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Calcitonin-loaded liposomes: stability under acidic conditions and bile salts-induced disruption resulting in calcitonin–phospholipid complex formation

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Abstract

Calcitonin-loading in liposomes composed of phosphatidylcholine, cholesterol and stearylamine or dipalmitoyl phosphatidylglycerol was studied at low pH values and in the presence of bile salts to check whether liposomal entrapment could be a possible means of protecting the peptide against the aggressive conditions present in the gastrointestinal tract. The association of calcitonin with the lipidic vesicles was monitored using radioactive labelling of the peptide and gel-filtration separation of the free and liposome-associated fractions. The results show that for all phospholipid compositions tested, loading was preserved in light acidic or basic buffers, and that only a slight disruption was observed at pH 2.5. Cholate caused a significant but only partial release of calcitonin even when the cholate-to-phospholipid ratio was increased. To understand the mode of calcitonin entrapment in the vesicles, the release of liposome-entrapped calcein was monitored concomitantly and taken as a stability criterion. Liposome integrity appears to be resistant at low pHs but to be totally destroyed by 4 mM cholate in a manner quasi-independent of the phospholipid concentration. These results strongly suggest that bile salts induce a disruption of the liposomes which results in the formation of new lipidic structures involving calcitonin and probably cholate.

Key words: Calcitonin; Calcein; Liposome; pH variation; Bile salt; Lipid-protein complex formation

1. Introduction

The introduction of molecules other than phospholipids into the lipid bilayer is often used to modify the physicochemical properties of liposomes [1–6]. In this way, proteins and peptides have been incorporated into the lipid bilayer. Modifications are observed in phase transition enthalpy, and transition temperature as has been noticed after the incorporation of cyclosporin A [7] and numerous amphipathic α-helical peptides [8,9]. Amphipathic peptides such as mellitin have also been shown to solubilize the vesicle membrane with the formation of mixed micelles or discoidal lipidic structures [10], but proteins may also stabilize liposomes, as has been observed by Liu et al. [11] for Apo A1.

Salmon calcitonin (CT), a peptide of 32 amino acids, contains a hydrophobic sequence from residue 8–22. This sequence can form an amphipathic helix. Owing to its well-defined single chain polypeptide structure, CT has been studied from a fundamental point of view to investigate some aspects of the interactions of proteins with lipids [9]. The hormone is also often used in the therapy of calcium disorders and cardiac diseases [12,13]. Recently, attempts have been made to encapsulate CT in liposomes in order to achieve oral administration of the hormone, namely to protect the protein from the aggressive conditions present in the gastrointestinal tract [12]. A hypocalcemic effect was observed 1 h after oral administration of PC/Chol/SA liposomes loaded with 1 mg CT. Although, large doses of CT were encapsulated, only small changes in blood calcuim were observed. To improve these results and

Abbreviations: Chol, cholesterol; CT, calcitonin; DMPG, dimyristoylphosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; PC, phosphatidylcholine; PS, phosphatidylserine; SA, stearylamine.

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to rationalize the strategy of liposomal administration of CT, a fundamental understanding of the way the protein associates with the liposomal system under these conditions is required.

In this study, we have investigated the association of CT with several types of liposomes in the presence of bile salts and acidic pHs in relation to liposome stability, as detected by calcein release. The extent to which protein entrapment by liposomes can be attributed to encapsulation in the aqueous volume is discussed.

2. Materials and methods

2.1. Materials and buffers

Egg phosphatidylcholine, phosphatidylserine, dipalmitoylphosphatidylglycerol, cholesterol, stearylamine, salmon calcitonin, calcein and cholate were provided by Sigma. The 99% purity of the lipids was verified by thin-layer chromatography as described by New [14]. The lipids were run on silica gel 60 plates (Merck) with chloroform/methanol/water (65:25:4, v/v) as the mobile phase. The clearly defined spots were visualized by means of iodine. The radioactive marker $Na^{125}I$ (1 mCi/5 μl) was obtained from Cis-International. Sephadex G-50 (fine) and G-100 (fine) were provided by Pharmacia. Iodobeads (*N*-chlorobenzenesulfonamide as sodium salt derived on non-porous polystyrene beads) were obtained from Pierce. All other chemicals were reagent grade and provided by Merck.

The buffer, Tris 20, used for the preparation and dilution of CT-loaded liposomes, contained 5 mM Tris and 20 mM NaCl and was adjusted to pH 7.4. For the fluorescence assays a buffer, Tris 150, was used which contained 5 mM Tris and 150 mM NaCl adjusted to pH 7.4. For the radioiodination of CT, we prepared a phosphate buffer containing 0.5 M Na_2HPO_4 which was adjusted to pH 7.4 by the addition of 0.5 M KH_2PO_4.

2.2. Radioiodination of calcitonin

CT was radioiodinated by the use of iodobeads as described elsewhere [15]. Briefly, 1 mCi carrier-free $Na^{125}I$ was incubated for 5 min at room temperature in the presence of 500 μl phosphate buffer containing 4 iodobeads. Subsequently, 20 μg CT were added and the reaction was continued for 20 min before the reaction was stopped by removing the solution from the reaction vessel containing the iodobeads. The free ^{125}I was removed from the CT-fixed ^{125}I by column chromatography over a 1×60 cm column of Sephadex G-50 equilibrated with phosphate buffer containing 30 μM albumin. The specific activity of the labelled CT was determined to be 11 μCi/μg CT.

2.3. Preparation of liposomes

Liposomes were prepared by a modification of the method described elsewhere [16]. Briefly, small unilamellar vesicles were prepared by drying 100 μmol PC, 28 μmol Chol and 14 μmol SA, DPPG or PS, in order to obtain positively or negatively charged liposomes. The thin film obtained was rehydrated with 1 ml Tris buffer. Subsequently, the vesicles were sonicated for 8 min using a probe type sonicator (Ultrasons Annemasse). Following sonication the liposomes were incubated at 40°C for 1 h to anneal any structural defects. The Ti-fragments and multilamellar vesicles were removed by centrifugation at $15\,000 \times g$ for 20 min.

CT loading within the liposomes was achieved by rehydrating the lipid film with 1 ml Tris 20 buffer containing 10 μg CT and its radioiodinated tracer ($3 \cdot 10^6$ cpm). The non-encapsulated CT was removed by applying the liposomal suspension to a 1×30 cm Sephadex G-100 column equilibrated with Tris 20 buffer.

To determine the stability of the liposomes by fluorescence, self-quenched calcein was encapsulated in the vesicles by dissolving the lipid film with 5 mM Tris (pH 7.4) containing 175 mM calcein. Free calcein was removed by column chromatography over a 1×10 cm column of Sephadex G-50 equilibrated with Tris 150 buffer, which rendered the liposomes osmotically stable [17].

2.4. Determination of calcitonin release

CT release was evaluated by incubating the CT-loaded vesicles for 30 min at room temperature in Tris 20 buffer adjusted to various pHs with 0.1 M HCl or 0.1 M NaOH; or in the presence of cholate prepared in Tris 20 buffer. After incubation the suspensions were applied to a 0.5×20 cm Sephadex G-100 column in order to separate the released from the entrapped ^{125}I-labelled CT. The radioactivity of each fraction was determined for 1 min in a region between 15 keV and 75 keV by a Cobra II gamma counter (Packard Instruments). Background radioactivity was 33 cpm. The liposomal peak was detected in fractions 4–6, while the free CT left the column in fractions 9–13. The percentage of CT released was determined from the area under the peaks.

2.5. Determination of vesicle stability by fluorescence

Efflux of self-quenched calcein was determined on a Spex Fluoromax spectrofluorimeter (John Yvon) equipped with a thermostated cuvette holder and a magnetic stirring device. Excitation and emission wavelengths were set at 490 and 520 nm, respectively. The excitation and emission slits provided a 2 nm band

pass. Temperature (25°C) was programmed and controlled (0.1°C) by a SLM 8000 controller.

Calcein, which is self-quenched within liposomes owing to its high concentration, becomes intensely fluorescent after leakage from the vesicles and dilution into the dispersion medium. In most cases the rate of release, R, is directly related to the fluorescence intensity of the sample under the given experimental conditions (F), and to the fluorescence intensity of the same sample after total calcein release by detergent treatment (F_T). The fluorescence of the entrapped calcein is usually considered as negligible compared to the fluorescence of that released so that R becomes:

$$R = \frac{F}{F_T}$$

This simple analysis, however, is not applicable under conditions of acidic pH values. Indeed, a severe decrease in calcein fluorescence quantum yield has been observed when pH is decreased below 6 [18]. This has to be taken into account in the analysis of the results. For this purpose, we drew up the following. The fluorescence intensity, F, of a calcein sample of concentration, c, which is sufficiently low to avoid fluorescence quenching, may be written as:

$$F = \alpha_1 \cdot p \cdot c \qquad (I)$$

where α_1 is the experimental proportionality constant given by the slope at the origin of the curve relating the fluorescence intensity of calcein solutions, recorded in standard conditions, to their concentration. It should be noted that α_1 has to be determined for each experimental set-up. p is the correction factor taking into account the pH effect; it may be defined as the ratio of the fluorescence intensity of a calcein solution at a given pH to the fluorescence intensity of the same calcein solution at pH 7. This factor was also determined experimentally (see Section 3). Similarly, the fluorescence intensity (F'') of a calcein solution of concentration c'' higher than 100 mM, i.e. a solution for which quenching is maximal, may be written as:

$$F'' = \alpha_2 \cdot p \cdot c''$$

where α_2 is determined using a liposome sample loaded with highly concentrated calcein (175 mM). The fluorescence F'' is recorded under the usual standard conditions. The sample was subsequently treated with Triton X-100 to release calcein. In accordance with Eq. (I), the fluorescence F', then recorded, was equal to:

$$F' = \alpha_1 \cdot p \cdot c''$$

thus α_2 can be obtained from:

$$\alpha_2 = \frac{F''}{F'} \cdot \alpha_1 \qquad (II)$$

Then, the fluorescence of a sample of liposomes loaded with a high calcein concentration c_i, in the presence of a released calcein concentration c_o outside the vesicles, is equal to:

$$F = \alpha_1 \cdot p_o \cdot c_o + \alpha_2 \cdot p_i \cdot c_i \cdot \phi \qquad (1)$$

where p_i and p_o characterize the internal and external pH, respectively, and ϕ is the volume fraction of the liposomes in the sample. Mass conservation implies that:

$$c_T = c_i \cdot \phi + c_o \qquad (2)$$

where c_T is the total calcein concentration. c_T is also given by the fluorescence intensity of the sample treated with Triton X-100 (F_T):

$$c_T = \frac{F_T}{\alpha_1 \cdot p_0} \qquad (3)$$

The rate of release (R) is:

$$R(\%) = \frac{c_o}{c_T} \cdot 100 \qquad (4)$$

or from Eqs. (1), (2) and (3):

$$R(\%) = \frac{F \cdot \alpha_1 \cdot p_o - F_T \cdot \alpha_2 \cdot p_i}{F_T \cdot (\alpha_1 \cdot p_o - \alpha_2 \cdot p_i)} \cdot 100 \qquad (5)$$

3. Results

3.1. Release of liposomal-entrapped calcitonin

3.1.1. Effect of pH on [^{125}I]CT release

The release of CT, loaded in various types of liposomes, was determined after 30 min incubation in Tris 20 buffer at room temperature. During this period, release reached a maximal value which no longer varied when the incubation time was increased to 60 min. Varying the pH between 5 and 9 did not result in any significant release of [^{125}I]CT from the PC/Chol (7:2, mol/mol) or PC/Chol/SA (7:2:1, mol/mol) liposomes (Table 1). However, when these vesicle types were incubated at pH 2, a small but significant release of CT was observed: about 5% [^{125}I]CT was released after 30 min incubation at pH 2. Liposomes composed of PC/Chol/DPPG (7:2:1, mol/mol) showed no significant variation in stability with pH variation, although the vesicles were in general less stable than the other liposome preparations studied.

3.1.2. Release of calcitonin by cholate

The release of CT in the presence of different cholate concentrations was studied (Table 1). At 2 mM cholate, only a small release of [^{125}I]CT was observed and no distinction in stability could be made between the different vesicle preparations studied except that

Table 1
Effect of pH and cholate on the release of entrapped [^{125}I]CT from liposomes

Incubation buffer	Percentage of [^{125}I]CT release (%)		
	PC/Chol	PC/Chol/SA	PC/Chol/DPPG
pH 2	5.8±1.1	4.1±0.2	15.1±2.7
pH 5	0.2±0.2	0.1±0.1	10.5±2.1
pH 7	2.8±0.9	1.1±0.9	14.2±2.8
pH 9	3.7±1.4	0.7±0.3	16.3±3.1
2 mM cholate pH 7.2	4.8±1.0	4.6±1.4	13.0±3.4
10 mM cholate pH 7.2	38.9±1.3	9.1±1.1	23.7±3.9

10 mM liposomes composed of PC/Chol (7:2, mol/mol), PC/Chol/SA (7:2:1, mol/mol) or PC/Chol/DPPG (7:2:1, mol/mol) entrapping 2.9 μM ^{125}I-CT were incubated for 30 min at room temperature in Tris 20 buffer with a pH varying from 2 to 9. The effect of bile salts was determined by incubating the liposomal suspension with an equal volume of 4 or 20 mM cholate prepared in Tris 20 buffer. Release was determined by column chromatography as described in Section 2. Each value represents the mean ± S.E. of 2 experiments.

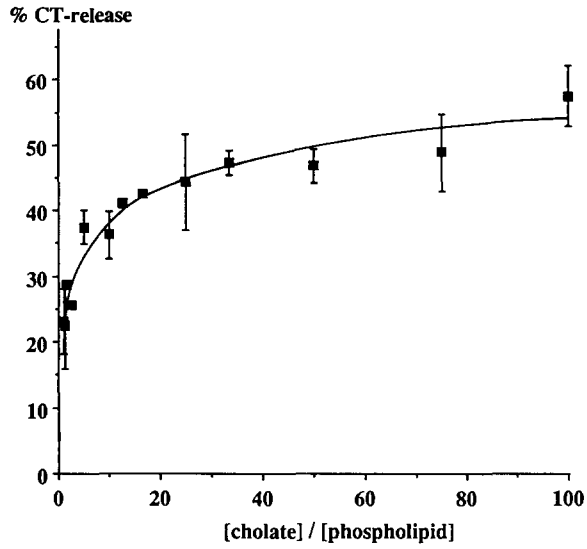

Fig. 1. Influence of the phospholipid concentration on the disruption of CT-loaded liposomes by 10 mM cholate. Varying amounts of PC/Chol liposomes (7:2, mol/mol) loaded with 2.9 μM CT and its radioactive tracer were incubated for 30 min at room temperature in the presence of 10 mM cholate, prepared in Tris 150 buffer. Release was determined by column chromatography as described in Section 2. Vertical bars represent S.E. of three determinations.

the liposomes containing DPPG were more permeable, as already noted in the absence of cholate. When the cholate concentration was increased to 10 mM, a significant release of CT was observed: the CT release increased 14-fold for the neutral PC/Chol liposomes, 8-fold for the PC/Chol/SA liposomes and only 2-fold for the PC/Chol/DPPG liposomes. These factors embody the basic CT release of each type of liposomes in the absence of cholate at pH 7, so they obey a different hierarchy than the absolute values of release.

Varying the vesicle concentration drastically influenced the release of CT induced by a given concentration of cholate (Fig. 1). At low molar ratios of cholate-to-phospholipid, e.g. cholate/phospholipid = 1, 10 mM cholate induced only 23% of CT release. Increasing the molar ratio resulted in an increase in the CT-released fraction. However, the data in Fig. 1 clearly show that the percentage release levelled off at a value close to 50% when the molar ratio reached a value of 40. No further significant increase in release was observed for higher values of the ratio. Total release was never observed.

3.2. Liposome stability measured by calcein release

3.2.1. Determination of coefficients α_1 and α_2

α_1 and α_2, the coefficients indicating the fluorescence of calcein solutions in relation to their concentration in monomeric and auto-associated states, respectively, were determined as described in Section 2. Fig. 2 shows fluorescence intensity as a function of calcein concentration, varying from 0 to 6.4 μM. Up to a concentration of $5 \cdot 10^{-7}$ M calcein, the intensity increased linearly with concentration. The slope of the linear part of the curve, giving α_1, was $1.2 \cdot 10^{13}$ cps/mol.

At high calcein concentrations, the light beam was absorbed by the calcein molecules in such a way that α_2 had to be determined from a freshly separated liposome suspension containing 175 mM self-quenched calcein, as explained in Section 2. The value of α_2 was

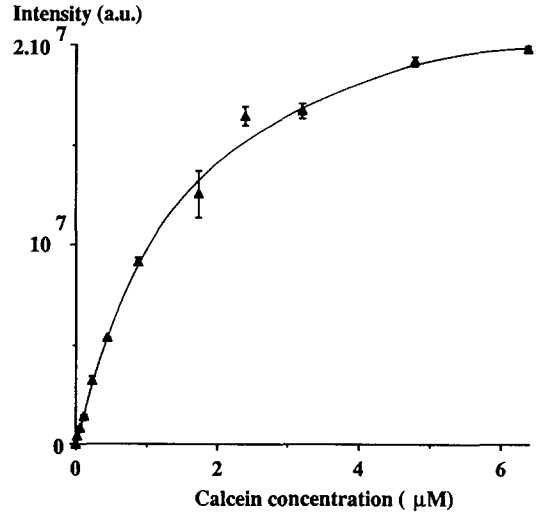

Fig. 2. Influence of the calcein concentration on the intensity of the fluorescent signal. Calcein was prepared in 2 ml Tris 150 buffer. Emission spectra were taken at 25°C with an excitation wavelength of 490 nm and emission wavelength of 520 nm. Vertical bars represent S.E. of two experiments.

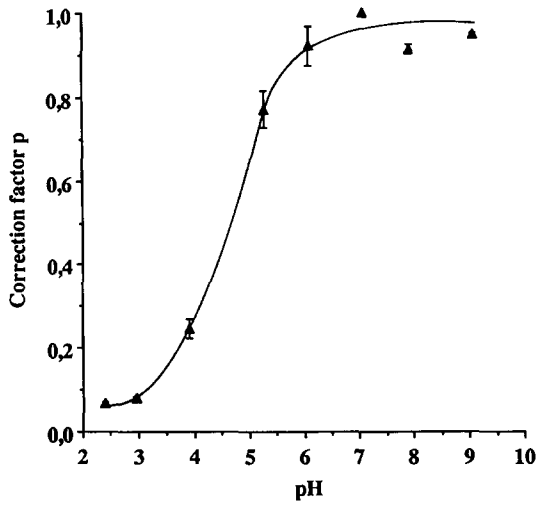

Fig. 3. Influence of pH on the fluorescence of calcein. Calcein was diluted in 2 ml Tris 150 buffer to obtain a concentration of $8.75 \cdot 10^{-7}$ M calcein. The pH of the buffer was ajusted to various pHs by the addition of 0.1 M HCl or 0.1 M NaOH. Emission spectra were taken at 25°C with excitation and emission wavelengths set at 490 nm and 520 nm, respectively. Vertical bars represent S.E. of two experiments.

then obtained from Eq. (II). The experiments were performed with 20 μM PC/Chol calcein-loaded liposomes and provided an α_2 value equal to $1.1 \cdot 10^{11}$ cps/mol.

3.2.2. pH effect on calcein fluorescence

The fluorescence of a $8.75 \cdot 10^{-7}$ M calcein solution was measured as the pH of the sample was changed. The results in Fig. 3 show that fluorescence intensity was stable for pH values ranging from 9 to 6, but that it severely decreased for pH values from 6 to 2. The correction factor p was obtained from this calibration curve and taken to be equal to the ratio of the fluorescence of the calcein solution at a given pH to the fluorescence of the same solution at pH 7. For instance, we obtained $p = 1$ for $6 < \text{pH} < 9$ and $p = 0.07$ for pH = 2.

3.2.3. Effect of pH on release of calcein by liposomes

The stability over time of different liposome preparations was evaluated for pH values ranging from 2.3 to 9 by measuring the efflux of the self-quenched calcein throughout the vesicles. Fig. 4 shows a typical kinetic profile of the release of liposomal-encapsulated calcein at pH 2.3. Before the addition of 0.1 M HCl, the fluorescence corresponding mainly to the low level of external calcein was stable. When hydrochloric acid was added, an immediate decrease in fluorescence was observed owing to the acidification of the external medium. This was followed by a slight increase in fluorescence due to the fast release of calcein throughout the vesicles. Afterwards fluorescence diminished again and stabilized after a few minutes. This slow decrease in fluorescence could be attributed to two different processes: either to the slow leakage and immediate acidification of calcein moving from the interior to the exterior of the vesicles; or to the entry of protons into the liposomes and acidifying the interior of the vesicles in such a way that the small fluorescence of the quenched calcein, loaded within the vesicles, decreased. The percentage release was calculated in the two cases by considering the following extreme situations: first $p_i = 1$, since the pH on the inner side of the vesicles could be equal to 7.4, and p_o is the pH of the experiment (Fig. 5A), second $p_i = p_o$ because of the proton influx equilibrating the internal and external pHs (Fig. 5B). The fluorescence F was measured at equilibrium after the addition of HCl, and F_T was determined after the addition of Triton X-100.

The results obtained according to the first hypothesis (Fig. 5A) for the various liposome types studied showed only a low instability in the presence of the most acidic pH, while no significant release was observed for the pH varying from 5 to 9. Analysis with the second hypothesis of proton influx provided slightly higher values of calcein release, especially at the lowest pH; however, they never exceeded 20%. No variation was observed for pHs ranging from 5 to 9. The release profiles obtained for the liposomes composed of PC/Chol/SA and PC/Chol/PS were very similar. The non-charged liposomes composed of PC and Chol showed a lower release at pH 2.3, but increasing the

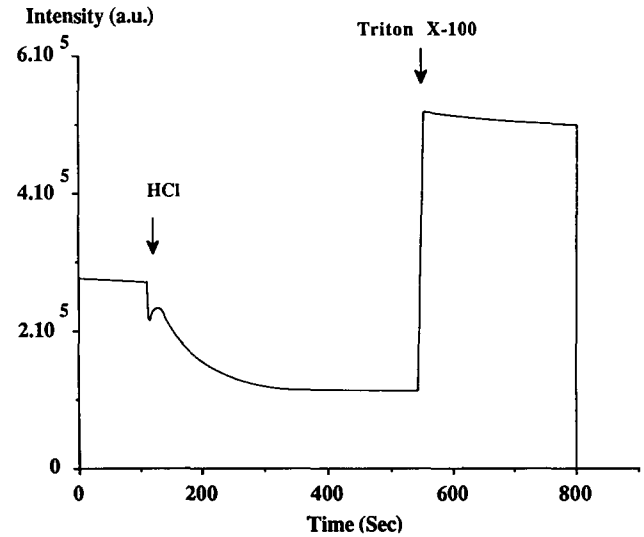

Fig. 4. Kinetic profile of calcein release from negatively charged liposomes under the influence of pH. 20 μM PC/Chol/PS liposomes (7:2:1, mol/mol) were incubated in Tris 150 buffer. At 150 s 0.1 M HCl was added to obtain a pH 2.3. The increase in fluorescence intensity caused by the release of the quenched calcein (175 mM) under the influence of a low pH was monitored for 8 min before 100 μl 10% Triton X-100 was added to disrupt the remaining liposomes.

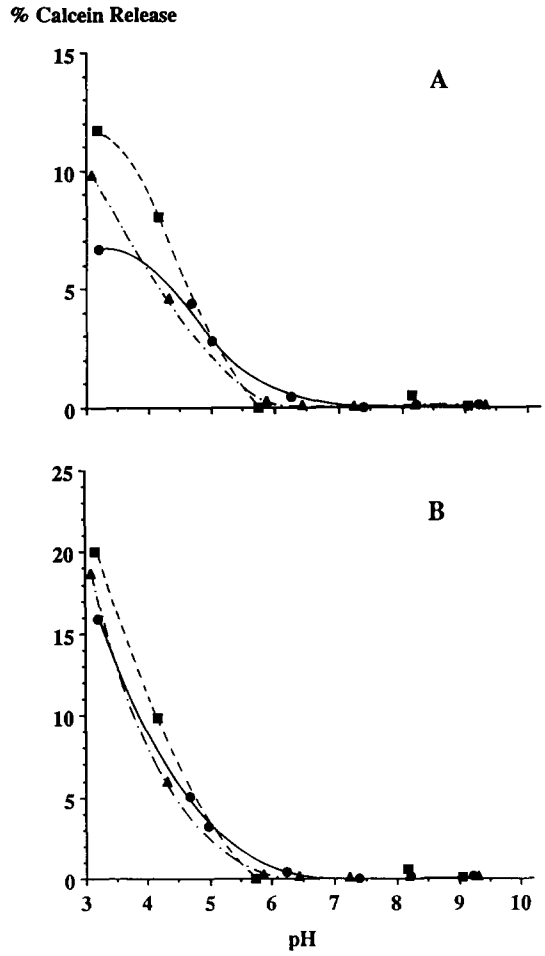

Fig. 5. Release of calcein from the liposomes as a function of pH. 20 μM liposomes composed of (●) PC/Chol (7:2, mol/mol), (■) PC/Chol/SA (7:2:1, mol/mol) or (▲) PC/Chol/PS (7:2:1, mol/mol) were incubated in 2 ml Tris 150 buffer with a pH ranging from 2.3 to 9. The release of the quenched calcein (175 mM) was monitored for 10 min before Triton X-100 was added to disrupt the remaining liposomes. Release was determined on the assumption that the protons entered vesicles (B) or not (A).

pH by 1 unit resulted in a similar release profile as that obtained for the charged liposomes containing SA or PS.

3.2.4. Effect of cholate on calcein release from liposomes

The fluorescence intensity from calcein-loaded liposome samples was monitored as time elapsed after the addition of various cholate concentrations. The addition of cholate at concentrations above 2 mM induced a drastic increase in fluorescence intensity as shown in Fig. 6. The fraction of release (R) was calculated from the kinetic profiles as indicated in Section 2 and plotted in Fig. 7 against the cholate concentration. Fluorescence F and F_T were measured at equilibrium after the addition of cholate and Triton X-100, respectively. A remarkable point was the similarity of the reponse obtained with the three liposome types studied. Whatever their surface charge, the liposomes were totally disrupted by a cholate concentration higher than 4 mM, i.e. for a molar ratio of cholate-to-lipid (cholate/phospholipid) equal to 200. Increasing the phospholipid concentration, i.e. decreasing the cholate/phospholipid molar ratio, had no significant influence on the effect of cholate up to a lipid concen-

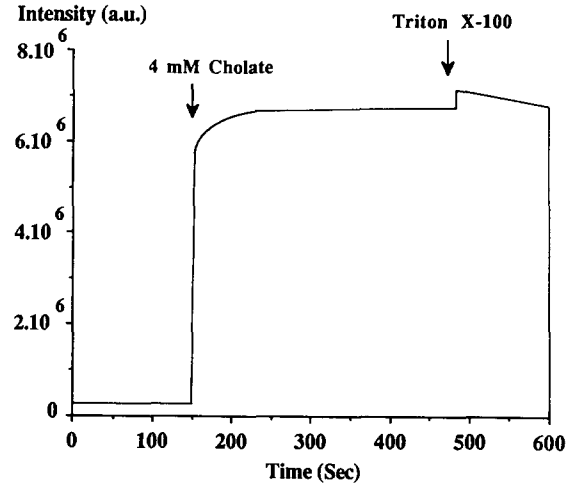

Fig. 6. Kinetic profile of the calcein release from liposomes under influence of cholate. 20 μM PC/Chol/PS liposomes (7:2:1, mol/mol) were incubated in Tris 150 buffer. At 150 s cholate was added to obtain a final concentration of 4 mM. The increase in the fluourescence intensity caused by the release of the quenched calcein (175 mM) was monitored until equilibrium was obtained. Subsequently 100 μl Triton X-100 was added to disrupt the remaining liposomes.

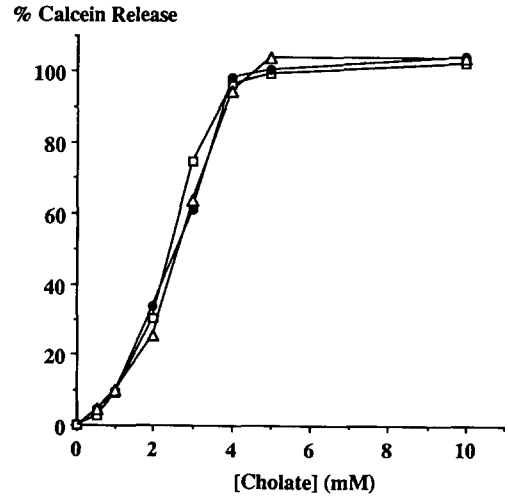

Fig. 7. Effect of cholate concentration on the release of self-quenched calcein from different liposome preparations. 20 μM liposomes composed of (□) PC/Chol (7:2, mol/mol), (●) PC/Chol/SA (7:2:1, mol/mol) or (△) PC/Chol/PS (7:2:1, mol/mol) were incubated with varying concentrations of cholate prepared in Tris 150 buffer. The release of the quenched calcein (175 mM) was monitored until equilibrium was reached before 0.5% Triton X-100 was added to disrupt the liposomes still intact. The percentage release was determined as described in Section 2.

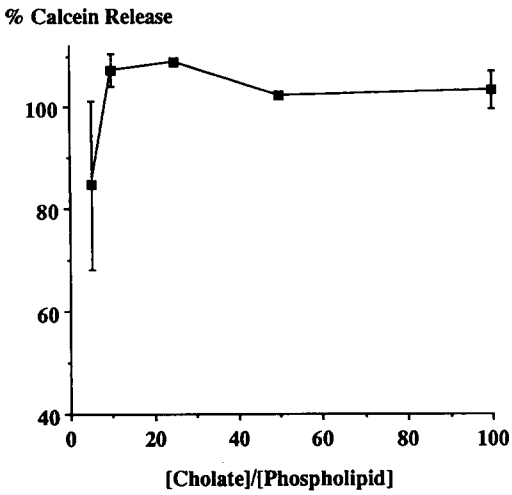

Fig. 8. Influence of the phospholipid concentration on the disruption of calcein-loaded liposomes by 5 mM cholate. Different amounts of PC/Chol liposomes (7:2, mol/mol) entrapping 175 mM calcein were incubated at 25°C in the presence of 5 mM cholate. Release of the encapsulated calcein was monitored for 10 min by fluorimetry before 0.5% Triton X-100 was added to disrupt the liposomes still intact. Vertical bars represent the S.E. of at least 2 experiments. The value for cholate/phospholipid = 5 was the mean ± S.E. of eight determinations.

tration equal to $5 \cdot 10^{-4}$ M, i.e. a cholate/phospholipid molar ratio equal to 10 (Fig. 8). Release was slightly lower when the molar ratio came down to 5. However, it should be noted that at these low ratios, high lipid and subsequently high total calcein concentrations had to be used, thereby making the fluorescence analysis somewhat aleatory.

4. Discussion

The structure of the amphipathic α-helix of CT makes it possible to predict interactions of the polypeptide with lipidic membranes, as shown by workers such as Epand et al. [9], who demonstrated that lipid solubilization particularly occurs with negatively charged lipids. This raises the question of the mode of CT encapsulation and release from the liposomes. In this study we have investigated the association of salmon CT with small unilamellar liposomes composed of PC, cholesterol and positively or negatively charged phospholipids in the presence of various external pH values and bile salts.

Varying the pH of the incubation medium had almost no effect on the rate of remaining CT associated with the liposomal fraction. This was clearly observed for neutral and charged liposomes for pH values ranging from 5 to 9, while pH 2 seemed to induce a minimal release of the protein. DPPG-containing liposomes appeared to release higher amounts of [^{125}I]CT under all pH conditions. This basic instability might be due to the less-than-ideal miscibility of the different phospholipids in this liposome composition. Indeed, DPPG exists in the gel phase at the temperature of the experiment; this could result in clustering or lateral phase separation rendering the bilayer less-tightly packed.

The concomitant analysis of liposome-entrapped calcein leakage, which was taken as a probe of bilayer stability, also demonstrates that none of the liposomes of the compositions tested were significantly affected by changing the external pH values in the range 5 to 9. Some leakage was observed with the lowest pH values tested. However, the induced leakage did not exceed 15%, thereby suggesting that even when the bilayer membrane becomes more permeable, no drastic disruption of the membrane occurs. Previous results have shown that an increased permeability of the liposome membrane at low pH could be attributed to phospholipid polar head protonation, which renders them less likely to form tight bilayer structures [19]. However, it should also be noted that at the lowest pHs tested, the calcein protonation degree is increased [18], and this is also likely to increase its membrane diffusion coefficient, as has previously been shown with carboxyfluorescein [20].

However, our results with calcein and CT show that the liposomal membrane barrier resists a low pH and that the acidic medium does not abolish the entrapment of molecules in the liposomes. This can be deduced from the integration of the calcein response at low pHs in our fluorescence analysis. Such pH values have until now been considered a hindrance to the use of the marker under acidic conditions.

The situation is different when the effect of bile salts, such as cholate, on both CT and calcein release is considered. Partial CT release is induced by 10 mM cholate to a degree dependent on the lipid concentration. The CT release never exceeded 50% of the total amount of the peptide initially encapsulated. In contrast, our calcein release experiments show that total liposome disruption was obtained with a 4 mM cholate concentration. CT and calcein are therefore obviously released according to different patterns. From the calcein release results, it clearly appears that cholate had a detergent effect with all types of liposomes tested. This effect was total at 4 mM cholate; yet at the same time and for even higher cholate concentrations, a significant proportion of CT remained associated with a fraction shown to be liposomal according to gel-filtration criteria. This strongly suggests that as liposomes are solubilized by cholate, new complexes are formed involving CT, lipids and very probably cholate. These structures could be analogous to those formed during membrane protein reconstitution in liposomes in the presence of detergent [21]. These structures appeared to differ in nature according to the lipid-to-detergent

ratio [22]: mixed micelles, bilayer disks or elongated structures. Disked-shaped particles with sizes varying from 5 to 50 nm have also been observed previously with DMPG in the presence of CT [9]. The interactions leading to these structures could be established through the amphipathic α-helix of the CT molecule and mediated by cholate. The present experiments do not allow speculation about the nature of the complexes. However, what may be considered is the possible occurrence of non-liposomal structures containing CT and lipids large enough to be eluted with the void volume of the column as intact liposomes. This could explain why CT is never completely released from the lipidic fraction, whereas calcein, the aqueous phase marker, is totally recovered in the external medium under the same conditions.

It now remains to be determined if the suspected protein–lipid complexes constitute a satisfactory carrier for CT, and if a certain degree of protection of the molecule is achieved within these complexes.

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