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Cadmium Causes Vesicle Leakage Under Conditions which Favor Reconstitution of Tissue Factor-Vesicle Complexes

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Summary. Cadmium has a singular ability to promote the functional reconstitution of tissue factor (coagulation factor III) apoprotein with vesicles formed from mixed bovine brain lipids. Cadmium causes rapid release of carboxyfluorescein from lipid vesicles which chromatograph in the included volume of Bio Gel A5m, and also promotes reconstitution of tissue factor with these vesicles. With larger vesicles, which are excluded on Bio Gel A5m, cadmium produces only low levels of reconstitution and leakage. Calcium has been shown to promote only low levels of tissue factor-vesicle reconstitution, and produces only low levels of carboxyfluorescein leakage when compared to cadmium. These results demonstrate that cadmium and calcium interact differently with the small mixed lipid vesicles, and suggest that the cadmium-promoted reconstitution of tissue factor vesicle-complexes accompanies cadmium-induced alterations in vesicle structure. The results also show that cadmium affects large and small vesicles differently.

Key Words tissue factor · coagulation factor III · cadmium · membrane reconstitution · vesicles

Introduction

Addition of $CdCl_2$ to mixtures of tissue factor apoprotein (coagulation factor III) and bovine brain lipids used for protein-membrane reconstitution has been shown to promote the association of the apoprotein and lipids (Carson & Konigsberg, 1980*a, b*). Furthermore, tissue factor apoprotein and lipid vesicles, which do not interact to form stable complexes upon mixing, will form stable complexes with procoagulant activity when an appropriate amount of $CdCl_2$ is added (Carson & Konigsberg, 1981).

The mechanism by which cadmium promotes association of tissue factor apoprotein and lipid vesicles remains unknown. It is puzzling that $CaCl_2$, which has been extensively investigated because of its ability to interact with membranes (e.g., Papahadjopoulos et al., 1977; Ingolia & Koshland, 1978; Papahadjopoulos & Portis, 1978), does not enhance the reconstitution of tissue factor-vesicle complexes nearly as efficiently as does $CdCl_2$ (Carson & Konigsberg, 1980*a*, 1981). This distinction is even more curious because Ca^{2+} and Cd^{2+} are divalent cations of similar atomic radius, both produce equivalent fusion of vesicles composed of mixed phosphatidic acid and phosphatidylcholine (Liao & Prestegard, 1980), and X-ray diffraction spectra of their complexes with dipalmitoylphosphatidic acid reveal similar repeat spacings (Liao & Prestegard, 1981). The work presented here further characterizes the mixed lipid vesicles used for tissue factor reconstitution, and examines the ability of cadmium to induce leakage of vesicular contents.

Materials and Methods

The mixed brain lipids were extracted from acetone-dehydrated bovine brain using heptane-butanol as described by Pitlick and Nemerson (1976). Dipalmitoylphosphatidyl-[^{14}C]-choline (0.21 mCi/mg) from New England Nuclear was added to give 1 μCi/mg of mixed lipids. The weight of mixed lipids was originally determined by phosphate analysis, assuming phosphorus to be 4% of the lipid weight (Pitlick & Nemerson, 1976). The compositional analysis of the mixed lipid preparation was kindly performed in the laboratory of Dr. Robert K. Yu, using the method of Macala and Yu (1982). Carboxyfluorescein was Eastman 6-carboxyfluorescein, lot C4Q. $CdCl_2$ was "99.999%" from Aldrich, and sodium deoxycholate was purchased from Cal-Biochem. Bovine serum albumin was "essentially fatty acid-free" (Sigma). The buffer used throughout these studies (Tris-saline) was 0.05M Tris, 0.1 M NaCl, 0.01% NaN_3, pH 7.6, prepared from a 10X concentrated stock solution. The purified bovine tissue factor was provided by Dr. Ron Bach (Bach, Nemerson & Konigsberg, 1981).

Lipid vesicles were prepared by combining 0.2 ml of mixed lipids suspended at 2 mg/ml in Tris-saline, 0.25% deoxycholate, with 0.7 ml water and 0.1 ml of Tris-saline 10X concentrate (containing bovine serum albumin at 10 mg/ml). For experiments with carboxyfluorescein-charged vesicles, 0.4 ml of water was replaced by 0.4 ml of 0.5 M carboxyfluorescein. The mixture was gel filtered on a column (1.5 × 21 cm) of Bio Gel A5m. Fractions of approximately 1 ml were collected and a 20 μl aliquot of each was assayed for ^{14}C by scintillation counting. Lipid concentrations were determined from the [^{14}C]-phosphatidylcholine measured, assuming uniform distribution of label

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among all vesicles. Vesicles were prepared prior to each series of experiments, which were generally completed within 6 hr. A 10-fold concentrated lipid stock was examined on a Perkin-Elmer Model 370 atomic absorption spectrophotometer and found to contain 0.03 mM Ca^{2+}, 0.1 mM Mg^{2+}, and 0.005 mM Zn^{2+}.

Release of vesicle contents was determined by following the increase in fluorescence ($\lambda_{ex}=480$ nm; $\lambda_{em}=520$ nm) due to dilution of released carboxyfluorescein (Weinstein et al., 1977). Fluorescence measurements were made at room temperature using a Turner model 430 spectrofluorometer and a 5 mm × 5 mm quartz cell. Complete release of vesicle contents (100% fluorescence) was established by adding sodium dodecyl sulfate (final concentration 2%). The maximum releasable fluorescence varied among vesicle preparations, but was consistent within each experimental series. Under the conditions of the experiments cadmium chloride did not significantly affect the fluorescence spectrum of the carboxyfluorescein. Vesicles (0.5 ml) were added to the cuvette and fluorescence set at 0%. The test solution was added, and the cuvette was quickly covered with parafilm and inverted to mix; measurements were begun approximately 15 sec after addition of the test reagent. Stocks of $CdCl_2$ and $CaCl_2$ were 50 mM, dissolved in water. Tissue factor reconstitution with vesicles was accomplished as described (Carson & Konigsberg, 1981), using purified bovine brain tissue factor and vesicular lipids. Tissue factor activity was determined with a two-stage coagulation assay (Pitlick & Nemerson, 1976).

Results

The lipid vesicles were characterized in terms of lipid composition and vesicle size. The lipids present in the brain extract, and their relative concentrations, are presented in Table 1. As expected from earlier work (Nemerson, 1968), phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin were major constituents of the extract. In addition, considerable amounts of cerebroside, sulfatide, and phosphatidylinositol were also present. Removal of deoxycholate by chromatography of the lipids on Bio Gel A5m produced vesicles which were resolved into two size classes. Large vesicles eluted at the column excluded-volume while small vesicles chromatographed in the included-volume of the gel (Fig. 1). The method of Ackers (1964), which has been successfully used by others for determining vesicle sizes (e.g., Huang, 1969), was employed to estimate the size of the smaller vesicles. The included-volume of the column was estimated from the elution of mannitol and carboxyfluorescein and the bacteriophage ϕX174 (diameter 29 nm) was used as a partially included standard. The calculated mean vesicle diameter was 19 nm (range 14 to 24 nm). This calculated vesicle diameter is consistent with the 20 nm vesicle diameter measured in another study using electron microscopy (Carson & Konigsberg, 1981).

Table 1. Composition of bovine brain lipid extract

Lipid	Percent of Total
Phosphatidylcholine	23.4
Phosphatidylethanolamine	22.5
Phosphatidylserine	17.0
Phosphatidylinositol	3.4
Sphingomyelin	9.5
Cerebroside	16.2
Sulfatide	8.1
Cholesterol	None Detected

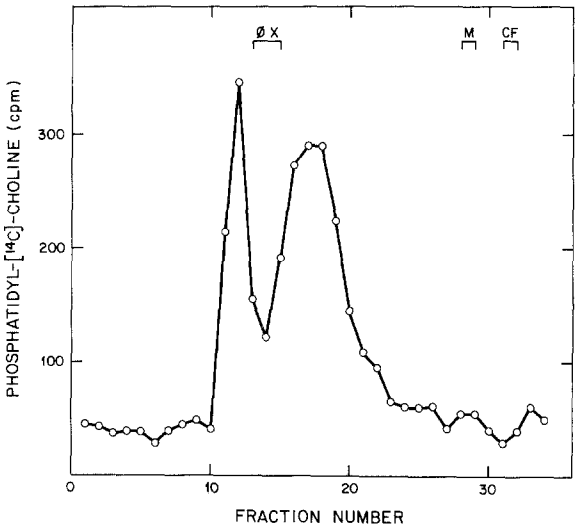

Fig. 1. Elution profile of vesicles chromatographed on Bio Gel A5m (column 1.5 × 21 cm). Fractions contained approximately 1 ml, of which 20 µl was taken for scintillation counting. Bacteriophage ϕX174 (ϕX), diameter 29 nm, was used to calibrate the pore radius (Ackers, 1964), and ^{3}H-mannitol (*M*) and carboxyfluorescein (*CF*) were used to estimate the included-volume of the column. ϕX174 was detected using a plaque assay

Table 2. Tissue factor vesicle reconstitution

Vesicles	Cation	TF Activity (units/ml)
Small	None (Tris-saline)	2.2
	Ca^{++}	3.3
	Cd^{++}	10.3
Large	None (Tris-saline)	<0.5
	Ca^{++}	<0.5
	Cd^{++}	1.0

Cadmium chloride promoted functional reconstitution of tissue factor with small vesicles, but the procoagulant activity recovered using large vesicles was relatively insignificant. The results shown in Table 2 were obtained by combining 90 µl of the vesicle preparation (34 µg lipid/ml) with 5 µl of tissue factor solution (11.4 ng/ml) and

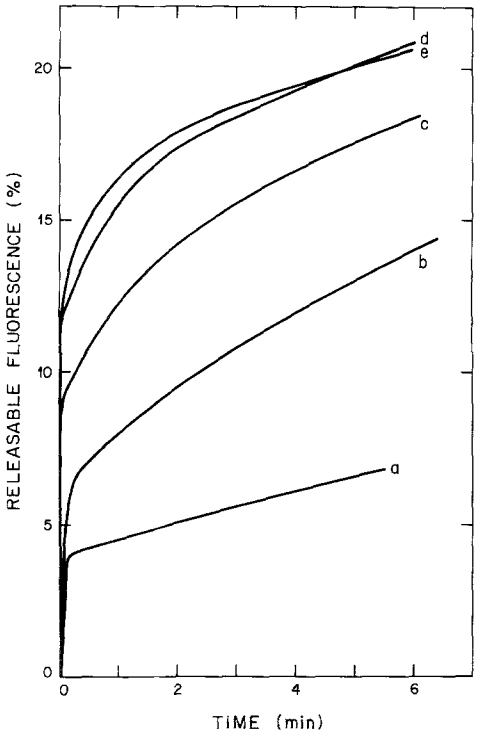

Fig. 2. Fluorescence increase due to release of vesicular carboxyfluorescein in response to different concentrations of $CdCl_2$. The $CdCl_2$ concentrations were (*a*) 0.98 mM, (*b*) 2.38 mM, (*c*) 4.55 mM, (*d*) 6.52 mM, (*e*) 8.33 mM. On the time scale, $t=0$ is taken as the time when measurements were begun; the actual addition of $CdCl_2$ occurred about 15 sec before measurements were initiated. The initial lipid concentration was 30 μg/ml

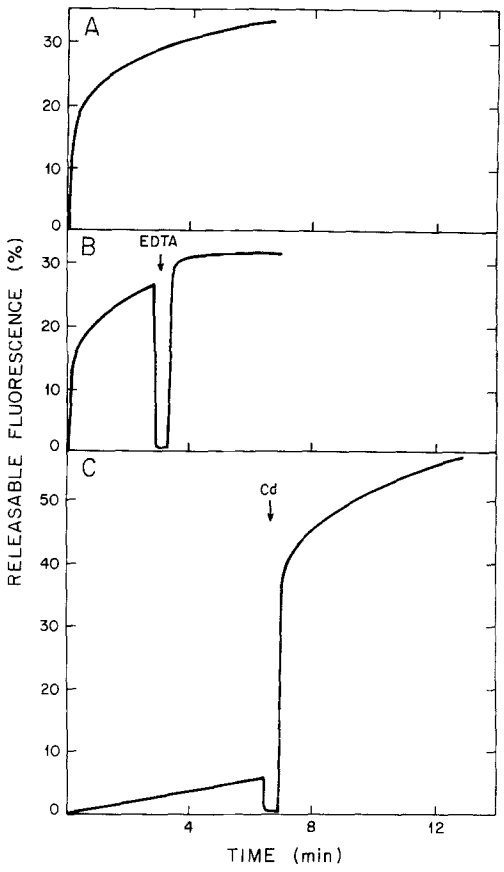

Fig. 3. Carboxyfluorescein release from vesicles in response to (*A*) 4.55 mM $CdCl_2$; (*B*) 4.55 mM $CdCl_2$, then at 3 min 25 μl 0.1 M EDTA was added; (*C*) 4.55 mM $CaCl_2$, then at 6.5 min 50 μl of 50 mM $CdCl_2$ was added. In *B* and *C*, the temporary drop in fluorescence corresponds to the time the fluorometer was off while EDTA or Cd was added to the cuvette

10 μl of $CdCl_2$ (50 mM), $CaCl_2$ (50 mM), or Tris-saline buffer. Procoagulant activity was determined after 10 min. As shown in Table 2, the activity of tissue factor reconstituted with $CdCl_2$ and small vesicles was about 10 times more than that obtained with large vesicles. Furthermore, the activity reconstituted with $CdCl_2$ and the large vesicles was less than the "spontaneous" activity which appeared with the small vesicles and Tris-saline.

The addition of $CdCl_2$ to small vesicles containing carboxyfluorescein caused release of vesicular contents into the bulk solution. Figure 2 presents a titration of vesicle leakage versus $CdCl_2$ concentration. Release of carboxyfluorescein appeared to occur in two stages: one very fast which was almost complete when fluorescence measurements were begun, and one which was much slower. At any time of the experiment, the release of carboxyfluorescein increased with increasing $CdCl_2$ concentration, up to 6.52 mM $CdCl_2$. In two experiments with large vesicles (lipid concentration 32 μg/ml), 4.55 mM $CdCl_2$ released only 4.2% and 3.4% of the carboxyfluorescein at 4.5 and 4.7 min, respectively. Thus $CdCl_2$ produced considerably less leakage from the large vesicles than from the small vesicles.

Further experiments with small vesicles demonstrated that calcium did not cause carboxyfluorescein release of the same magnitude as that caused by cadmium, and that EDTA was capable of halting the cadmium-induced slow release of carboxyfluorescein. As shown in Fig. 3, cadmium caused leakage of carboxyfluorescein from this vesicle preparation (Fig. 3, panel *A*) similar to that expected from earlier results (e.g., Fig. 2). When cadmium was complexed with EDTA after initiation of vesicle leakage (Fig. 3, panel *B*), the rate of fluorescence increase fell rapidly to zero, indicating that the presence of "free" cadmium was necessary for the observed slow phase of carboxyfluorescein release. Calcium produced only a slow release of carboxyfluorescein (Fig. 3, panel *C*), and did not interfere with the ability of $CdCl_2$ to cause a rapid and dramatic release of carboxyfluorescein.

Indeed, the amount of carboxyfluorescein released on addition of $CdCl_2$ (Fig. 3, panel *C*) suggested that the calcium potentiated the cadmium-induced vesicle leakage.

Discussion

This work has extended the chemical and physical characterization of phospholipid vesicles used for reconstituting tissue factor activity and has provided further information regarding the ability of cadmium to promote that reconstitution. The lipid classes constituting the brain extract have been quantitated with current methodology (Macala & Yu, 1982). The proportions of phosphatidylcholine, phosphatidylserine, and phosphatidylinositol agreed to within 3% with results previously reported by Nemerson (1968). In addition, the present method resolved considerable amounts of cerebroside and sulfatide which were not previously reported as constituents of the lipid mixture, and found lesser proportions of phosphatidylethanolamine and sphingomyelin.

Gel filtration removal of deoxycholate from the mixed lipids produced two vesicle size classes, similar to phosphatidylcholine vesicles prepared by sonication and gel filtration on Sepharose 4B (Huang, 1969). These mixed lipid vesicles were resolved into large structures which were excluded on Bio Gel A5m, and small vesicles with a mean calculated diameter of 19 nm. The vesicles were thus very similar to those prepared for tissue factor reconstitution by deoxycholate dialysis and characterized by gel filtration on Bio Gel A15m and by electron microscopy (Carson & Konigsberg, 1981).

Previous studies revealed that, whereas tissue factor apoprotein did not form stable associations with vesicles on mixing, $CdCl_2$ would promote functional reconstitution of stable protein-lipid complexes (Carson & Konigsberg, 1981). Gel filtration resolved reconstituted complexes with procoagulant activity and showed that the cadmium had promoted association of tissue factor with small (20 nm) vesicles as well as with larger vesicles (Carson & Konigsberg, 1981). This finding demonstrated that, in addition to any fortuitous entrapment of tissue factor which may have occurred during vesicle-vesicle fusion, cadmium must produce association of tissue factor with small vesicles which have not undergone fusion. It was not clear whether this cadmium-promoted reconstitution was the same for large and small vesicles, or whether the tissue factor activity was present in large vesicles as a result of fusion of reconstituted small vesicles. The present report has clearly resolved this question by demonstrating that cadmium promotes significantly more tissue factor reconstitution with the small vesicles than with large vesicles. This conclusion is valid even if the assumption of uniform lipid distribution among the vesicle sizes is incorrect. What cannot be concluded in view of this assumption is whether the specificity of the cadmium effect is strictly due to vesicle size and surface curvature or if it may at least in part be the result of uneven lipid distribution. This question will be addressed in future experiments utilizing pure preparations of individual phospholipids.

Parallels between the ability of cadmium to induce vesicle leakage and to promote tissue factor-vesicle reconstitution suggest that the two effects may be mechanistically related. Tissue factor-vesicle reconstitution was optimal near 5 mM $CdCl_2$ (Carson & Konigsberg, 1980*b*, 1981), and maximum carboxyfluorescein release was obtained when the $CdCl_2$ concentration was greater than 4.55 mM but less than (or equal to) 6.52 mM (Fig. 2). Furthermore, $CdCl_2$ produced both reconstitution and release of contents with the small vesicles to a much greater extent than with the large vesicles. Finally, compared to cadmium, calcium produced only low levels of tissue factor-vesicle reconstitution (Carson & Konigsberg, 1980*a*, 1981) and low levels of carboxyfluorescein release. It is thus reasonable to suggest that cadmium-induced leakage of vesicular contents and cadmium-promoted reconstitution of tissue factor with these lipid vesicles are related events. The rapid loss of vesicular contents indicates that cadmium causes a reorganization of lipids within the bilayer and a concomitant loss of vesicular integrity. Incorporation of tissue factor into the vesicles probably occurs in conjunction with this alteration in vesicle structure.

While only cadmium produced a dramatic and rapid release of carboxyfluorescein from the small vesicles, both cadmium and calcium produced slow leakage. Since both cadmium and calcium have been shown to fuse lipid vesicles (Liao & Prestegard, 1979, 1980), the slow release of carboxyfluorescein may be due to low-level spillage during vesicle-vesicle fusion. This suggestion is consistent with the elimination of carboxyfluorescein release when EDTA was added several minutes after cadmium (Fig. 3). Fusion has been observed as an increase in vesicle size when small vesicles exposed to cadmium were rechromatographed (S.D. Carson, *unpublished*).

In conclusion, these studies have shown that although the interactions of cadmium and calcium

with lipid vesicles may bear many similarities, the initial effects of these metals on small vesicles of mixed brain lipids must be considerably different. Cadmium has the singular ability to promote tissue factor association with the vesicles under conditions which also lead to pronounced vesicle leakage. A model is proposed suggesting that carboxyfluorescein leakage and tissue factor incorporation into the bilayer occur during cadmium-induced reorganization of vesicular lipids. While no physiological role is implied for such a mechanism involving cadmium, these results show that functional assembly of proteins into membranes can occur during physical perturbation of the membrane.

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