Cholate-Induced Disruption of Calcitonin-Loaded Liposomes: Formation of Trypsin-Resistant Lipid-Calcitonin-Cholate Complexes

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Purpose. The work was performed to obtain a better understanding why the oral administration of calcitonin (CT)-loaded liposomes to rats results in a hypocalcemia, while liposomes are normally disrupted in the gastro-intestinal tract and cannot protect the hormone from enzymatic digestion. ***Methods.*** *In vitro* comparisons between the stability of calcein and CT-loaded liposomes in the presence of cholate solutions led to an interpretation of the results observed. By means of gel filtration, turbidimetry, and fluorescence measurements, the interactions between CT and lipids were studied after sonicated liposomes had been broken down by cholate. ***Results.*** Experiments showed that CT in the external medium of a liposome suspension had no effect on the vesicles. Gel filtration of cholate-treated liposomes loaded with calcein and CT resulted in a total separation of calcein from the lipid fraction for detergent concentrations higher than 4 mM. However, 50% of the CT was reencapsulated even when the cholate-to-phospholipid molar ratio was increased up to 100. Incubation of cholate-solubilized liposomes with 1% trypsin resulted in a partial CT-breakdown. ***Conclusions.*** These results strongly suggest that during membrane solubilization by cholate, lipid-CT complexes are formed which retain most of the CT initially embedded in the liposomal membrane, and which offer some protection to CT under the action of trypsin. The existence of these complexes could be one of the reasons for the reported hypocalcemia in rats after oral administration of CT-loaded liposomes.

KEY WORDS: calcitonin; liposomes; cholate solubilization; lipid-protein complexes; enzymatic protein degradation.

INTRODUCTION

Fukunaga (1) observed in the rat a hypocalcemic effect 1 h after the oral administration of liposomes loaded with 1 mg calcitonin (CT). This result is surprising as it means that although liposomes are not stable enough to resist the action of bile salts (2–4), they partially protect the peptide from enzymatic digestion. One may hypothesize that molecular lipid-CT complexes are formed which persist after liposome disruption, and which are able to protect the CT from the action of proteases. To assess this hypothesis we used cholate and trypsin as models for bile salt and intestinal protease (5) actions, respectively, and studied the stability of CT-containing liposomes in the presence of these products.

Fluorescent measurements allowed us to follow the cholate-induced disruption of calcein-loaded liposomes (6). The disruption of CT and calcein-loaded liposomes was determined by gel filtration, which separated the free calcein and CT from the CT-containing liposome fraction, since gel filtration of lipid-detergent mixtures is known to be a method to reform liposomes (7). The resistance of CT-lipid complexes to proteases was tested by incubating them in the presence of trypsin. The results suggest the formation of lipid-CT complexes which offers some protection to the hormone from enzymatic digestion.

MATERIALS AND METHODS

Salmon calcitonin was provided by Bachem. Egg phosphatidylcholine (PC), cholesterol (Ch), stearylamine (SA), calcein, and cholate were obtained from Sigma. The absence of lipid hydrolysis during storage was verified by thin layer chromatography as described by New (7).

Preparation of Liposomes

Small unilamellar liposomes were prepared as described before (8). Briefly, 100 μmol PC, 28 μmol Ch and 14 μmol SA were dried to a thin film on the wall of a round-bottomed flask. The film was rehydrated with 1 ml Tris 20 buffer (5 mM Tris(hydroxymethyl)aminomethane pH 7.4 containing 20 mM NaCl) and sonicated for 30 min to obtain a 100 mM SUV suspension (suspension A). CT was loaded in the liposomes by rehydrating the lipid film with 1 ml Tris 20 buffer comprising 10 μg CT and its radioiodinated tracer (3.10^6 cpm) (suspension B). To determine the stability of the liposome by fluorescence, self-quenched calcein was encapsulated in the vesicles with CT (suspension C) or without CT (suspension D) by dissolving the lipid film with 1 ml 5 mM Tris pH 7.4 containing 175 mM calcein with or without 10 μg CT. To remove the non-encapsulated molecules, the liposome suspensions were applied on the top of a Sephadex G-50 column (1 × 30 cm), which was pre-equilibrated with Tris 20 (suspension B) or Tris 150 buffer (5 mM Tris pH 7.4 containing 150 mM NaCl; suspension C and D). These different NaCl concentrations were used to render the liposomes osmotically stable (9).

Liposome Stability in CT Solutions

Direct fixation of CT to the vesicle membrane was studied by incubating radioactive ^{125}I-CT (23000 cpm) with 100 μl of a 5-fold diluted liposome suspension A for periods of time ranging from 1 to 24 h. Subsequently, the suspensions were applied on a Sephadex G-100 column (0.5 × 20 cm) to separate the liposomes fixing CT from the protein still in its free form.

The influence of the peptide on liposomal membrane integrity was studied either by fluorescence or by spectrophotometry. For the fluorescence measurements, 20 μg CT were added to a 1000-fold diluted suspension D and the efflux of calcein was monitored for 75 min with a Spex Fluorimax spectrofluorimeter (John Ivon). Similarly, turbidity changes at λ = 400 nm were measured for 1 h with a Uvikon 820 spectrophotometer (Kontron) after the addition of 2.9 μM CT to 17 mM liposomes of suspension A.

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Liposome Stability in Cholate Solutions

The stability of the vesicles to cholate was tested either by gel filtration or by fluorescence. For the gel filtration experiments, vesicles of suspensions B and C were incubated for 30 min at room temperature in the presence of various cholate concentrations (0–20 mM). Subsequently, the mixtures were applied to a 0.5 × 20 cm Sephadex G-100 column to separate the released from the entrapped molecules. The presence of ^{125}I-CT in each fraction was determined with a Cobra II gamma counter (Packard Instruments), while the calcein concentration was determined spectrophotometrically. To test the liposome stability to cholate by fluorescence, freshly separated liposomes of suspensions C and D were diluted 1000-fold in Tris 150 buffer. After measurement of the initial fluorescent signal various cholate concentrations were added to the incubation buffer and the efflux of the self-quenched calcein was monitored for 10 min. Subsequently, Triton X-100 was added to disrupt the vesicles still intact. The percentage calcein release could then be calculated from:

$$R = \frac{F}{F_t} \cdot 100$$

where R, F, and F_t represent percentage release, fluorescence intensity after cholate treatment, and fluorescence intensity after Triton X-100 treatment respectively. Statistical significance between the percentage release values was determined by Student's t-test.

Enzymatic Digestion of CT by Trypsin

To determine the extent to which the membrane fragments formed after liposome disruption with cholate are able to protect CT from digestion with trypsin, 150 μl liposomes of suspension B were disintegrated by 10 mM cholate and were subsequently incubated for 30 min at 37°C in the presence of 1% trypsin. Next, the mixture was applied on a Sephadex G-50 column (0.5 × 20 cm) to separate the membrane fragments containing ^{125}I-CT from the digested ^{125}I-CT fragments.

RESULTS

Liposome Stability in CT Solutions

Gel separation of a liposome mixture incubated with 23000 cpm ^{125}I-CT for 1, 3, 6, or 24 hours showed that the fixation of ^{125}I-CT was low, about 8%, and that prolonging the incubation time had no influence. Monitoring the efflux of calcein from the vesicles after the addition of CT to the incubation buffer revealed no significant variation in vesicle stability: liposomes incubated in buffer alone released 1% of the entrapped molecule, while incubation in a buffer containing 2.9 μM to 5.8 μM CT caused less than a 2% release. Studying turbidity changes up to 1 h after the addition of 2.9 μM CT to the liposome suspension A did not reveal any modifications in the vesicle size, and increasing CT concentrations (up to 20 μg CT/ml) had no influence on the absorbance of the empty vesicles.

Liposome Stability in Cholate Solutions

Fluorescence Measurements

Cholate-induced calcein release from liposomes in suspensions C and D was studied by fluorescence. Irrespective of the presence of CT in the liposomes, a total release of the entrapped calcein was observed as soon as cholate concentrations reached 5 mM (Figure 1). Varying the phospholipid concentration had no influence on the percentage of calcein released by 5 mM cholate. In fact, no variation in calcein release was observed when the cholate-to-phospholipid molar ratio was varied from 10 to 100.

Gel Filtration

Gel filtration of cholate-disrupted vesicles was performed in the absence of cholate in the eluent in order to reform vesicles during the passage of the liposome-cholate mixture through the gel as in the "detergent removal" preparation method of liposomes (7). In this way, the small hydrophilic molecules are retained by the gel while the phospholipid fragments reform vesicles and are eluted in the void volume (V_0) of the column.

Treatment of liposome suspension C with cholate showed that over 95% of the calcein was eluted in the total volume (V_t) of the column for cholate concentrations higher than 10 mM. Therefore this small hydrophilic molecule was not reencapsulated in the vesicles formed during the passage of the liposome-cholate mixture through the gel (Figure 2). On the contrary, CT was eluted at the same time as the lipidic fraction; more than 80% of the small peptide remained fixed to the lipid fragments formed after treatment of the liposomes with 20 mM cholate, and so was probably reencapsulated within the liposomes during gel separation. This phenomenon was observed irrespective of the presence of calcein in the CT-loaded liposomes (Figure 2).

Increasing the molar ratio of cholate to phospholipid by

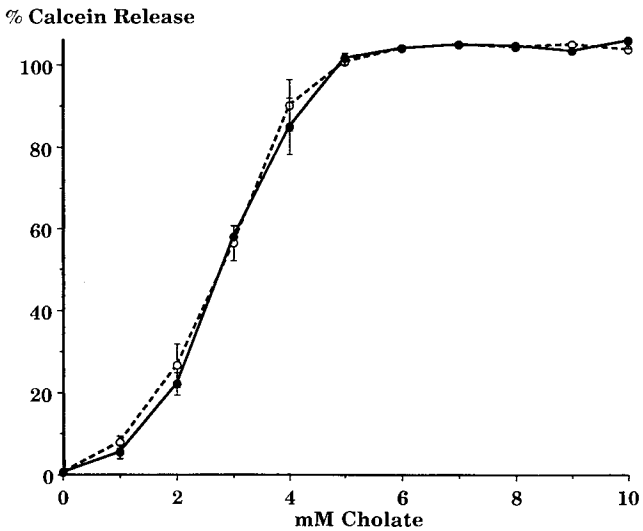

Fig. 1. Fluorimetric determination of the percentage release of calcein from liposomes in the 1000-fold diluted suspensions C (—●—) and D (--○--) after treatment with various cholate concentrations. Vertical bars represent the S.D. of the mean of 2 determinations.

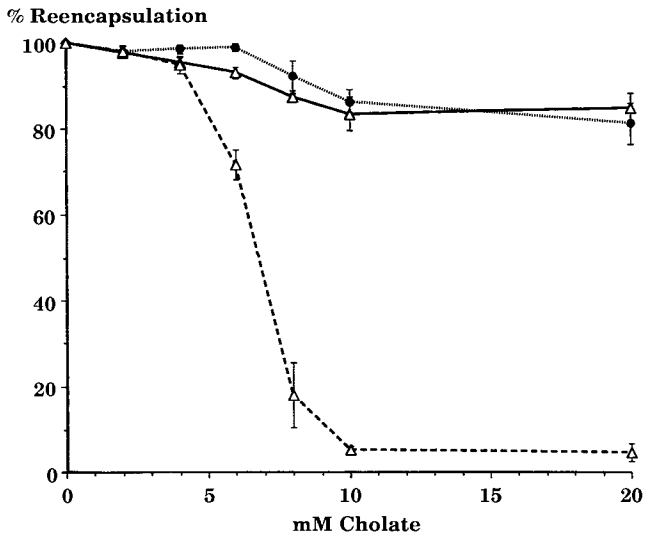

Fig. 2. Percentage reentrapment of CT (———) and calcein (---) from the 10-fold diluted liposome suspensions B (●) and C (△) by gel chromatography. The percentage reencapsulation represents the reentrapment of the molecules released by cholate only. Vertical bars represent the S.D. of the mean of at least 6 determinations.

changing the vesicle concentration resulted in a decrease in the fraction of CT fixed to the phospholipids after gel filtration (Figure 3). However, for cholate-to-phospholipid molar ratios higher than 40, the decrease in percentage CT-reencapsulation slowed down, and about 50% of the CT remained fixed to the liposomes for cholate-to-phospholipid molar ratios as high as 100.

Enzymatic Digestion of CT by Trypsin

Incubating liposomes solubilized with 10 mM cholate in the presence of 1% trypsin did not result in the total enzymatic degradation of CT. Figure 4a shows a second peak at the V_t of the column, corresponding to ^{125}I-CT fragments formed after trypsin digestion. Calculation of the percentage of CT remaining intact after trypsin digestion from the surface under the peaks showed that about 26% of the CT resisted enzymatic degradation by 1% trypsin. However, when CT was incubated in its free form with 1% trypsin, total CT degradation was obtained, as shown in Figure 4b. The peak corresponding to intact CT has totally disappeared and has been replaced by a peak leaving the column at V_t, corresponding to totally digested CT.

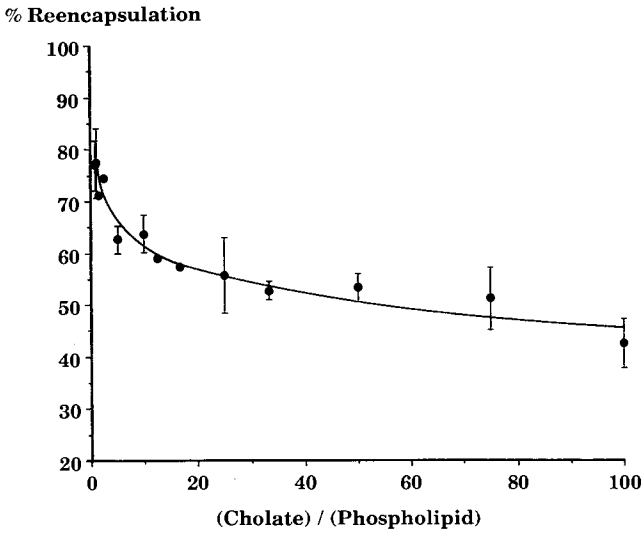

Fig. 3. Influence of the molar ratio of cholate to phospholipid on CT reencapsulation. Various concentrations of CT-loaded liposomes (PC/Ch 7/2 mol/mol) were incubated with 10 mM cholate and the released molecule was separated from the lipid-fixed CT by gel chromatography. Vertical bars represent the S.D. of the mean of 3 determinations.

DISCUSSION

Incubating CT in the presence of empty liposomes resulted in a low fixation of the hormone to the liposomal membrane, which did not modify either the permeability or the size of the vesicles. So even if CT interacts with the lipid membrane, the interactions are limited to non-disturbing surface interactions. Fluorescence measurements of the cholate-disrupted liposomes were performed with 1000-fold

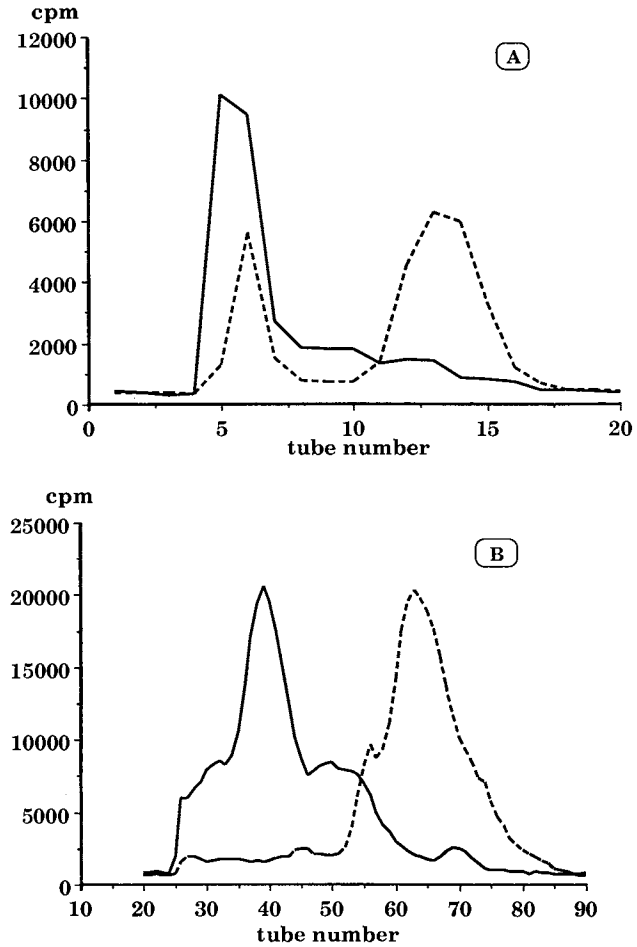

Fig. 4. (A) Gel chromatography on a Sephadex G-50 column (0.5 × 20 cm) of CT-lipid fragments obtained by cholate solubilization of the 10-fold diluted liposome suspension B, treated (---) or not (———) with 1% trypsin. (B) Separation on a Sephadex G-25 column (1 × 60 cm) of 2.9 μM CT digested (---) or not (———) by 1% trypsin and 5 mM cholate after 30 min incubation at 37°C.

diluted suspensions to avoid self-quenching of the released calcein in the medium. This extensive dilution diminished the phospholipid-to-cholate molar ratio and favoured the disruption of the lipid-CT complexes. However, we observed that the presence of CT did not modify the pattern of calcein release or the resistance of the liposomes to the action of cholate. Gel filtration of CT and calcein-loaded liposomes disrupted by cholate induced only a small elimination of CT from the lipid fraction (14% CT at 10 mM cholate), while a total elimination of the calcein was obtained for cholate concentrations higher than 5 mM. This large discrepancy is probably due to the fact that CT is reencapsulated in the liposome membrane. Indeed, though we did not observe any significant CT fixation to the preformed vesicles, CT was shown to interact with the hydrophobic region of certain phospholipids by the presence of its amphipathic α-helix (10). So during sonication, when contact between the hydrophobic regions of the lipids and CT becomes possible, lipid-CT complexes are probably formed which are subsequently embedded in the liposomes without changing their stability.

The formation of a lipid-CT complex fits well with the results obtained from the gel filtration experiments. When the CT and calcein-loaded liposomes were incubated in 20 mM cholate solutions, a total disruption of the vesicles was obtained (11–12). The separation of these mixtures over columns equilibrated with buffer containing no cholate resulted in the reformation of vesicles (4,6) which contained much of the CT (over 80%), while all calcein was eluted in the V_t of the column. However, if the CT-lipid complexes had been totally disrupted by the cholate, CT would have been eluted together with calcein in the V_t of the column, as the small peptide has a molecular weight of only 3432.

Incubations of solubilized CT-loaded liposomes in the presence of 1% trypsin resulted only in partial degradation of the hormone. In contrast, when the hormone was incubated in its free form with the enzyme, total CT degradation was observed. This difference was probably due to the fact that the lipids in the lipid-CT complexes mask the sites for cleavage in the hormone from the enzyme, which cleaves the peptide bonds in the protein near the basic amino acids, arginine and lysine (5). Thus, protection of these sites by lipids could result in a higher protease resistance.

CONCLUSION

The present experiments show that the formation of lipid-CT-cholate complexes offers the hormone a certain degree of protection from digestion by trypsin. This type of preservation may exist *in vivo* and could partially explain the hypocalcemic effect noted by Fukunaga (1) in rats after the oral administration of CT-loaded liposomes.

ABBREVIATIONS

Ch, cholesterol; CT, calcitonin; DPPC, dipalmitoyl phosphatidylcholine; EDTA, ethylene diamine tetraacetic acid disodium salt; PC, egg phosphatidylcholine; SA, stearylamine; SUV, small unilamellar vesicle; Tris, tris(hydroxymethyl)aminomethane.

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