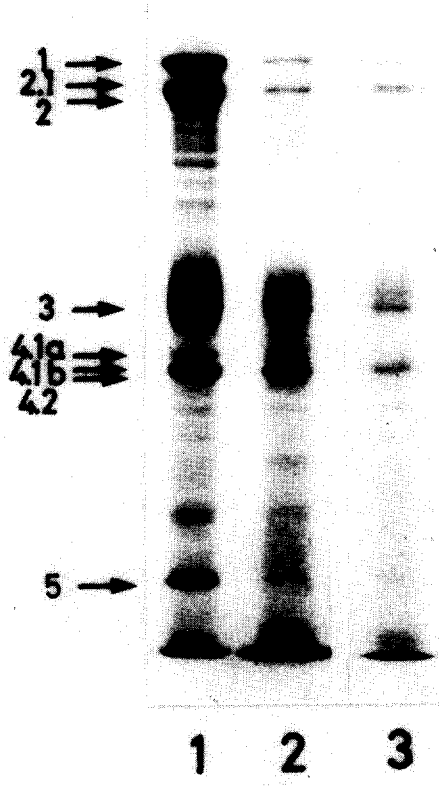

Fig. 4. Electrophoretogram of ghosts and vesicles. Untreated ghosts (lane 1), vesicles from ATP-depleted red cells (lane 2) and vesicles from Cd^{2+}-treated ghosts (lane 3) were analyzed according to the method of Laemmli [18].

Decreased solubilization of cytoskeletal proteins at low ionic strength produced by Cd^{2+}

When ghosts are incubated in 0.3 mM sodium phosphate buffer, bands 1, 2 and 5, major components of the cytoskeletal network, are preferentially solubilized [25]. To examine whether Cd^{2+} mediates interactions of ghost protein each other, the Cd^{2+}-loaded ghosts were incubated at low ionic strength. In stead of phosphate buffer, 0.6 mM Tris-HCl (pH 7.4) was used as incubation buffer to avoid a possible interaction between phosphate and Cd^{2+}. The proteins solubilized from untreated ghosts consisted mainly of bands 1, 2 and 5 (Figs. 6A and 6B, lane 7). The amount of solubilized proteins decreased dependent on the Cd^{2+} concentration used for incubation (Fig. 5). At 0.1 mM Cd^{2+} the amount of solubilized proteins decreased to almost a minimum. $CaCl_2$ and $MgCl_2$ also decreased a solubilization of proteins. The concentrations of Cd^{2+}, Ca^{2+} and Mg^{2+} to achieve a 50% inhibition of solubilization were 50, 180 and 200 μM, respectively. These results show that whether Cd^{2+} mediates an association of cell membrane proteins with cytoskeletons or Cd^{2+} crosslinks cytoskeletal proteins each other leading to decreased solubilization of cytoskeletal proteins at low ionic strength.

Association of cell membrane proteins with cytoskeletons produced by Cd^{2+}

The cytoskeletons prepared by washing ghosts

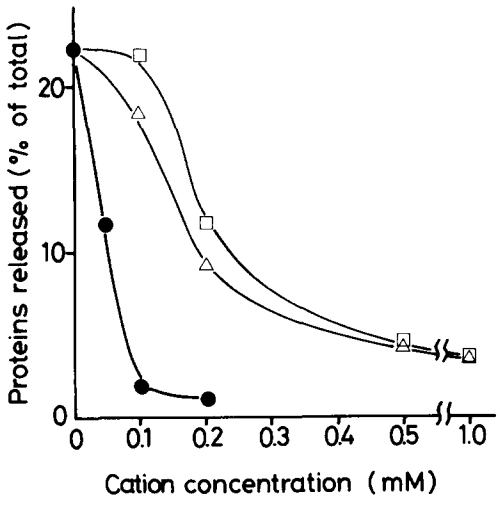

Fig. 5. Release of cytoskeletal proteins at low ionic strength from ghosts incubated with either Cd^{2+}, Mg^{2+} or Ca^{2+}. The ghosts incubated either with Cd^{2+} (●), Mg^{2+} (□) or Ca^{2+} (△) for 30 min at 37°C were treated with 0.6 mM Tris-HCl (pH 7.4) and the released proteins were analyzed as described in Materials and Methods. The amounts of released proteins were expressed as percentages of the total protein in the incubation mixtures.

with a Triton X-100 solution almost depleted bands 3, 4.2 and 4.5 (Figs. 6A and 6B, lane 2). Band 2.1 was extracted partially from ghosts. This protein like that of human red cells remained largely to be associated with spectrin-depleted inside-out vesicles (Figs. 6A and 6B, lane 6). Human red cell band 2.1 as ankyrin is found within band 2 in Laemmli's gel system [17], while it is found just below band 2 in Fairbanks' gel system [18]. Rat red cell band 2.1 migrated in a similar fashion in Fairbanks' gel system (Fig. 6A, lane 6), although it migrated between bands 1 and 2 in Laemmli's gel system (Fig. 6B, lane 6). The cytoskeletons prepared from Cd^{2+}-treated ghosts clearly retained cell membrane proteins, bands 2.1, 3, 4.2 and 4.5 (Figs. 6A and 6B, lanes 3 to 5). Periodic acid-Shiff reagent-reactive proteins (glycophorins) also were increased by Cd^{2+}-treatment in cytoskeletons (Fig. 7). The amount of cell membrane proteins associated with cytoskeletons appeared to increase dependent on the Cd^{2+} concentration. Since the addition of 2-mercaptoethanol to cytoskeletons did not affect electrophoretic patterns, no covalent-disulfide bond was formed during incubation with Cd^{2+} (data not shown).

Fig. 8 shows the effect of Cd^{2+} concentration on the association of bands 3 and 4.2 with cytoskeletons. The association of bands 3 and 4.2 saturated at 0.2 and 0.1 mM Cd^{2+}, respectively. The maximum association of bands 3 and 4.2 was

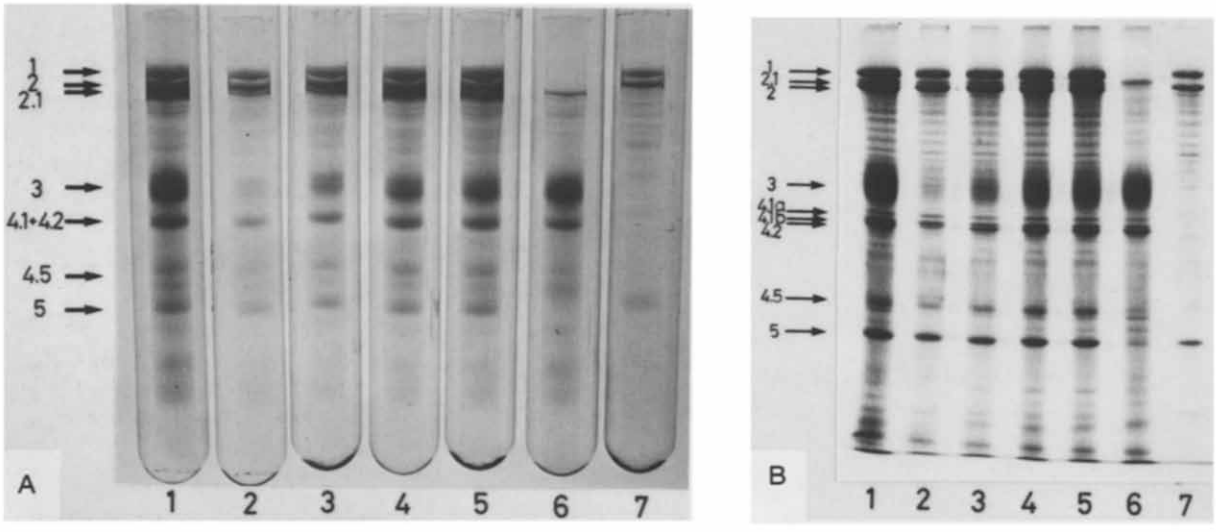

Fig. 6. Electrophoretogram of ghosts and the cytoskeletons prepared from Cd^{2+}-treated ghosts. Electrophoresis was performed according to the methods of Fairbanks et al. [19] (A) and Laemmli [18] (B). Untreated ghosts (lane 1), cytoskeletons prepared from the ghosts incubated with 0 (lane 2), 0.1 (lane 3), 0.2 (lane 4) and 0.5 mM $CdCl_2$ (lane 5) for 30 min at 37°C, spectrin-depleted inside-out vesicles (lane 6) and the proteins solubilized from ghosts at low ionic strength (lane 7).

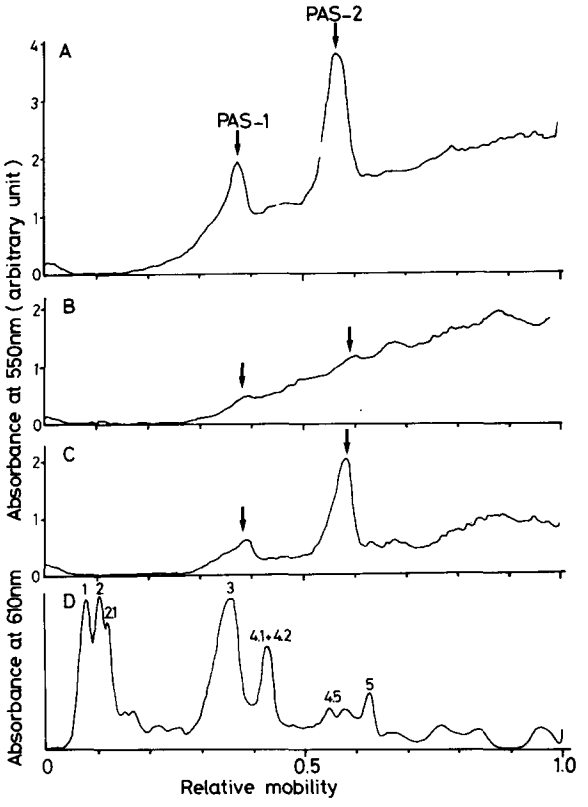

Fig. 7. Densitometric patterns of periodic acid-Schiff reagent-stained gels of ghosts and the cytoskeletons prepared from Cd^{2+}-treated ghosts. Electrophoresis was performed according to the method of Fairbanks et al. [19]. Untreated ghosts (A) and cytoskeletons prepared from the ghosts incubated with 0 (B) and 0.2 mM $CdCl_2$ (C) for 30 min at 37°C. Densitometric pattern of Coomassie-blue stained gel of untreated ghosts (D) was also presented.

74% and 100% of those present in the ghosts, respectively. Concomitantly, the amounts of band 3 and 4.2 extracted with Triton X-100 decreased. On the other hand, phospholipids were extracted to a similar extent in the presence of Cd^{2+} (data not shown). Cd^{2+} also was bound to cytoskeletons almost in proportion to an association of band 3 (Fig. 8). It can be concluded that Cd^{2+} mediated an association of cell membrane proteins with cytoskeletal components.

Effects of divalent cations and sulfhydryl-reactive compounds on association of cell membrane proteins with cytoskeletons

Divalent cations, $HgCl_2$, $PbCl_2$ and $ZnCl_2$ at

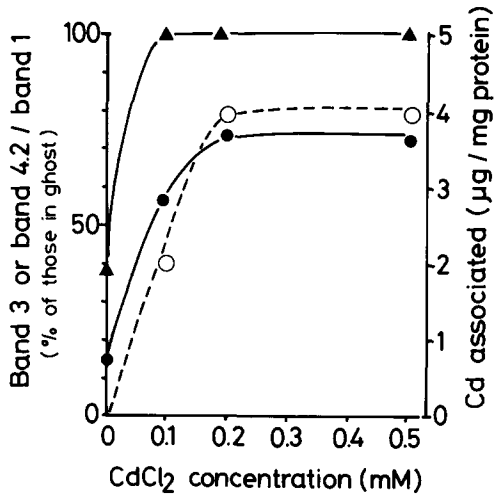

Fig. 8. Effect of Cd^{2+} concentration on the association of bands 3 and 4.2 with cytoskeletons. The protein composition of cytoskeletons prepared from Cd^{2+}-treated ghosts was analyzed by SDS-polyacrylamide gel electrophoresis and densitometry as described in Materials and Methods. The amounts of bands 3 (●) and 4.2 (▲) in the cytoskeletons were calculated as the ratio of their peak areas to that of band 1 and expressed as percentages of the ratio in untreated ghosts. The amount of Cd^{2+} bound to cytoskeletons (○) was also presented.

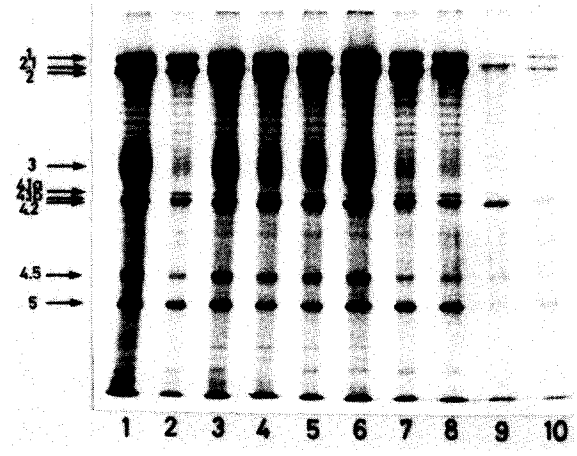

Fig. 9. Electrophoretogram of cytoskeletons prepared from the ghosts incubated with divalent cations and sulfhydryl-reactive compounds. Electrophoresis was performed according to the method of Laemmli [18]. Untreated ghosts (lane 1) and cytoskeletons prepared from the ghosts incubated with no addition (lane 2), $CdCl_2$ (lane 3), $HgCl_2$ (lane 4), $PbCl_2$ (lane 5), $ZnCl_2$ (lane 6), $CaCl_2$ (lane 7), $MgCl_2$ (lane 8), PCMB (lane 9) and *N*-ethylmaleimide (lane 10) at 0.2 mM each.

0.2 mM each also induced an increased association of cell membrane proteins with cytoskeletons, whereas $CaCl_2$ and $MgCl_2$ did not (Fig. 9). PCMB and *N*-ethylmaleimide, which are known to solubilize cytoskeletal proteins [8], destroyed the cytoskeletal network.

Discussion

The present study indicates that Cd^{2+} is bound to rat red cell ghosts resulting in association of cell membrane proteins with cytoskeletons. Hg^{2+}, Pb^{2+} and Zn^{2+}, like Cd^{2+}, are sulfhydryl-reactive divalent cations and induced an increased association of cell membrane proteins with cytoskeletons, whereas Ca^{2+} and Mg^{2+} did not (Fig. 9). In addition, Cd^{2+} prevented the dissociation of cytoskeletal network at low ionic strength at several-fold lower concentration than those of Ca^{2+} and Mg^{2+} (Fig. 5), which are known to precipitate spectrin molecules [27]. Apparently, Cd^{2+} as sulfhydryl-reactive divalent cation can mediate tight interactions between cell membrane proteins and cytoskeletons.

There is no simple explanation for the Cd^{2+} mediated interaction of cell membrane proteins with cytoskeletons. In the last years molecular features of the interaction of lipid bilayer membranes with cytoskeletal networks in human red cells have been characterized in some detail. Spectrin (bands 1 and 2) is a major structural element of the cytoskeleton. Band 2.1 (ankyrin) is associated tightly with band 3 and has additional function of anchoring these proteins to the cytoskeleton [28,29]. Human red cells contain about $1.1 \cdot 10^5$ copies of band 2.1 [30] and about $1.2 \cdot 10^6$ band 3 molecules or $3 \cdot 10^5$ tetramers [31]. Even if tetrameric band 3 is maximally connected with cytoskeletons through band 2.1 as proposed by Hargreaves et al. [29], only 37% of the total band 3 is estimated to be associated with cytoskeletons through band 2.1 and remaining 63% of band 3 still exists in unbound form. In our results, 74% of band 3 was associated with cytoskeletons in the presence of 0.2 mM Cd^{2+} (Fig. 8). If the content and function of band 2.1 in rat red cells are similar to those of human ones, Cd^{2+} could produce an association of band 3 with cytoskeletons without mediation of band 2.1. In support of this assumption, Liu et al. [5] have suggested the formation of spectrin-band 3 complexes following acid-induced disulfide bond formation in red cell ghosts.

Bennett and Stenbuck [28] showed that human red cell band 4.2 is associated with band 3. At 0.1 mM Cd^{2+}, 100% of band 4.2 and 56% of band 3 were associated with cytoskeletons (Fig. 8). The association of band 3 further proceeded in parallel to the binding of Cd^{2+}, and reached a maximum at 0.2 mM Cd^{2+}. Recently, we found that purified bands 3 and 4.2 alone were associated with isolated cytoskeletons in the presence of Cd^{2+} (unpublished data). Based on these results, it seems probable that band 4.2 is directly associated with cytoskeletons and band 3 too is associated with cytoskeletons in part through band 4.2, although further investigations are required for understanding Cd^{2+}-induced interactions of cell membrane proteins with cytoskeletons.

Cadmium induced the release of small vesicles from red cell ghosts depending on the temperature and Cd^{2+} concentration (Fig. 3). The cytoskeletal network plays an important role in stabilizing the cell membrane and preventing microvesiculation through the interaction with the cell membrane [3]. ATP-depleted red cells release the vesicles containing lipids and cell membrane proteins and almost depleting cytoskeletal components [15]. Incubation at high temperature and with sulfhydryl-reactive compounds also provokes the vesiculation of ghosts [6,7]. It has been postulated that the dissociation of cell membrane proteins from the cytoskeletal network enables formation and release of small vesicles [3]. The Cd^{2+}-induced vesicles showed the protein composition similar to that from ATP-depleted red cells (Fig. 4). However, the results presented here show that Cd^{2+} tightly fixed cell membrane proteins to cytoskeletons. It seems likely that Cd^{2+} mediates the linkage between cell membrane proteins and cytoskeletons, which may produce protein-poor region in cell membranes leading to the vesicle formation.

Higher concentrations of Cd^{2+} suppressed vesicle release drastically (Fig. 3C). This suppression seems, in part, due to cosedimentation of formed vesicles with ghosts. It seems also likely that the binding of a larger amount of Cd^{2+} to cell membranes inhibit a production of protein-poor region in cell membranes resulting in suppression

of the vesicle release. The Cd^{2+}-induced vesicles were similar in the protein composition but differed in the phospholipid composition to those from ATP-depleted red cells (Table I). The ratio of PS and PE, innerleaflet phospholipids of cell membranes, were lower in Cd^{2+}-induced vesicles than in those from ATP-depleted red cells. This seems to imply a strong interaction between cytoskeletons and these phospholipids, which is mediated by Cd^{2+} at the cytoplasmic surface.

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