Amphipathic Helix and Its Relationship to the Interaction of Calcitonin with Phospholipids†

R. M. Epand,* R. F. Epand, R. C. Orlowski, R. J. Schlueter, L. T. Boni, and S. W. Hui

ABSTRACT: Salmon, porcine, and human calcitonins interact with phosphatidylglycerol to form water-soluble complexes, but these peptides do not interact with the zwitterionic lipids phosphatidylcholine or sphingomyelin. The calcitonins are more helical in the presence of dimyristoylphosphatidylglycerol than in its absence, but human calcitonin is considerably less helical than the other two, particularly in the presence of the lipid. This may explain the previously reported faster rate of degradation of human compared with salmon calcitonin in vivo. The ability of human calcitonin to solubilize dimyristoylphosphatidylglycerol and to alter the phase transition properties of this phospholipid while maintaining a low content of helix indicates that the presence of an amphipathic helix is not a requirement for these effects. The binding of salmon calcitonin to dimyristoylphosphatidylglycerol has been studied by determining the dependence of the circular dichroism properties of the peptide on the concentration of lipid. At 25 °C, salmon calcitonin binds to five molecules of dimyristoylphosphatidylglycerol with an affinity constant of 1×10^5 M^{-1}. Little change in these parameters is observed at 38 °C, and the complex is stable over a wide range of temperatures both above and below the phase transition temperature. The rate of reaction of salmon calcitonin with dimyristoylphosphatidylglycerol is rapid at or above the phase transition temperature of the lipid but not at low temperatures. Salmon calcitonin also interacts with egg phosphatidylglycerol. These results demonstrate that salmon calcitonin can react with phosphatidylglycerol at or above its phase transition temperature to form complexes which are at least kinetically stable both above and below the phase transition temperature. Salmon calcitonin can solubilize mixtures of dimyristoylphosphatidylglycerol and dimyristoylphosphatidylcholine containing 25% or more of the former phospholipid. The helical content of the peptide in the presence of these lipid mixtures is dependent on the fraction of the lipid which is phosphatidylglycerol, with larger fractions of this lipid leading to the formation of a higher helical content. At 25% phosphatidylglycerol, salmon calcitonin can solubilize the lipid mixture without much increase in the helix content of the peptide, again demonstrating that an amphipathic helical structure is not required for the solubilization of phospholipids. Ionic bonding appears to be an important component in the binding of the cationic calcitonins to phospholipids. Salmon calcitonin binds to the acidic phospholipids phosphatidylinositol and phosphatidic acid, but not to zwitterionic phospholipids. In addition, high concentrations of NaCl cause the dissociation of the complex between salmon calcitonin and dimyristoylphosphatidylglycerol. Nevertheless, some specificity is exhibited by the interaction of salmon calcitonin and lipids, with only small effects on the conformation of the peptide being observed with the negatively charged sulfatide, cardiolipin, or phosphatidylserine. Calcitonin has no effect on the phase transition properties of dimyristoylphosphatidylcholine, but it greatly broadens the phase transition and lowers the transition enthalpy of dimyristoylphosphatidylglycerol. The effect of salmon calcitonin on the phase transition properties of mixtures of phosphatidylglycerol and phosphatidylcholine indicates that if the peptide induces any lateral phase separation of the lipids it is not very extensive.

Proteins are major constituents of biological membranes. Since proteins contain many polar side chains, a protein which can interact with the hydrophobic environment of membrane lipids must be arranged in such a manner that its polar groups are either exposed to the aqueous environment or associate with other polar groups to form ion pairs or hydrophilic channels. This requires a spacial segregation of the hydrophobic and hydrophilic groups of a membrane protein. The hydrophobic groups of many integral membrane proteins are segregated as a result of the primary structure of the protein in which a portion of the sequence contains a high percentage of hydrophobic residues, but many other proteins which can interact with lipids segregate their hydrophobic groups as a result of the folding of the peptide chain. An example of this latter type of protein is the apolipoprotein C-I, which is believed to segregate its hydrophobic residues as a result of the formation of an amphipathic helix (Segrest et al., 1974). Other apolipoproteins (Segrest, 1977), amyloid A (Segrest et al., 1976), glucagon (Jones et al., 1978), and mellitin (Terwilliger et al., 1982), can also form amphipathic helices, and it may be this property which enables them to solubilize phospholipids.

Lipids may be directly involved in the binding of many drugs and hormones to specific cell-surface receptor sites. There is evidence that binding to lipids is required for the activity of cholera toxin (Critchley et al., 1981; Lai, 1980), enkephalin (Law et al., 1978), and thyrotropin (Kohn et al., 1980). Many peptide hormones, including glucagon (Epand et al., 1977), β-endorphin (Wu et al., 1979, 1981a), and growth hormone (Segrest & Feldmann, 1977) as well as secretin, parathyroid hormone, calcitonin, and others, may interact with lipids through the formation of an amphipathic helix. This property is a feature of many membrane-active peptides and may be related to their biological activity (Epand, 1980, 1983; Hammonds et al., 1982; Taylor et al., 1983). In addition, these hormones provide relatively small, well-defined systems with which to study an aspect of protein–lipid interactions.

Calcitonin is a particularly interesting hormone for the study of the association of peptides with lipids. Calcitonin is a single

† From the Department of Biochemistry, McMaster University Health Sciences Centre, Hamilton, Ontario L8N 3Z5, Canada (R.M.E. and R.F.E.), the Armour Pharmaceutical Company, Kankakee, Illinois 60901 (R.C.O. and R.J.S.), and the Department of Biophysics, Roswell Park Memorial Institute, Buffalo, New York 14263 (L.T.B. and S.W.H.). *Received January 28, 1983; revised manuscript received May 23, 1983.* This work was supported by the Medical Research Council of Canada (Grant MA-7654).

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polypeptide chain of 32 amino acids which contains a sequence from approximately residue 8 to residue 22 which can form an amphipathic helix. The sequence undergoes considerable species variation but maintains a very regular spacing of hydrophobic amino acids in this region. Three predictive methods for calculating the secondary structure of proteins indicate that the helix-forming potential of ultimobranchial calcitonins (e.g., salmon) is much greater than that of thyroidal calcitonins (e.g., human and porcine) (Merle et al., 1979). This has been suggested as an explanation for the greater biological potency of the former calcitonins (Merle et al., 1979). The sequences of the calcitonins used in this work are shown as follows:

human calcitonin

Cys^{+}-Gly-Asn-Leu-Ser-Thr-Cys-*Met*-*Leu*-Gly-Thr-*Tyr*-Thr-Gln-Asp^{-}-*Phe*-Asn-Lys^{+}-*Phe*-$His^{\delta+}$-Thr-*Phe*-Pro-Gln-Thr-*Ala*-*Ile*-Gly-Val-Gly-Ala-Pro-NH_2

porcine calcitonin

Cys^{+}-Ser-Asn-Leu-Ser-Thr-Cys-*Val*-*Leu*-Ser-*Ala*-*Tyr*-Trp-Arg^{+}-Asn-*Leu*-Asn-Asn-*Phe*-$His^{\delta+}$-Arg^{+}-*Phe*-Ser-Gly-*Met*-Gly-Phe-Gly-Pro-Glu^{-}-Thr-Pro-NH_2

salmon calcitonin

Cys^{+}-Ser-Asn-Leu-Ser-Thr-Cys-*Val*-*Leu*-Gly-Lys^{+}-*Leu*-Ser-Gln-Glu^{-}-*Leu*-$His^{\delta+}$-Lys^{+}-*Leu*-Gln-Thr-*Tyr*-Pro-Arg^{+}-Thr-Asn-Thr-Gly-Ser-Gly-Thr-Pro-NH_2

The Cys residues 1 and 7 are bonded by a disulfide linkage. Groups carrying a charge at neutral pH are indicated, and hydrophobic residues of a putative amphipathic helix are italicized.

Experimental Procedures

Materials

Synthetic salmon calcitonin (sCT),[1] natural porcine calcitonin (pCT), and synthetic human calcitonin (hCT) were prepared by Armour Pharmaceutical Co., Kankakee, IL. Glucagon was purchased from the Elanco Corp. The amino acid analysis of these peptides agreed well with the composition expected from their known sequences.

Dimyristoylphosphatidylglycerol (DMPG) and dipalmitoylphosphatidylglycerol (DPPG) were purchased from Avanti Polar Lipids, Inc., or from Calbiochem Corp. Dimyristoylphosphatidylcholine (DMPC) was from Calbiochem, and dipalmitoylphosphatidylcholine (DPPC), bovine brain sphingomyelin, bovine heart cardiolipin, and phosphatidylglycerol and phosphatidic acid both prepared from egg yolk lecithin were from Sigma Chemical Co. Bovine sulfatide was from Analab and yeast phosphatidylinositol from Serdary Research Labs. Bovine brain phosphatidylserine was prepared by the procedure of Papahadjopoulos & Miller (1967). The purity of the lipid preparations was confirmed by thin-layer chromatography and/or differential scanning calorimetry (DSC).

Methods

Buffer. Unless otherwise indicated, the buffer used was 20 mM Pipes, 1 mM EDTA, and 150 mM NaCl containing 0.02 mg/mL NaN_3, pH 7.40. For the gel filtration experiment and the solubilization experiment, 0.1 M ammonium acetate at pH 7.4 was used as buffer, and for some circular dichroism (CD) measurements, 0.005 M acetic acid or the Pipes buffer containing 1.5 M NaCl was used.

Formation of Lipid–Protein Complexes. Lipid was first deposited as a film from a 2:1 (v/v) chloroform:methanol solution by solvent evaporation under nitrogen followed by removal of traces of solvent for at least 1 h in a vacuum oven at 40 °C with a liquid nitrogen trap. In some cases where only a single lipid was used which was solubilized by the calcitonin, the lipid was added as a solid. To the dry lipid was added a solution of calcitonin, and the mixture was vortexed above the phase transition temperature of the lipid. The mixture was then heated and cooled between 10 and 40 °C to aid in equilibration.

Peptide Concentration. The concentration of calcitonin was determined from its weight concentration or its ultraviolet absorption spectrum or by amino acid analysis using an internal standard of norleucine. The peptide concentration was obtained from the ultraviolet spectra by using molar extinction coefficients calculated from values given for the absorption characteristics of *N*-acetyl methyl esters of the aromatic amino acids and of cystine (Sober, 1970). The observed absorption maxima of sCT, pCT, and hCT were 275, 272, and 275 nm, respectively, with corresponding extinction coefficients calculated to be 1515, 6817, and 1531 cm^{-1} M^{-1} for the three peptides at their absorption maxima. The concentration of sCT or hCT calculated from the absorption spectra was routinely used and agreed to within 10% of that calculated on the basis of peptide weight or from amino acid analysis. In the case of pCT, the peptide was not very soluble in the Pipes buffer at pH 7.40. The pCT solution was clarified by centrifugation and the peptide concentration in the supernate determined from the absorption spectra and amino acid analysis. The peptide is more soluble at high pH, but the pH was maintained at 7.4 to be comparable with other studies. For solubilization and gel filtration experiments, a modified form of the Folin–Lowry assay with sodium dodecyl sulfate was employed (Hess et al., 1978) with calcitonin itself as a standard. This allowed for increased sensitivity as well as the avoidance of light scattering and spectral perturbation caused by the presence of lipid.

Lipid Concentration. Lipid concentrations were calculated from the phosphate content of the solutions after digestion with perchloric acid (Bartlett, 1959), except for sulfatide whose concentration was estimated simply by weight.

Solubilization of Lipid Mixtures by sCT. A calcitonin solution (90 μM) in 0.1 M ammonium acetate, pH 7.4, was added to lipid films containing varying amounts of DMPC and DMPG to give a final total lipid concentration of 1 mM. Controls were run in which the buffer was added to the lipid films in the absence of peptide. The mixtures were vortexed at about 35 °C for 15 s and cooled on ice. They were then centrifuged for 5 min in a Beckman Microfuge B at room temperature. Peptide and lipid concentrations of the supernate were determined.

Gel Exclusion Chromatography. A solution of sCT and DMPG was analyzed by gel filtration on a 1.25 × 50 cm column of Sepharose 4B with 0.1 M ammonium acetate, pH 7.4, as eluent as previously described (Jones et al., 1978).

Right-Angle Light Scattering. The rate of dissolution of a suspension of lipid was monitored by right-angle light scattering by using a Perkin-Elmer MPF-44 spectrofluorometer with both excitation and emission monochromators set

[1] Abbreviations: sCT, salmon calcitonin; pCT, porcine calcitonin; hCT, human calcitonin; DMPG, dimyristoylphosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; CD, circular dichroism; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid.

Table I: Circular Dichroism of Calcitonin Solutions[a]

peptide	peptide concn (μM)	buffer	$[\theta]_{222}$ (deg cm^2 dmol^{-1} $\times 10^3$)
sCT	57	HOAc	−4.07
sCT	284	HOAc	−4.08
sCT	100	Pipes	−4.16
sCT	2000	Pipes	−4.59
sCT	90	Pipes-NaCl	−3.94
hCT	100	Pipes	−1.87
pCT	10	Pipes	−5.20

[a] $[\theta]_{222}$ is the mean residue ellipticity at 222 nm. Temperature, 25 °C; buffer, 0.005 M acetic acid (HOAc), or 20 mM Pipes, 1 mM EDTA, and 0.15 M NaCl containing 0.02 mg/mL NaN_3, pH 7.40 (Pipes), or the above Pipes buffer containing 1.5 M instead of 0.15 M NaCl (Pipes-NaCl).

at 450 nm, using 1-nm slit widths and a standard 1-cm path-length sample cell. The sample was maintained at constant temperature and was continually stirred by means of a small magnetic stirring bar placed in the cuvette below the light path.

Fluorescence Emission Spectra. Fluorescence spectra were measured with a Perkin-Elmer MPF-44 spectrofluorometer in the ratio mode using 4-nm slit widths. Spectra of identical solutions of peptide in the presence and absence of lipid were compared. Peptide concentrations were sufficiently low to make the inner filter correction small.

Circular Dichroism (CD). CD spectra were recorded in the wavelength range 250–190 nm on a Cary 61 spectropolarimeter equipped with a thermostated cell holder. The CD data were expressed as the mean residue ellipticity by using 107.4, 112.6, and 106.8 as the mean residue weights of sCT, pCT, and hCT, respectively.

Differential Scanning Calorimetry (DSC). DSC scans were performed with a Microcal MC-1 calorimeter (Microcal Inc., Amherst, MA). A sample volume of 0.9569 mL was used. The scan rate was accurately measured and was always 0.49–0.51 K/min. The instrument was calibrated electrically. Pipes buffer was always used for the reference cell. A flat base line was observed over the temperature range 10–70 °C with buffer in both sample and reference cells or with 100 μM sCT solution in the sample cell.

Electron Microscopy. The sample was divided into two aliquots. One aliquot was used for negative staining with 1% ammonium molybdate. The other aliquot was used for freeze–fracture preparation. The rapid freezing procedure was described previously (Epand et al., 1981). Both preparations were performed at room temperature.

X-ray Scattering. For X-ray scattering studies, the samples in 1.5-mm glass tubes (Charles Supper, Natick, MA) were exposed to a line-focused X-ray beam which was generated from a copper target in a Jarrel-Ash generator. The scattering patterns were recorded by Kodak X-ray film.

Results

Conformation of Calcitonin in Aqueous Solution. All of the calcitonins studied have very little, if any, helical content. This is demonstrated by the low magnitude of the ellipticity at 222 nm (Table I). The CD spectrum of sCT in acid is not dependent on peptide concentration over the range studied, and no dependence of the conformation on the pH of the buffer was noted (Table I). There is a small temperature dependence of the CD spectrum of sCT (Figure 1). The peptide appears to become more ordered at higher temperatures, suggesting hydrophobic bonding. The CD spectrum observed for pCT was similar to that previously reported for this peptide in acetic

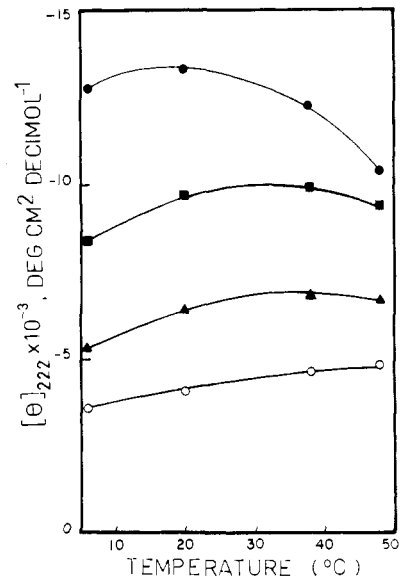

FIGURE 1: Temperature dependence of the circular dichroism of sCT in the presence and absence of DMPG. Pipes buffer, peptide concentration 100 μM. Peptide alone (○); DMPG:sCT molar ratios of 27 (●), 4 (■), and 1.5 (▲).

acid solution (Brewer & Edelhoch, 1970). hCT has a somewhat less ordered structure, as revealed by CD, than the other two calcitonins.

Interaction of Calcitonin with Zwitterionic Phospholipids. The addition of a 100 μM solution of sCT or hCT to DMPC to give a final lipid concentration of 1 mM did not result in any change in the CD spectrum of the peptide, and the mixture remained turbid. No change in the morphology of this lipid in the presence of sCT was observed with electron microscopy (data not shown). Sonication of the sCT–DMPC mixture also did not result in any change in the CD spectra. Addition of a 10 μM solution of pCT to DMPC to give a final lipid concentration of 0.4 mM likewise did not alter the CD spectra, nor was any solubilization of the lipid apparent. A 90 μM solution of sCT was added to a lipid film of bovine brain sphingomyelin to give a final lipid concentration of 900 μM. The magnitude of $[\theta]_{222}$ of the peptide at 25 °C increased to −5870 deg cm^2/dmol. This small increase could result from a contribution of sphingomyelin to the CD spectrum as this lipid has a large negative ellipticity band centered at 197 nm.

The same suspensions used to measure the CD properties of the peptides in the presence of DMPC (unsonicated) were used for DSC analysis. In general, no effect of the peptides on the position or magnitude of either the major transition or the "premelt" transition was noted (Table II). However, there was a small lowering of the enthalpy of DMPC in the presence of pCT. The results from the CD, electron microscopy, and DSC analyses indicate that none of the calcitonins interacts strongly with zwitterionic lipids.

Interaction of Calcitonin with Phosphatidylglycerol. In contrast to the zwitterionic lipids, the acidic phospholipid, DMPG, has a large effect on the conformations of sCT and pCT, although hCT still has a very low helix content even in the presence of this lipid (Table III). Addition of further increments of DMPG to the hCT solution, up to a lipid:peptide molar ratio of 35:1, did not cause any further change in the CD spectrum of this peptide. The shape of the CD curve of sCT in the presence of DMPG demonstrated the formation of a peak at 222 nm, indicative of an increased helical content (Figure 2). As expected, a sonicated sample of DMPG in the absence of peptide showed no CD properties in this

Table II: Phase Transition Enthalpy from Differential Scanning Calorimetry for Phospholipids with and without Calcitonin[a]

lipid(s)	peptide	T_c (°C)	ΔH (kcal/mol of lipid)
DMPC	none	14.9	1.0
		23.63	5.25
DMPC	sCT	14.2	0.9
		23.61	5.31
DMPC	hCT	14.2	0.8
		23.62	4.8
DMPC	pCT	14	nd[b]
		23.60	4.2
DMPG	none	22.5	4.2
DMPG	sCT	23.1	1.8
DMPG	hCT	21.13 (with high-temp shoulder)	1.2
DMPG	pCT	23	1.5
DPPG	none	32.94	0.73
		39.8	9.9
DPPG	sCT	37.4, 39.25, and 40.2 (overlapping)	4.85
52% DMPC, 48% DPPG	none	30.75	5.4
52% DMPC, 48% DPPG	sCT	31.13	5.7
48% DPPC, 52% DMPG	none	32.5	6.9
48% DPPC, 52% DMPG	sCT	31.1 and 34.5 (overlapping)	6.9

[a] Buffer, 20 mM Pipes, 1 mM EDTA, and 0.15 M NaCl containing 0.02 mg/mL NaN_3, pH 7.40; peptide concentration, when present, 100 μM; total lipid:peptide molar ratio, 10 (except for a pCT peptide concentration of 10 μM with a lipid:peptide molar ratio of 55). T_c is the temperature of the peaks in the DSC curves and is recorded to the precision of the instrument. [b] nd, not determined.

Table III: Circular Dichroism of Calcitonin in the Presence of Phospholipids[a]

peptide	lipid	$[\theta]_{222}$ (deg cm^2 dmol^{-1} × 10^3)
sCT	DMPG	−12.35
sCT	DMPG-NaCl[b]	−6.76
hCT	DMPG	−2.24
pCT	DMPG	−8.13
sCT	DPPG	−8.99
sCT	egg phosphatidylglycerol[c]	−6.70
sCT	bovine brain sulfatide	−4.90
sCT	bovine heart cardiolipin	−5.42
sCT	bovine brain phosphatidylserine	−5.69
sCT	yeast phosphatidylinositol	−13.10
sCT	phosphatidic acid[c]	−14.60

[a] Temperature, 25 °C; buffer, 20 mM Pipes, 1 mM EDTA, and 0.15 M NaCl containing 0.02 mg/mL NaN_3, pH 7.40; peptide concentration, 100 μM; lipid:peptide molar ratio, 10 (except for a pCT peptide concentration of 10 μM with a lipid:peptide molar ratio of 55). [b] Buffer containing 1.5 M instead of 0.15 M NaCl. [c] Made from egg phosphatidylcholine.

wavelength range. Increasing the NaCl concentration from 0.15 to 1.5 M has little effect on the CD properties of sCT as a free peptide (Table I), but it markedly diminishes the enhanced helical content observed in the presence of DMPG (Table III). The increased salt concentration also led to the appearance of turbidity in the solutions of the sCT–DMPG complex, suggesting the importance of ionic interactions in the maintenance of the structure of this lipid–peptide complex. The temperature dependence of the CD of solutions of sCT in the presence of DMPG indicates that the maximum negative

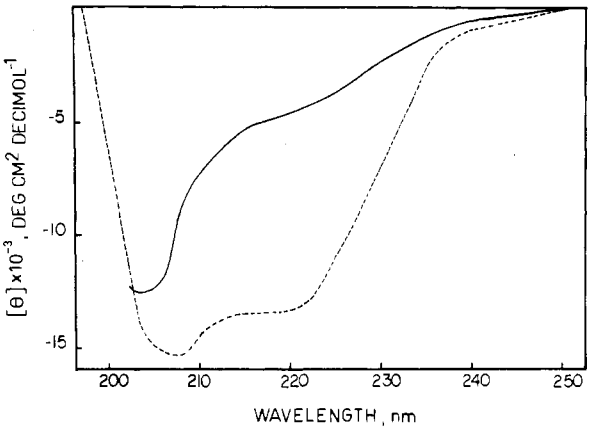

FIGURE 2: Circular dichroism spectra of sCT (100 μM) alone (solid line) or in the presence (dashed line) of 2.4 mM DMPG. Pipes buffer, 25 °C.

ellipticity occurs in the temperature region of 20–30 °C (Figure 1). The decrease with temperature was gradual, and no time dependence of the CD was observed over a period of approximately 30 min. Through the entire temperature range from 6 to 45 °C, the magnitude of the ellipticity was always considerably larger in the presence of lipid than in its absence (Figure 1).

In all cases, including hCT, the peptide appeared to solubilize the lipid, and the resulting DMPG–calcitonin solutions were transparent with no visible turbidity. The ability of the calcitonins to disrupt the multilamellar bilayer structure of DMPG was confirmed by electron microscopy. The complexes formed between sCT and DMPG show what seem to be rod-shaped objects which may represent side views of disk-shaped particles. At higher concentrations, which usually occurs during the negative-stain process, these particles tend to stack as shown in Figure 3A. Such behavior is also typical of high-density lipoprotein recombinants (Forte et al., 1971; Tall et al., 1977; Jonas et al., 1980). However, no diffraction lines, suggestive of regular stacking, were observed by X-ray scattering from a solution of the sCT–DMPG complex. Freeze–fracture electron micrographs of this preparation showed round-shaped particles of about the same dimension as that of the negatively stained particles (Figure 3B). Since the fracture plane did not cut through the particles, precise boundaries of the particles are difficult to define. The size of the sCT–DMPG complexes depends on their lipid to protein ratios. Electron micrographs of the products formed from different initial lipid:protein ratios showed that the size of the complex increased with increasing lipid:protein ratio. The mean length of the complex increased from 5 nm at a lipid:protein ratio of 0.7 to about 50 nm at a lipid:protein ratio of 68. hCT also destroys the multibilayer structure of DMPG to produce particles. At a lipid to protein ratio of 10, the particles shaped like partly twisted disks are about 50 nm in diameter, but with less tendency to show stacking by negative staining (Figure 3C). The calcitonin–DMPG particles are about 8 nm thick, similar to those of the lipoprotein conjugates and suggestive of a disk structure composed of a single bilayer. Thus, in the case of hCT, a discoidal lipoprotein particle appears to be formed without a large increase in the helix content of the peptide.

The relatively low molecular weight particle which is formed from unsonicated, multilamellar DMPG in the presence of sCT is also demonstrated by gel filtration. In the presence of DMPG a portion of the calcitonin is eluted in a higher molecular weight fraction along with the phospholipid (Figure

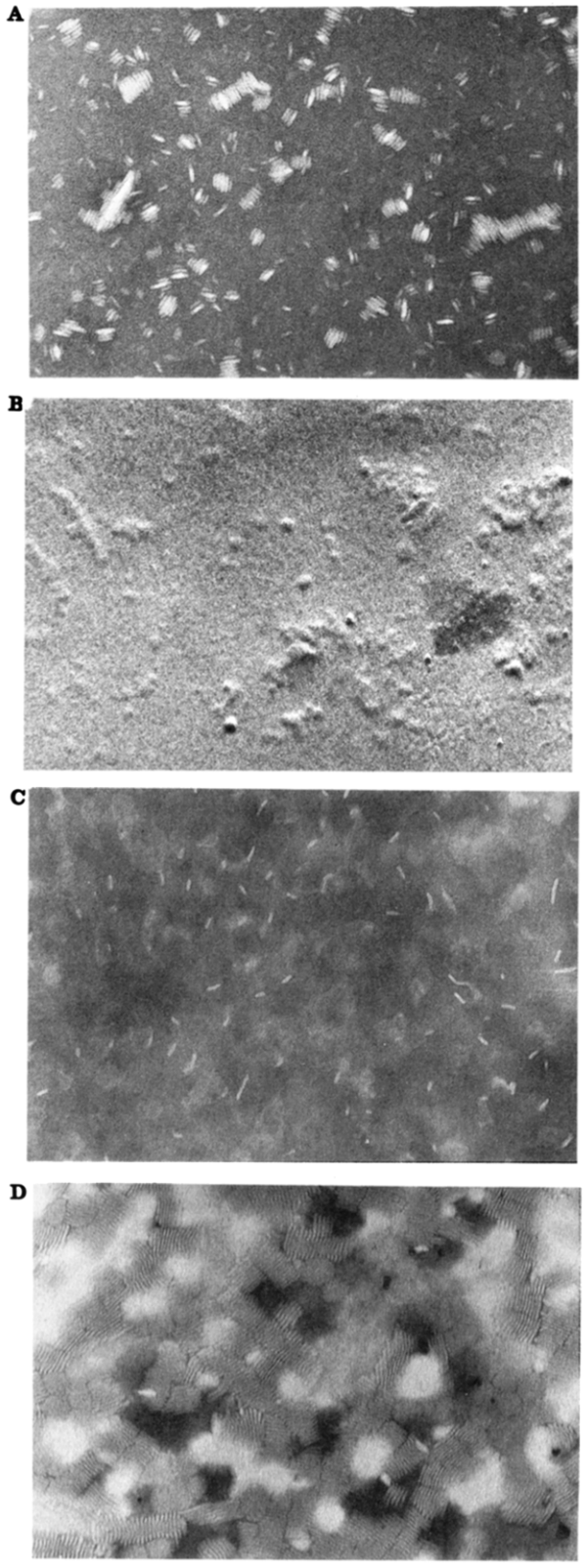

FIGURE 3: Electron micrographs of calcitonin lipoprotein particles. Samples prepared at room temperature. Magnification 96750×. (A) Negative stain of sCT (400 μM) and DMPG (4.4 mM); (B) freeze–fracture of same sCT–DMPG preparation; (C) negative stain of hCT (100 μM) and DMPG (1 mM); (D) negative stain of sCT (400 μM) and a mixture of DMPC (5 mM) and DMPG (1.25 mM).

4). This fraction has a Stokes radius which is probably somewhat less than that of sonicated DMPG. The peptide in the high molecular weight fraction elutes slightly behind the lipid. As a result, the lipid to peptide stoichiometry is not uniform through the peak but decreases with increasing elution

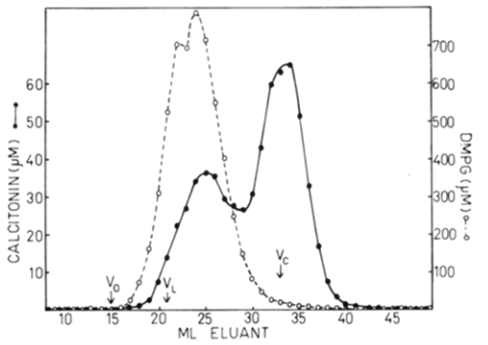

FIGURE 4: Sepharose 4B chromatography of sCT–DMPG. The 1.25 × 50 cm column was eluted with 0.1 M ammonium acetate (pH 7.4) at 4 °C at 6 mL/h. A solution containing 0.6 μmol of sCT and 7 μmol of DMPG in ammonium acetate was applied to the column. One-milliliter fractions were collected. V_O, V_L, and V_C mark the elution positions of multilamellar liposomes, sonicated DMPG, and sCT, respectively, when each was applied individually.

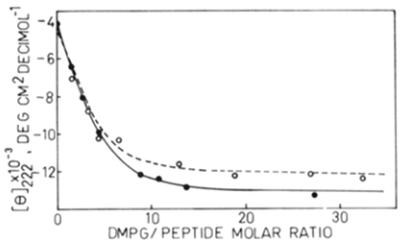

FIGURE 5: Dependence of the mean residue ellipticity of sCT (90 μM) at 222 nm on the molar ratio of DMPG to sCT. Pipes buffer, measurements at 25 °C (closed circles) and 38 °C (opened circles). Experimental points for both 25 and 38 °C were fitted to a single association constant of 1.03×10^5 M^{-1} for the binding of sCT to 5.3 molecules of DMPG. Solid and dashed lines correspond to 25 and 38 °C measurements, respectively.

volume. Samples taken from different fractions of the column eluent showed, by negative staining, decreasing size with increasing fraction number, confirming that particles with a lower lipid to peptide ratio have a lower molecular weight and are therefore more retained on the column. The average stoichiometry of the high molecular weight fraction (18–27 mL) is 23 molecules of DMPG per sCT. This suggests that sCT can solubilize more lipid than that which is required for inducing helix formation.

The dependence of the CD of a 100 μM solution of sCT on the concentration of DMPG fits a simple Scatchard analysis for single, noninteracting binding sites. At 25 °C, the affinity constant is 1.0×10^5 M^{-1} for the binding of sCT to a cluster of 5.3 ± 0.2 molecules of DMPG (Figure 5). The ellipticity measurements of sCT in the presence of DMPG at 38 °C can also be described by the same binding parameters as used for measurements at 25 °C, but the data show somewhat more scatter (Figure 5). The affinity of sCT for DMPG is thus independent of temperature, within experimental error, between 25 and 38 °C. We have not performed this titration at lower temperatures since the rate of reaction of sCT with DMPG is very slow under these conditions (vida infra) and it would be less certain that the equilibrium has been established.

Of the three calcitonins used, the only one which contains a tryptophan residue is pCT. The emission maximum of this residue for the peptide alone is similar to that for free tryptophan in solution and is independent of peptide concentration between 5 and 20 μM. DMPC (550 μM) does not alter the emission spectrum of pCT (10 μM), but DMPG (550 μM) causes the emission intensity to increase 1.7-fold and the emission maximum to be shifted from 349 nm for the pure

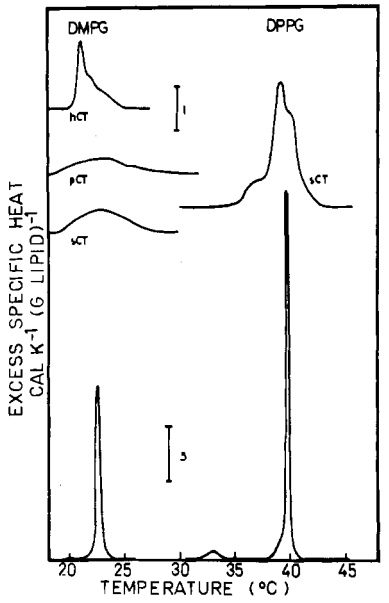

FIGURE 6: Differential scanning calorimetry curves. Pipes buffer, scan rate 0.5 K/min. Curves labeled DMPG and DPPG are for the pure phospholipids at concentrations of 0.8 and 1.0 mM, respectively. Note that the curves for the pure lipids (lowermost curves) are drawn to a different scale than those from the lipid–peptide solutions. The calibration mark for the pure lipids is the one given for 5 cal K^{-1} (g of lipid)$^{-1}$ while the 1 cal K^{-1} (g of lipid)$^{-1}$ calibration mark is for the remaining curves from samples containing calcitonin. The sCT curve around 23 °C is from 100 μM sCT–1 mM DMPG while the sCT curve around 40 °C is from 100 μM sCT–1 mM DPPG. The pCT sample contains 10 μM pCT and 0.55 mM DMPG. The hCT sample contains 100 μM hCT and 1 mM DMPG.

Table IV: Effect of Lipid:Peptide Ratio on the Thermotropic Transition of sCT–DMPG Mixtures[a]

sCT (μM)	DMPG (mM)	DMPG: sCT molar ratio	ΔH (kcal/mol of DMPG)	$\Delta T_{1/2}$ (°C)	T_c (°C)
0	0.8	∞	4.2	0.4	22.5
98	6.7	68	2.9	4.8	23.2
98	2.9	30	2.5	5.4	23.2
86	1.3	15	1.8	6.3	23.1
384	1.5	4	*b*	*b*	*b*

[a] Buffer, 20 mM Pipes, 1 mM EDTA, and 0.15 M NaCl containing 0.02 mg/mL NaN_3, pH 7.40. T_c is the temperature of maximum excess heat capacity, and $\Delta T_{1/2}$ is the width of the transition at half-height. [b] Transition not detectable.

peptide to 335 nm in the presence of DMPG. This suggests that the tryptophan residue of pCT is incorporated into a more hydrophobic environment in the presence of DMPG. The fluorescence emission from the tyrosine residue of sCT or hCT, excited at 275 nm, is centered at 304 nm, and its position is not altered by the presence of DMPG. The lipid causes an increase of 20% in the intensity of the emission from hCT but does not alter the emission intensity from sCT.

The major effect of sCT on the phase transition properties of DMPG was to increase the width of the phase transition, indicating a marked reduction in the cooperativity in the presence of sCT as well as the other calcitonins (Figure 6). There was also a reduction of the transition enthalpy and a slight increase in the transition temperature (Table II). This small increase in transition temperature also occurs for the hCT–DMPG complex even though there is also a sharper lower temperature component for the complex with this calcitonin (Figure 6). Decreasing the lipid–protein molar ratio with solutions of DMPG and sCT results in a gradual reduction in the enthalpy of the transition with little change in the transition temperature, T_c (Table IV).

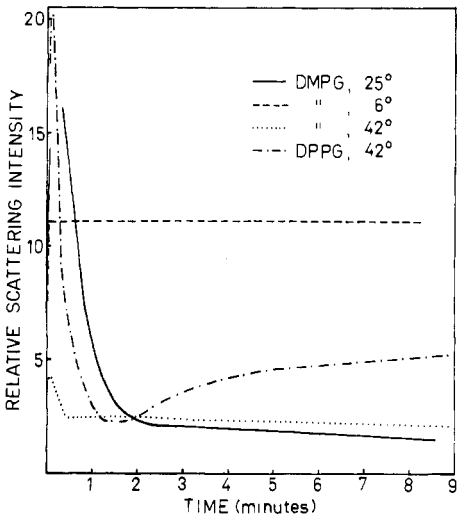

FIGURE 7: Time dependence of the right-angle light scattering at 450 nm of suspensions of 1 mM phosphatidylglycerol in Pipes buffer with 100 μM sCT. DMPG, 25 °C (—); DMPG, 42 °C (···); DPPG, 42 °C (–·–); DMPG, 6 °C (– – –).

The light scattering from a suspension of DMPG is reduced when it is mixed isothermally at 25 °C with sCT (Figure 7). The formation of the transparent solution at 25 °C occurs rapidly and appears to pass through an initially more aggregated stage during the first half minute of reaction. A highly aggregated state of the glucagon–DMPC complex at temperatures slightly above the phase transition temperature of the lipid has also been previously observed (Epand, 1978). At 6 °C, a suspension of DMPG in the gel state is not clarified by sCT, although some interaction of the lipid and peptide appears to occur, leading to increased light scattering (Figure 7). The increased light scattering observed with the lipid suspension in the presence of sCT may occur because the cationic peptide can shield the negative surface charge of DMPG. At 42 °C, the peptide again solubilizes DMPG. The reaction occurs rapidly but without the formation of a more highly aggregated intermediate (Figure 7). The initial scattering from the lipid at zero time is much lower than that for the other cases because of the known dependence of light scattering intensity on the phase of lipids in suspension, including phosphatidylglycerols (Findlay & Barton, 1978). At the same temperature of 42 °C, where DMPG is in the liquid-crystalline state, the DPPG–sCT complex is in the region of its phase transition (Figure 6). The rate of reaction of sCT with DPPG at 42 °C resembles that of sCT with DMPG at 25 °C, indicating that the rate of reaction and the reaction path are determined by the phase of the lipid rather than directly by the temperature. At 42 °C, continued incubation with sCT and DPPG results in the slow appearance of increased light scattering (Figure 7). Similar effects were observed with solutions of glucagon and DMPC mixed isothermally at temperatures slightly above the phase transition temperature of the pure phospholipid (Epand & Epand, 1980). Some other peptides and proteins such as glucagon (Epand & Epand, 1980) and apolipoprotein A-I (Pownall et al., 1978) react rapidly with DMPC only in the temperature region of the phase transition. We have also measured the rate of reaction of glucagon (20 μM) with DMPG (1 mM) by measuring the time dependence of both the fluorescence and light scattering of mixtures of glucagon with DMPG. In contrast to the case of sCT, glucagon reacts rapidly with DMPG, as it does with DMPC, only in the region of the phase

Table V: Circular Dichroism of sCT with Lipid Mixtures[a]

lipids used	mol % PG	temp (°C)	$[\theta]_{222}$ (deg cm^2 dmol^{-1} × 10^3)
DMPC-DMPG	77	25	−11.4
DMPC-DMPG	53	25	−8.0
DMPC-DMPG	27	25	−5.3
DMPC-DMPG	27	6	−5.1
DMPC-DMPG	27	38	−4.9
DMPC-DPPG	50	25	−4.8
DMPC-DPPG	50	40	−4.6
DPPC-DMPG	52	25	−8.4
DPPC-DMPG	52	40	−5.5

[a] Buffer, 20 mM Pipes, 1 mM EDTA, and 0.15 M NaCl containing 0.02 mg/mL NaN_3, pH 7.40; peptide concentration, 100 μM; lipid:peptide molar ratio, 10.

transition and not at higher temperatures where the complex is not stable.

The difference between glucagon and sCT in their ability to form complexes with phosphatidylglycerol in the liquid-crystalline state is also demonstrated by their abilities to form complexes with egg phosphatidylglycerol. This lipid preparation has a phase transition temperature of about −15 °C (Ladbrooke & Chapman, 1969) and is therefore in the liquid-crystalline phase in water. Addition of a solution of sCT to a lipid film of egg phosphatidylglycerol at a lipid to peptide molar ratio of 10 leads to an apparent solubilization of the lipid at room temperature although the solution is slightly hazy in contrast to the crystal-clear solutions formed by DMPG and sCT. Negative-stain electron micrographs of this solution of egg phosphatidylglycerol with sCT reveal individual disks of dimensions 50 × 7 nm as well as multilamellar vesicles, presumably of undissolved egg phosphatidylglycerol. The vesicles account for the turbidity of the solution. Egg phosphatidylglycerol can also enhance the helix content of sCT, although it is not as effective as the synthetic phosphatidylglycerols (Table III). In contrast, glucagon (20 μM) shows no apparent solubilization of egg phosphatidylglycerol (1 mM). There is also no apparent increase in the helix content of glucagon in the presence of this phospholipid nor is there a shift in the fluorescence emission spectrum of glucagon induced by egg phosphatidylglycerol. These results demonstrate than an acidic phospholipid which remains in the liquid-crystalline state can form complexes with sCT but does not do so with glucagon.

Interaction of sCT with Lipid Mixtures. Because sCT is able to form complexes with phosphatidylglycerol but not with phosphatidylcholine, it was of interest to determine the behavior of sCT with mixtures of the two phospholipids. DMPG and DMPC both have about the same phase transition temperature and transition enthalpy. The former lipid is solubilized by sCT and induces an increased helical content in this peptide while no effect is observed with DMPC. Mixtures of DMPC and DMPG containing 25% or more DMPG were quantitatively solubilized by sCT while less than 50% of the lipid remained in solution after centrifugation in the absence of the peptide (see Methods for criteria of solubilization). The extent of helix formation induced in the presence of lipid was very dependent on the amount of DMPG in the mixture, with more helix being induced by lipid mixtures containing a higher fraction of DMPG (Table V). There was very little temperature dependence of the CD of sCT with 27% DMPG and 73% DMPC (Table V). Mixtures of equimolar concentrations of DMPC with DPPG and of DPPC with DMPG were also solubilized by sCT but resulted in the induction of less helical content in the peptide than occurs with the pure phosphatidylglycerol. DMPG promotes a higher helical content is sCT than DPPG (Table III). Similarly, the former phospholipid, when mixed with phosphatidylcholine, promotes a higher helical content in sCT than does DPPG mixed with phosphatidylcholine (Table V). In the case of DMPG–DPPC mixtures, there is a marked temperature dependence of the CD, with the complex becoming less stable above the phase transition temperature of the lipid (Table V) similar to the case of the glucagon–DMPC complex (Epand et al., 1977).

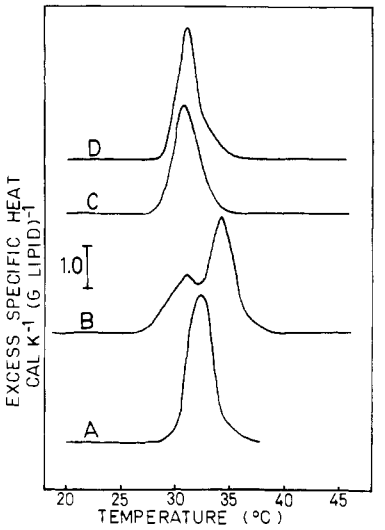

FIGURE 8: Differential scanning calorimetry curves for lipid mixtures. Pipes buffer, scan rate 0.5 K/min. Curve A, 0.5 mM DPPC–0.5 mM DMPG; curve B, same lipid mixture as (A) with 100 μM sCT; curve C, 0.5 mM DMPC–0.5 mM DPPG; curve D, same lipid mixture as (C) with 100 μM sCT.

sCT had less effect on the phase transition properties of the lipid mixtures than it did on pure DMPG or DPPG. The enthalpy of the transition was not altered by sCT (Table II), and the breadth of the transition and its temperature were only slightly affected at this particular peptide:lipid ratio (Figure 8). The peptide appeared to have more effect on the transition properties of DMPG–DPPC than on DMPC–DPPG, in keeping with the larger effect of the former phospholipid mixture on the helix content of sCT (Table V).

The ability of sCT to disrupt multibilayer structures of a 25% DMPG–75% DMPC mixture, despite the small increase in the apparent helical structure of the peptide in the presence of this lipid mixture, is demonstrated by electron microscopy. Negative staining shows the typical stacked-discoidal structure of lipoprotein recombinants (Figure 3D). In this case, the average plate thickness is 5 nm, slightly thinner than the complex with pure DMPG, but the diameter increases to 40 nm.

Interaction of sCT with Other Phospholipids. In addition to egg phosphatidylglycerol, several other lipids from natural sources were tested for their effect on the helical content of sCT. Both phosphatidylinositol and phosphatidic acid were solubilized by sCT, leading to the formation of a complex in which the peptide had a higher helical content (Table III). However, several other acidic phospholipids including sulfatide, cardiolipin, and phosphatidylserine had relatively little effect on the circular dichroism of sCT (Table III). Negative-staining electron microscopy of mixtures of sCT with phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, or phosphatidic acid shows the presence of at least some multilamellar structures together with varying amounts of individual particles, indicating that the extent of solution with these naturally occurring lipids is not as complete as that with

DMPG. Disk-shaped particles were found when sCT was mixed with either phosphatidylinositol, phosphatidylserine, or phosphatidylglycerol while with phosphatidic acid the complexes appeared round. Many complexes were seen at various stages of separation from multilamellar vesicles of the added lipid. Thus, phosphatidylglycerol is not unique among phospholipids in its ability to react with calcitonin.

Discussion

Methods for the estimation of the secondary structure of proteins predict that sCT should have a higher helical content than pCT (Merle et al., 1979). This conclusion, however, is not supported by our CD studies (Table I). It is therefore unlikely that the increased biostability of sCT over the other calcitonins (Habener et al., 1972; Newsome et al., 1973; Otani et al., 1977) is a result of differences in their conformation when free in solution. However, sCT does acquire considerably more helix content than pCT and much more than hCT in the presence of DMPG (Table III). Thus, the increased biostability of sCT may be a result of its greater degree of folding in the presence of certain phospholipids.

Calcitonin can interact with several negatively charged lipids but not with the zwitterionic lipids DMPC or sphingomyelin. The three calcitonins studied are all positively charged at pH 7.4, the pH used for most of the studies reported here. The estimated isoelectric points of hCT, sCT, and pCT are 8.7, 10.4, and 11.0, respectively (Maier et al., 1977), and these peptides carry an overall charge of approximately 1+, 3+, and 2+, respectively, at pH 7.4. This suggests that electrostatic interactions are a component of the noncovalent bonding between calcitonin and acidic phospholipids. High salt concentrations are able to decrease the strength of this bonding and thus lead to a destabilization of the sCT–DMPG complex (Table III). Electrostatic interactions may be of particular importance for the induction of increased helix content in the presence of lipid. Thus, hCT which has an overall charge of 1+ forms less helix in the presence of the negatively charged DMPG than does sCT with an overall charge of 3+ at pH 7.4. In addition, the negatively charged DMPG induces more helix in sCT than do mixtures of DMPG and the zwitterionic DMPC. The importance of electrostatic interactions for the induction of helical structures in peptide–detergent systems has previously been suggested (Wu et al., 1981b). However, not all negatively charged lipids induce a higher helical content in sCT (Table III). The reason for the stronger interaction of sCT with certain acidic phospholipids cannot be fully explained at this time but is probably a result of competition between lipid–lipid and lipid–peptide interactions. The results do demonstrate that there is some specificity in the interaction between calcitonin and lipids and that it is not due solely to nonspecific adsorption to a negatively charged surface. In general, other proteins such as cytochrome *c* oxidase (Cable & Powell, 1980), (Na,K)-ATPase (Brotherus et al., 1981), lipophilin (Boggs et al., 1977), and rhodopsin (Watts et al., 1979) have also been found to interact more strongly with negatively charged lipids than with zwitterionic ones, although an exception to this generalization has been found with the lipovitellin–phosvitin complex (Birrell et al., 1982).

The ability of calcitonin to solubilize lipid may result from its sequence in which hydrophobic residues are found regularly spaced at every third or fourth residue along a portion of the chain. Such sequences endow proteins with the ability of forming amphipathic helices. This is illustrated (Figure 9) for residues 8–22 of the calcitonins by the helical wheel projections (Schiffer & Edmundson, 1967) which indicate how the hydrophobic and hydrophilic residues are grouped together

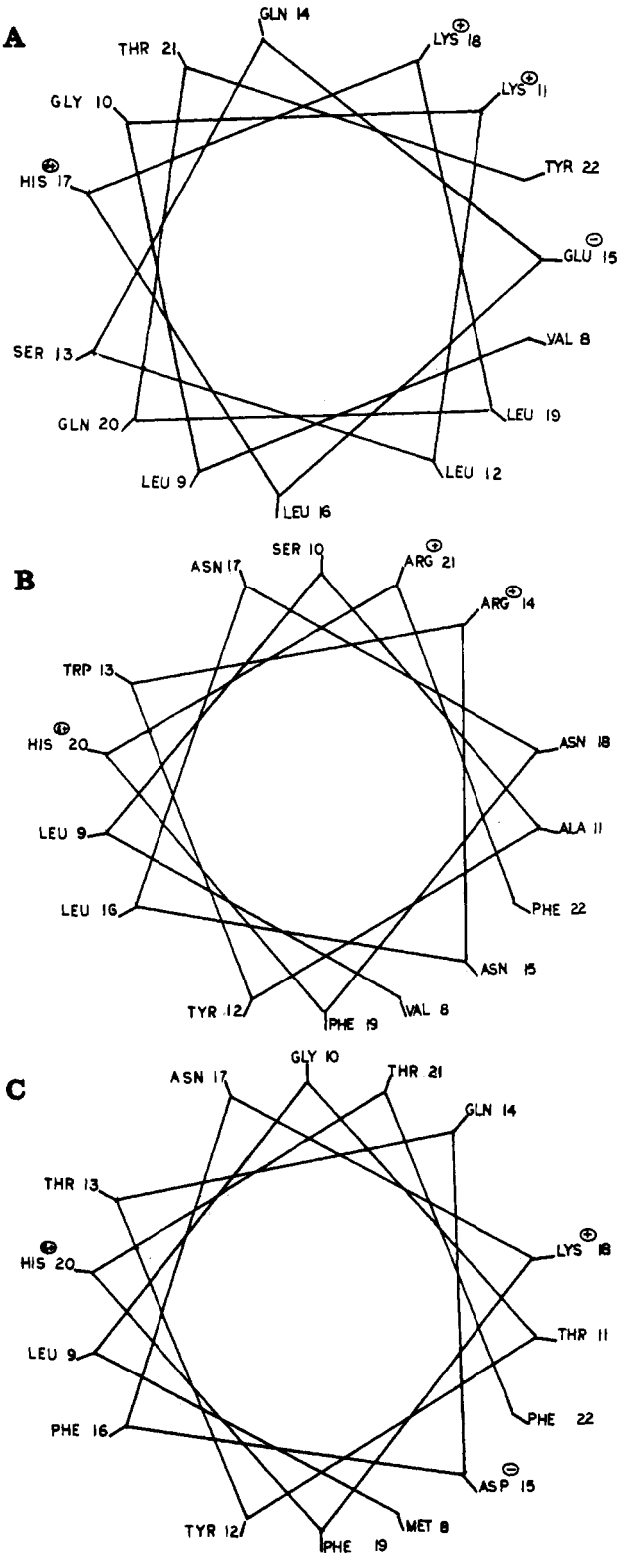

FIGURE 9: Helical wheel representations of residues 8–22 of sCT (A), pCT (B), and hCT (C).

on opposite faces of the helix. If the calcitonins were to form an amphipathic helix, the extent of the spacial segregation of hydrophobic and hydrophilic groups can be estimated by calculating the hydrophobic moment (Eisenberg et al., 1982). For the segment from residues 8–22 in sCT, pCT, and hCT, the hydrophobic moments are 0.35, 0.20, and 0.21, respectively. The value for the hydrophobic moment of residues 8–25 or 9–26 is lower for sCT (0.27) but about the same for the other two calcitonins. The mean hydrophobicities (Janin, 1979) for

residues 8–22 in sCT, pCT, and hCT are −0.24, −0.11, and −0.13. Thus, sCT has a somewhat greater tendency than pCT or hCT to be amphiphilic when folding into an α-helix, although it has a lower overall hydrophobicity. The formation of an amphipathic helix in hCT may also be less probable because of the presence of a negative charge at residue 15 which occurs in the hydrophobic side of the helix (Figure 9) and as a result of the proline residue at position 23 which would inhibit the peptide from forming α-helical structures near the carboxyl terminus, although sCT also has these features. The values for the hydrophobic moments and the mean hydrophobicities of the calcitonins are not much different from those of an 18-residue segment of mellitin (0.25 and −0.15, respectively) or residues 18–29 of glucagon (0.28 and −0.13, respectively), both lipid-solubilizing peptides.

However, hCT is capable of solubilizing DMPG and altering the phase transition properties of this lipid in a manner similar to the other calcitonins (Table II) but without a large increase in the helical structure of the peptide (Table III). In addition, sCT can solubilize lipid mixtures of 25% DMPG–75% DMPC or 50% DPPG–50% DMPC without a large increase in the helical content of the peptide. Although calcitonin does not necessarily attain a highly helical structure in the presence of lipid, it probably becomes amphipathic by virtue of a conformational change which segregates hydrophobic amino acid side chains so that they can then interact with lipid. The side chain of the tryptophan residue of pCT is buried in a more hydrophobic environment in the presence of DMPG, for example. In fact, the conformation of the peptide may be that of a distorted helix. Perhaps pCT and hCT, which have lower hydrophobic moments than sCT, must form more distorted helices in order to be amphiphilic in the presence of lipid. Although CD has been extensively used to estimate the helical content of proteins, the magnitude of the rotatory strength of the n–π^* transition is highly dependent on helix length and dihedral angles (Vournakis et al., 1968). In addition, some nonhelical conformations may give rise to an α-helix-like CD spectrum (Bandekar et al., 1982). Thus, although we have demonstrated that peptides can solubilize lipids without forming an amphipathic α-helix, we cannot state how different from an α-helix the conformation of the lipid-bound peptide is.

The calcitonin–DMPG complex appears to be stable over a wide range of temperatures and is not greatly affected by the change in phase of the lipid. This is in contrast with the glucagon–DMPC complex which is markedly affected by changes in the phase of the lipid (Epand & Epand, 1980). This also explains the ability of sCT, but not glucagon, to interact with egg phosphatidylglycerol. However, there may be some lipid mixtures, such as DMPG–DPPC (Table V), where even sCT appears to interact more strongly with the gel-state lipid. Glucagon binds fewer lipid molecules as the temperature is raised through the phase transition (Epand & Epand, 1980), but neither the equilibrium binding constant nor the number of DMPG molecules bound to sCT greatly changes above the phase transition temperature (Figure 5). Another manifestation of the stability of sCT–DMPG complexes above the phase transition temperature is the ability of sCT to rapidly react with DMPG at 42 °C (Figure 7). Some peptides such as glucagon (Epand & Epand, 1980) and apolipoprotein A-I (Pownall et al., 1981) do not react rapidly with DMPC above its phase transition temperature while other smaller peptides, such as the synthetic peptide LAP-20, can react with DMPC in its liquid-crystalline state (Pownall et al., 1981). The properties of the peptide and lipid which are responsible for the stability of the complexes at higher temperatures are not known but must be dependent on how rapidly the number of lipids bound per peptide decreases with increasing temperature. The decrease in the number of bound lipids with increasing temperature probably occurs for all peptides and proteins but over a very narrow temperature range for some peptides such as glucagon (Epand & Epand, 1980), while for cytochrome *c* oxidase (Dahlquist et al., 1977; Longmuir et al., 1977) or the myelin proteolipid apoprotein (Curatolo et al., 1978) it occurs over a wide temperature range. Perhaps ionic interactions, which are of relatively greater importance to the binding of sCT to DMPG, lead to complexes which are stable over a wide range of temperatures. Ionic bonding appears to be of less relative importance in the stabilization of glucagon–DMPC or serum lipoprotein complexes since these complexes can be isolated by equilibrium density gradient centrifugation using gradients composed of high concentrations of KBr (Jones et al., 1978; Nelson et al., 1974).

The affinity constant for sCT binding to DMPG is remarkably similar to that for glucagon binding to DMPC (Epand & Epand, 1980) or for the binding of reduced and carboxymethylated apolipoprotein A-II to DMPC (Pownall et al., 1981a). The affinity of calcitonin for specific membrane receptors (Brown & Aurbach, 1980) is much greater than that for lipids, but direct binding to lipids could contribute significantly to the overall stabilization of the hormone–receptor complex. In certain cancer cell lines, it has been found that there is a large increase in the number of calcitonin receptors compared with normal cells (Findlay et al., 1980). It is possible that a change in the lipid composition of cancer cell membranes contributes to the increased expression of calcitonin receptors.

The addition of calcitonin to DMPG or DPPG leads to a gradual broadening of the phase transition of the phospholipid and a progressive lowering of the transition enthalpy with little change in the transition temperature (Tables II and IV, Figure 6). Such behavior is consistant with the peptide having equal solubility in the gel and liquid-crystalline phases and causing a reduction in the size of the cooperative unit of the phospholipid. This type of behavior is also observed with integral membrane proteins and with cholesterol, both of which presumably have a greater penetration into the bilayer than calcitonin. Transition broadening is also a prominent feature of the DMPC-solubilizing peptides glucagon (Epand & Sturtevant, 1981) and apolipoprotein A-I (Andrews et al., 1976; Tall et al., 1977). However, with increasing amounts of glucagon, the transition of the pure lipid gradually disappears and is replaced by the transition of the lipoprotein complex which is not altered by further additions of glucagon (R. M. Epand and J. M. Sturtevant, unpublished results). With sCT–DMPG mixtures, increasing sCT concentrations lead to a gradual reduction in the observed transition enthalpy until no transition can be detected (Table IV). This may be a result of the lower number of DMPG molecules (five) bound to sCT and of the small size (5 nm) for the DMPG–sCT complex at low lipid to peptide ratios. Twenty molecules of DMPC are bound to glucagon (Epand & Epand, 1980), forming a particle of 25-nm diameter (Jones et al., 1978).

The effects of sCT on the phase transition properties of phosphatidylcholine–phosphatidylglycerol lipid mixtures were examined at a lipid to peptide molar ratio of 10 (Table II, Figure 8). There is less effect of the peptide on the transition enthalpy, but the transition temperature is altered, particularly for the DPPC–DMPG mixture. In the case of the DPPC–DMPG mixture, the two peaks observed in the DSC analysis

are similar to those found with the DMPC–glucagon complex, with one component below the phase transition temperature of the lipid alone and the other major component above the lipid transition temperature (Epand & Sturtevant, 1981). In the present case, the appearance of a two-component transition could be explained by a peptide-induced lateral phase separation. Since sCT interacts with DMPG but not DPPC, it could cause enrichment of the DMPG concentration in its surroundings. Although this may occur to some extent, the lateral phase separation is not very extensive and may not be the cause for the observed shape of the phase transitions. Multiple-component transitions are observed even with the single pure phospholipid DPPG in the presence of sCT (Figure 6). In addition, there is no large change in the transition enthalpy as would be expected if the peptide interacted with much of the DMPG. Therefore, the possibility of lateral phase separation cannot be eliminated, but if it occurs, it is not very extensive.

We conclude that calcitonin interacts with an acidic phospholipid but not with zwitterionic phospholipids. The interaction between this peptide and phosphatidylglycerol leads to the formation of a soluble lipoprotein complex which is stable over a wide range of temperatures both above and below the phase transition temperature of the lipid. hCT has a lower helical content in the presence of DMPG than does sCT. This may explain its decreased biostability (Habener et al., 1972; Newsome et al., 1973; Otani et al., 1977) and brings into question the requirement of an amphipathic helix for the solubilization of phospholipids.

Added in Proof

A biologically active, synthetic analogue of calcitonin, based on an amphiphatic helical model, has been recently reported by Moe et al. (1983).

Acknowledgments

We are grateful to Dr. M. A. Moscarello for the use of his differential scanning calorimeter, to Dr. D. Wood for running the amino acid analyses, and to Andy Epand for writing a computer program to calculate hydrophobic moments.

Registry No. Human calcitonin, 21215-62-3; dimyristoylphosphatidylglycerol, 61361-72-6; dimyristoylphosphatidylcholine, 13699-48-4; salmon calcitonin, 47931-85-1; porcine calcitonin, 12321-44-7.

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Dependence of Phosphatidylcholine Phosphorus-31 Relaxation Times and $^{31}P\{^1H\}$ Nuclear Overhauser Effect Distribution on Aggregate Structure†

R. A. Burns, Jr., R. E. Stark, D. A. Vidusek, and M. F. Roberts*

ABSTRACT: Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) relaxation rates and $^{31}P\{^1H\}$ nuclear Overhauser effects (NOEs) have been obtained for a variety of phosphatidylcholine monomers, micelles, and sonicated vesicles. NOE measurements conducted with broad-band irradiation of the entire ^1H spectrum monitor the influence of aggregation state on both the overall time scale of molecular motions and the relative contributions of various spin-relaxation pathways. Selective NOE studies are in part consistent with earlier findings [Yeagle, P. L., Hutton, W. C., Huang, C.-H., & Martin, R. B. (1977) *Biochemistry 16*, 4344], which attributed enhanced ^{31}P signal intensity to *N*-methyl protons of the choline head group. In the present lecithin systems, the maximum NOE occurs when methylenes adjacent to the phosphate moiety are irradiated (monomeric lecithin or small nearly spherical micelles) or, in some cases, at a frequency intermediate between these methylenes and the choline methyl group (sonicated egg lecithin vesicles and large micellar species such as those formed by 1,2-dioctanoylphosphatidylcholine). With addition of cholesterol to vesicles or 1,2-dioctanoylphosphatidylcholine micelles, the position of the NOE maximum shifts further away from the choline methyl frequency. For model compounds in which an *N*-methyl group is covalently linked to phosphorus, substantial NOEs from these protons are observed, but contributions from other adjacent methylene protons are also important. Multiple sources of $^{31}P\{^1H\}$ NOEs in these model studies, as well as possible complications from proton cross relaxation in larger aggregates, result in serious ambiguities if such measurements are used to study intermolecular phospholipid head group interactions.

Phosphorus-31 nuclear magnetic resonance (NMR)[1] spectroscopy has been widely used in studies of the conformation and dynamics of phospholipid head groups. The spectral line shape has proven to be a powerful structural probe in partially ordered model-membrane systems (Browning, 1981), and valuable molecular information has been obtained in the solution state from ^{31}P spin-relaxation times (T_1's) and nuclear Overhauser effects (NOEs) (Yeagle, 1978; Yeagle et al., 1977; Moore et al., 1977; Castellino & Violand, 1979). If the entire ^1H spectrum is irradiated, the resulting enhancement in ^{31}P signal intensity allows separation of dipole–dipole (DD) contributions from other relaxation pathways (Noggle & Schirmer, 1971), so that overall relaxation rates may be interpreted in terms of the motional dynamics of the lipid head group (Viti & Minetti, 1981). If ^1H irradiation is applied at frequencies that correspond to single chemical groupings, then selective $^{31}P\{^1H\}$ NOEs are obtained. Since the magnitude of the enhancement depends inversely on the sixth power of the internuclear separation (r^{-6}_{PH}), experiments of this sort provide in principle a nonperturbing probe of phospholipid head group interactions in the solution state (Yeagle, 1978; Viti & Minetti, 1981).

For phosphatidylcholine and sphingomyelin bilayers, Yeagle et al. (1977) reported a $^{31}P\{^1H\}$ NOE profile with a maximum

† From the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (R.A.B., D.A.V., and M.F.R.), and the Department of Chemistry, Amherst College, Amherst, Massachusetts 01002 (R.E.S.). *Received March 3, 1983.* This work was supported by grants from the National Science Foundation (PCM-7912622) and the National Institutes of Health (BRSG S074407110). NMR experiments performed at the National Magnet Laboratory were supported by the National Institutes of Health (RR 00995) and the National Science Foundation (C-670).

[1] Abbreviations: NMR, nuclear magnetic resonance; T_1, spin–lattice relaxation time; NOE, nuclear Overhauser effect; 1,2-diacyl-PC or 1,2-diacylphosphatidylcholine, 1,2-diacyl-*sn*-glycero-3-phosphocholine; DD, dipole–dipole spin relaxation; CSA, chemical shift anisotropy relaxation; TLC, thin-layer chromatography; rf, radio frequency; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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