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Lipophilic derivatization and its effect on the interaction of cholecystokinin (CCK) nonapeptide with phospholipids

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N-terminal lipophilic derivatization of the fully active cholecystokinin analogue (Thr,Nle)-CCK-9 with the di-myristoyl-*raz*-thioglyceryl moiety leads to spontaneous self-aggregation of the lipopeptide into polydispersed vesicles at the liquid state. The high degree of fluidification of the vesicles favors a fast and net transfer of monomers to phospholipid bilayers even below the phase-transition temperature of the acceptor vesicles. Surprisingly, the process is accompanied by formation of peptide clusters. The peptide-rich domains exhibit a high affinity for Ca^{2+}, a fact which may be correlated to the biological function of CCK as this hormone is known to stimulate calcium outflux from reserves in the cell membranes. Moreover, induced membrane affinity allowed to study more precisely the interaction of the CCK peptide with lipid bilayers as mimicry of cell membranes. Differently from what was observed with a similar lipogastrin derivative in which the peptide tail was found to be permanently exposed to the aqueous phase, in the case of the lipo-CCK compound insertion of its C-terminal active site into more hydrophobic compartments of the bilayer is occurring, as well as a folding into β-type structures, thus confirming the role of cell membranes in displaying peptide hormones for specific receptor recognition.

Introduction

The peptides cholecystokinin (CCK) and gastrin (HG) have been recognized to play key roles in normal physiology as (neuro)hormones and neurotransmitters. They have been characterized in mammalians in various molecular sizes of which the minigastrin (HG-14) and CCK-8 represent the shortest fully active circulating forms of this family of hormones (for recent reviews, see Refs. 1–3). Although sequence identity is conserved in their C-termini with the common pentapeptide amide portion Gly-Trp-Met-Asp-Phe-NH_2, known to contain the message site, sufficient additional information seems to be encoded in the residual portions of the relatively short molecules to trigger specific hormonal responses. This, however, would imply that either the two homologous peptides are capable of adopting different bioactive conformations in the receptor-bound state or the structural elements located in the N-terminal extensions of the active site suffice for a selective recognition by receptors.

The N-terminal extension of both the gastrin and CCK molecule consists of characteristic structural elements as a tyrosine residue, the sulfation of which is essential for the CCK activity at least in the peripheral system, and a cluster of negative charges, i.e., a pentaglutamic acid sequence in gastrin and the Asp-Tyr(SO_3H) dipeptide in CCK-8. For the mechanism of receptor recognition of peptide hormones statistical collision with lipid bilayers of the cell membrane has been proposed as the first step with induction of preferred conformations and orientations to facilitate their binding to specific receptors [4,5]. Studies on the conformational properties of gastrin [6] in sodium dodecylsulfate (SDS) micelles as mimicry of the cell membrane lipid environments revealed interaction with the negatively charged micelles and thus, folding of the peptide into more ordered structures only upon protonation of the pentaglutamic acid sequence, i.e., at pH 2. Since gastrin is lacking a well-expressed primary or secondary amphiphilicity as one of the main prerequisites for

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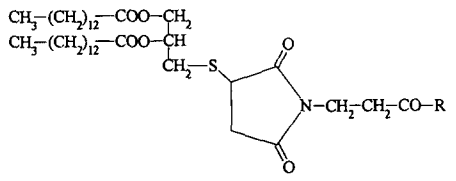
Abbreviations: CCK, cholecystokinin; HG, human gastrin; (Thr,Nle)-CCK-9, (Thr28,Nle31)-CCK-(25–33); DMPC, dimyristoyl-phosphatidylcholine; DM-CCK, dimyristoylmercaptoglycerol/N^{α}-maleoyl-β-alanyl-(Thr,Nle)-CCK-9 adduct; DM-gastrin, dimyristoylmercaptoglycerol/N^{α}-maleoyl-β-alanyl-(Nle15)-HG-(2–17) adduct; DMF, dimethylformamide; MeOH, methanol; BuOH, butanol; AcOH, acetic acid; AcOEt, ethyl acetate; TFE, trifluoroethanol; SDS, sodium dodecylsulfate; TLC, thin-layer-chromatography; HPLC, high-performance liquid chromatography; hs-DSC, high-sensitivity differential scanning calorimetry; CD, circular dichroism; SUV, small unilamellar vesicles.



R = Gly-Pro-Trp-Leu-(Glu)$_5$-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2, (Nle^{15})-HG-(2-17)

or

Arg-Asp-Tyr(SO_3H)-Thr-Gly-Trp-Nle-Asp-Phe-NH_2, (Thr,Nle)-CCK-9

Fig. 1. Structure of the lipo-CCK (DM-CCK) and lipogastrin (DM-gastrin) derivative.

lipid affinity, we have recently investigated the effect of a lipo-derivatization of this peptide on its interaction with differently charged surfactant micelles, as well as with phospholipid bilayers [7,8]. Despite the induced embedment of double-tailed lipogastrin derivatives into surfactant micelles and model phosphatidylcholine bilayers (DPPC, DMPC), clearly detectable conformational transitions towards more ordered structures were not observed by circular dichroism (CD) measurements except for the case where positively charged micelles were used to facilitate interactions with the negatively charged gastrin molecule. On the other hand, incorporation of lipogastrins into lipid bilayers was observed to occur readily, thus shifting the distribution equilibrium of the hormone molecule in favor of lipid bilayers. This induced lipid interaction, however, did not prevent hormonal activity, whilst the chain length of the lipid moieties did affect the potency possibly via differentiated two-dimensional migration rates of the membrane-bound peptides to the target receptors.

In view of the promising results obtained in these model studies with gastrin we have synthesized a similar, double-tailed lipophilic CCK derivative, shown in Fig. 1, in order to compare its mode of interaction with lipid bilayers to that of the lipogastrin derivative. For this purpose the lipid derivatization was performed at the N-terminus of the CCK molecule, since modifications at this sequence position are known to be without any effect on its biopotency [9,10]. However, to further assure a maximum of free conformational space and accessibility of the bioactive core of CCK via a larger size spacer, the (Thr,Nle)-CCK-9 analogue [11] was used. Besides an additional N-terminal extension of the CCK-8 molecule in sequence mode by the arginyl residue, the maleoyl-β-alanyl moiety, chosen as handle for a covalent linkage of the di-fattyacyl-mercaptoglycerol group [7] was expected to guarantee full flexibility of the peptide portion. Moreover, replacement of the two methionine residues in the CCK-sequence by threonine and norleucine, respectively, has been shown to enhance the stability of the CCK molecule by preventing the troublesome easy oxidation of the two thioether functions without affecting its bioactivity [11].

The characterization of the double-tailed lipophilic (Thr,Nle)-CCK-9 derivative in terms of aggregational and conformational properties as well as of its interaction with artificial membranes revealed substantial differences if compared with similar lipogastrin compounds. This could suggest distinct properties of the two homologous peptides already at the level of their interaction with cell membranes.

Materials and Methods

Materials

For thin-layer chromatography (TLC) precoated silica gel 60 plates (Merck, Darmstadt, Germany) were used; compounds were visualized with chlorine/tolidine and/or $KMnO_4$ solution. Amino-acid analysis of the acid hydrolysates (6 M HCl containing 2.5% thioglycolic acid; 110°C; 24 h) were obtained on a Biotronic analyzer (LC 6001). DMPC and reagents for synthesis were purchased from Fluka (Buchs, Switzerland) and used without further purification. For spectroscopic measurements trifluoroethanol (TFE) (UVASOL grade) and for fluorescence quenching experiments NaI (> 99%) were from Merck (Darmstadt, Germany).

Synthesis of CCK peptides

N-Hydroxysuccinimido maleoyl-β-alaninate. The title compound was prepared according to the procedure of Nielsen and Buchardt [12] modified as follows: β-alanine (4.45 g; 50 mmol) was reacted for 1 h at room temperature with maleic anhydride (4.9 g; 50 mmol) in DMF (100 ml). The resulting solution was cooled in an ice-bath and then *N*-hydroxysuccinimide (7.2 g; 62.6 mmol) was added followed by dicyclohexylcarbodiimide (20.65 g; 100 mmol). After 4 h stirring the precipitate was filtered off and the solution was evaporated to dryness. The residue was recrystallized twice from 2-propanol. Yield: 10 g (76%); m.p. 164–166°C; (165–167°C [12]; 168–170°C [13]). Analysis calculated for $C_{11}H_{10}N_2O_6$ (266.22): C, 49.62; H, 3.76; N, 10.53. Found: C, 49.14; H, 3.97; N, 10.52.

Na^{α}-maleoyl-β-alanyl-(Thr,Nle)-CCK-9. The derivatization of (Thr,Nle)-CCK-9 [11] was performed by the procedure described previously for other CCK-related peptides [14] as follows: to a solution of the CCK-peptide (65 mg; 0.046 mmol calculated for the peptide content of 86.7%) in water *N*-methylmorpholine was added (10 μl; 0.092 mmol) and the solution freeze-dried. The lyophilisate was dissolved in DMF (10 ml) and reacted with *N*-hydroxysuccinimido maleoyl-β-alaninate (75 mg; 0.282 mmol) in DMF (5 ml) overnight at room temperature. Then 0.5% AcOH (1.1 ml) was added and the reaction mixture evaporated to oily

residue. The precipitate formed on addition of AcOEt was filtered off and reprecipitated from DMF with AcOEt. Yield: 56 mg (73%); homogeneous on TLC (solvent system $CHCl_3$/ MeOH/ AcOH/ H_2O, 60:40:5:10) and HPLC (Nucleosil 300/C_{18}; eluent, CH_3CN/0.05 M perchlorate buffer (pH 6.5), linear gradient from 18 to 30% CH_3CN in 30 min; flow rate, 1.2 ml/min); amino-acid analysis of the acid hydrolysate: Asp, 2.01(2); Thr, 1.02(1); Gly, 1.02(1); Nle, 0.99(1); Tyr, 1.01(1); Phe, 0.99(1); Trp, 0.98(1); Arg, 1.00(1); β-Ala, 0.98(1); peptide content: 81.7% calculated for M_r = 1383.5.

3-{(3'Ξ)-3'-[(2RS)-1,2-Dimyristoyl-3-thioglyceryl] succinimidyl}propionyl-(Thr,Nle)-CCK-9 (DM-CCK). (2*RS*)-3-(*tert*-butyldithio-1,2-dimyristoyloxy)propane (74 mg; 0.120 mmol) was reduced at room temperature with tributylphosphine (31.3 mg; 0.155 mmol) in 95% aqueous TFE to produce the (2*RS*)-1,2-dimyristoyl-3-mercaptoglycerol [15]. The thiol compound was immediately dissolved in freshly degassed argon-saturated DMF (5 ml) and added to a solution of maleoyl-β-alanyl-(Thr,Nle)-CCK-9 (35 mg; 0.021 mmol calculated for the peptide content of 81.7%) in argon-saturated DMF (5 ml). The mixture was allowed to react for 15 h at room temperature under gentle stirring. Then, the solution was concentrated and the product precipitated with AcOEt/hexane (1:1). The precipitate was collected by centrifugation, washed extensively with AcOEt in order to remove the excess of the mercaptoglycerol derivative, and was then dried over KOH pellets. Finally, the solid residue was redissolved in *tert*-BuOH/H_2O and freeze-dried. The yield was 42 mg (95% calculated for the peptide content of 95%). Because of the two additional chiral centers, i.e., at the C-2 of the glycerol and C-3 of the succinimidyl moiety, HPLC (Nucleosil 300/C_4; eluent CH_3CN/2% H_3PO_4; linear gradient from 15% to 74% CH_3CN in 40 min at a flow rate of 1.2 ml/min) served only to assess the absence of starting reactants; on TLC (solvent system: CH_3Cl/MeOH/H_2O, 70:30:10) the compound was found to behave homogeneously as expected from the lower resolution power of this technique; amino-acid analysis of the acid hydrolysate: Asp, 2.02(2); Thr, 1.00(1); Gly, 1.00(1); Nle, 0.98(1); Tyr, 1.03(1); Phe, 0.99(1); Trp, 0.99(1); Arg, 0.99(1); β-Ala, 0.81(1); peptide content: 95% calculated for M_r = 1931.9; fast atom bombardment mass spectrometry: *m/z* 1852.4 (*M*-SO_3 + H^+), 1873.5 (*M*-SO_3 + Na^+).

Methods

Sample preparation. The peptide concentrations were determined by weight and peptide content of DM-CCK and further controlled by UV absorption measurements. All buffers were filtered through a 0.2-μm polycarbonate filter (Millipore). Aqueous solutions of (Thr,Nle)-CCK-9 and DM-CCK were prepared by dissolving the peptide samples in 0.1% ammonia (5% of the final volume) and diluting with 5 mM phosphate buffer containing 100 mM NaCl (pH 7.0). Stock solutions of DM-CCK were vortexed or sonicated as described below for the phospholipid preparations. The phospholipid preparations were done in 5 mM phosphate containing 100 mM NaCl (pH 7.0). In order to obtain small unilamellar vesicles (SUV) of DMPC, the dry lipid was weighed into glass test tubes, suspended in buffer and sonicated with a Branson titanium rod sonifier for short periods of time until optically transparent vesicle preparations were obtained. The SUV solutions were incubated at 30°C for 1 h and then centrifuged in order to remove titanium dust contamination from the sonifier rod.

Quasi-elastic light scattering. The measurements were performed at a scattering angle of 90°, a sample temperature of 20°C, a laser wavelength of 488 nm, and a laser power of 200 mW. The DM-CCK samples ($3.2 \cdot 10^{-4}$ M) were stored in 3-ml glass cylindrical cells. The apparatus for measuring the intensity and the autocorrelation function of the scattered light consisted of an argon ion laser (Coherent, Innova 70), a temperature-controlled scattering cell holder with toluene index matching bath, and a digital autocorrelator (ALV 3000, ALV-Laservertriebsgesellschaft, Langen, Germany) which sampled in 1024 channels. The data analysis was performed using a software provided by ALV and applying the cumulant method [16].

Fluorescence quenching experiments. Fluorescence quenching was measured on a Perkin-Elmer 650–40 spectrofluorimeter operating in the ratio mode and equipped with a thermostated cell holder. The measurements were performed at peptide concentrations of $1.0 \cdot 10^{-5}$ M, except for the (Thr,Nle)-CCK-9 ($1.8 \cdot 10^{-5}$ M) and at 30°C by using 2 nm excitation and emission bandwidths. The excitation wavelength was 280 nm and the emission wavelength 350 nm. Iodide [17] was used as aqueous quencher as follows: small aliquots (10 μl) of a 2 M solution of NaI containing 0.5 mM $Na_2S_2O_3$ were added to 0.5 ml sample solutions. The emission intensities were corrected for the dilution and for the scatter contribution of the blank solutions, i.e., of the lipids in absence of peptide. The data were analyzed according to the Stern-Volmer equation for collisional quenching [17].

High-sensitivity differential scanning calorimetry (hs-DSC). hs-DSC measurements were performed on DM-CCK vesicle preparations and on DM-CCK/DMPC mixtures (1:12 molar ratio) at a DM-CCK concentration of $2.5 \cdot 10^{-4}$M with an MC-2 microcalorimeter (Microcal, Amherst, MA, USA) at a scan rate of 30 C°/h (ascending temperature mode only) interfaced to an IBM AT microcomputer. Endotherms in presence of calcium were recorded at 25 mM $CaCl_2$ concentration. The data were stored and analyzed using the

DA-2 software provided by Microcal as described previously [8].

Circular dichroism (CD). The CD spectra were recorded on a Jobin-Yvon dichrograph Mark IV equipped with a thermostated cell holder and connected to a data station for signal averaging and processing. The CD measurements in the presence of DMPC were performed at 30°C; all other spectra were recorded at 20°C unless stated otherwise. All the solutions of DM-CCK and DM-CCK/DMPC were sufficiently optically clear to allow recording of the spectra in the far-UV region. Quartz cells with 0.1-cm optical path were used. The data averages are of 10 scans; the spectra are original computer-drawn CD spectra reported as ellipticity units per mol of amino-acid residue ($[\theta_R]$). The diastereomeric mixture of the 3-{(3′*Ξ*)-3′-[(2*RS*)-1,2-diacyl-3-thioglyceryl] succinimidyl}propionyl moiety does not contribute to the optical activity, since a negligible CD was detected in separate measurements for this group as propionic acid derivative [7].

Results

The aggregational and conformational state of DM-CCK

Similarly to what was observed with the lipogastrin derivative DM-gastrin, phospholipid-type aggregational properties were expected for the double-tailed DM-CCK, too. Dynamic light scattering measurements performed on vortexed or sonicated DM-CCK probes clearly revealed the presence of a polydispersed system of vesicles in both samples, but of significantly smaller size in the case of sonication. Thereby the presence of micelles at equilibrium with vesicles cannot be excluded.

By hs-DSC measurements on vortexed and sonicated DM-CCK probes no chain-melting transition could be detected between 5 and 40°C. This is strongly suggesting that, as in the case of DM-gastrin, even the DM-CCK vesicles are in the liquid state above 5°C, despite the shorter peptide chain as head group. A less stable aggregational state in comparison to DM-gastrin is further supported by the observation that simple vortexing is sufficient for clearance of the DM-CCK probes with formation of vesicles, whereas in the case of DM-gastrin sonication or extrusion were necessary for this purpose. Fluidification of the lipopeptide bilayer should not result from the racemic di-fattyacyl-thioglyceryl moiety as it is known that the stereoconfiguration of phospholipids is affecting only marginally their phase-transition temperatures [18–20].

Aggregation of DM-CCK into vesicles is accompanied by a blue shift of the fluorescence emission maximum from 360 nm for (Thr,Nle)-CCK-9 to 354–355 nm (see Table I) as resulting from a more hydrophobic environment of the tryptophan residue [21]. Fluorescence quenching experiments were performed on

TABLE I

Fluorescence properties and accessibility of the tryptophan residues of DM-CCK and DM-gastrin to iodide quenching in 5 mM phosphate buffer, 100 mM NaCl (pH 7.0)

Peptide	$\Delta\lambda_{max}$ (nm)	k_{sv} [a]
(Thr,Nle)-CCK-9	0	2.97
DM-CCK (vortexed)	5–6	1.20
DM-CCK (sonicated)	5–6	1.62
DM-CCK/DMPC (1:12)	18 and 22 [b]	0.85 and 0.80 [b]
(Nle^{15})-HG-(2-17)	0	5.89
DM-gastrin	4–5	1.78

[a] The relative error on k_{sv} amounts to 3%; [b] measured after 16 h equilibration at 30°C.

(Thr,Nle)-CCK-9 in solution and on DM-CCK vesicle preparations. The linearity of the Stern-Volmer plots confirmed the collisional nature of the quenching process [22]. The resulting quenching constants (k_{sv}) are reported in Table I in comparison to those of gastrin and DM-gastrin [7]. The k_{sv} of 2.97 for (Thr,Nle)-CCK-9 is half that of gastrin ($k_{sv} = 5.89$) as expected for the full accessibility of one and two tryptophan residues in the CCK and gastrin molecule, respectively. Sonication of DM-CCK preparations lowers the k_{sv} to 1.62, i.e., to approximately half the value of that of (Thr,Nle)-CCK-9. This fact would support formation of unilamellar vesicles as expected for this high energy technique. Conversely, a significantly lower k_{sv} of 1.2 was determined as average value for vortexed DM-CCK samples. It should reflect an additionally hindered accessibility of the fluorophore as possibly resulting from the presence of multilamellar vesicles. However, the polydispersed character and the relative instability of both vesicle preparations makes an unambiguous interpretation of the quenching constants difficult. Conversely, in the case of DM-gastrin the vesicle preparations were found to be remarkably more stable and thus, the decrease of the k_{sv} value from 5.89 to 1.78, i.e., to slightly more than one fourth of the value, was attributed to the fact that of the two tryptophan residues present in the molecule, the one located in the N-terminus, i.e., near the lipid moiety is largely buried into interior compartments of the bilayer, whereas the tryptophan residue in the C-terminus remains accessible on the bilayer surface [7]. This was further supported by CD measurements which indicated that a differentiated packing of the fatty-acid chains was affecting the display of the peptide head group at the water-lipid interphase and correspondingly, its conformational state.

The CD spectra of DM-CCK vesicles are reported in Fig. 2. The CD pattern of the sonicated and vortexed probes are similar, but both exhibit a surprisingly strong concentration-dependence. The spectrum of the vortexed probe is characterized by a weak negative

maximum at 236–238 nm, a strong maximum at 214 nm and a shoulder between 207–210 nm. Upon dilution the shoulder is blue shifted and enhanced in intensity to become the dominant maximum at 205 nm, whereas the maximum at 214 nm is reduced to a shoulder between 210–214 nm; concomitantly a remarkably overall loss of intensity to about half value is observed.

These relatively strong changes of the dichroic properties suggest a high degree of instability of the polydispersed vesicle systems; it may be correlated with different populations of mono- and multilamellar vesicles and possibly even of micelles and monomers at equilibrium. Upon diluting the solution, the CD pattern is shifted from a spectrum more indicative of extended β-type structures to a more α-helix-like spectrum, but of low intensity. This would suggest the presence of mixtures of ordered conformations, e.g., β-type turns, α-helix or γ-type turns [23–25], in equilibrium with aperiodic structures. The CD spectrum of the diluted probe strongly reminds that of (Thr,Nle)-CCK-9 in 70% TFE (Fig. 3), i.e., of the CCK-peptide in a more hydrophobic environment. This could result from multilamellar or larger size vesicles of lower packing density, but also from conversion of the polydispersed vesicle system into a dominant population of micelles. Unfortunately, light-scattering measurements on the diluted probes did not allow us to assess the aggregated state because of the insufficient sensitivity of this technique.

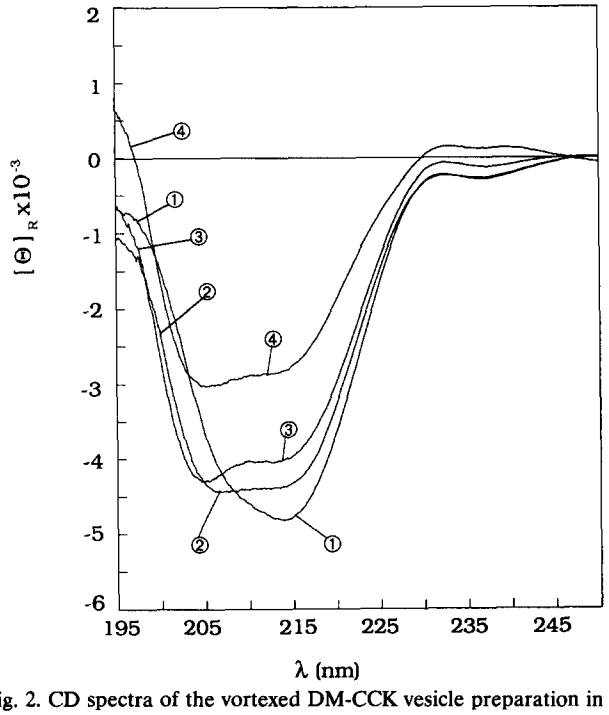


Fig. 2. CD spectra of the vortexed DM-CCK vesicle preparation in 5 mM phosphate buffer containing 100 mM NaCl (pH 7.0) at a peptide concentration of $3.4 \cdot 10^{-4}$ M (curve 1), $2.1 \cdot 10^{-4}$ M (curve 2), $1.4 \cdot 10^{-4}$ M (curve 3) and $6.0 \cdot 10^{-5}$ M (curve 4); the spectra were recorded at 20°C.

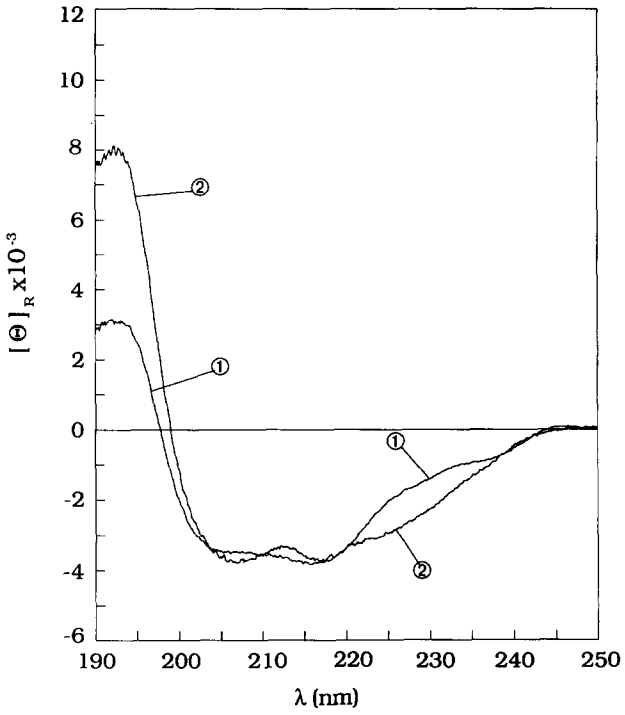


Fig. 3. CD spectra of (Nle,Thr)-CCK-9 (curve 1) and DM-CCK (curve 2) in TFE/5 mM phosphate buffer containing 100 mM NaCl (pH 7.0), 70:30 (v/v) at $3.2 \cdot 10^{-4}$ M peptide concentration and 20°C.

The relatively high instability of the aggregated state of DM-CCK towards environmental changes is also reflected by the CD changes induced on addition of TFE at increasing concentrations. Whilst for DM-gastrin gradual changes of the CD spectra were recorded by titrating its SUVs preparation with TFE [7], for DM-CCK inconsistent changes of CD intensities and patterns are observed to reach then at 70% TFE a CD spectrum which is very similar to that of (Thr,Nle)-CCK-9 at this TFE/buffer ratio (Fig. 3). This should confirm complete dissolution of the aggregates into DM-CCK monomers at this TFE content.

Interaction of DM-CCK with phospholipid bilayers

The transfer of DM-CCK vesicles to DMPC SUVs was found to occur rapidly and quantitatively even below the phase-transition temperature of the DMPC bilayer. As shown in Fig. 4, already the first endotherm of a 10-scan cycle performed at a scan rate of 30 C°/h shows that the 1:12 molar mixture of DM-CCK vesicles and DMPC SUVs reached its final state. The additional changes recorded in the following endotherms of the cycle represent only 5% of the total. Of the initial three peaks with T_c values of 16.32°C, 19.87°C and 24.97°C, respectively, two are slightly shifted to T_c values of 18.42°C and 20.24°C, whereas

the third peak remains constant. This weak T_c shift and intensity variation may derive from internal rearrangements of the system. The peak with $T_c = 24.97°C$ should correspond to a DMPC domain with statistically inserted minor amounts of DM-CCK, whereas the two peaks of the endotherm with lower T_c values should be related to differently enriched CCK-peptide domains. In order to confirm this interpretation the effect of Ca^{2+} ions on the transition temperatures was examined (Fig. 5). Reduction in electrostatic charge of the headgroups as a result of Ca^{2+} binding should induce the bilayer to condense, increasing the packing density in the gel phase and, thus, raising the transition temperature. Addition of Ca^{2+} to the system did not change the overall pattern of the endotherm, thus confirming the presence of a homogeneous vesicle population. But as monitored by the increased opalescence of the solution, neutralization of the electrostatic charges on the vesicle surfaces resulted in liposome aggregation, the driving force of which is presumably Van der Waals interaction. By depleting the bilayer surface of water, Ca^{2+} was found to provoke a parallel shift of the T_c values of the two CCK-9-rich domains by 5°C to higher values, i.e., at extents superior to the shift observed for the DMPC domain (3°C). This confirms the presence of different domains in the DMPC vesicles, but it also suggests a higher affinity of the CCK domains for Ca^{2+}. Formation of CCK domains upon transfer of DM-CCK to DMPC vesicles raises the question of whether the transfer process occurs via monomer migration or via collision of the vesicles and thus, by fusion. Therefore, the transfer process was examined at a 2-fold dilution in order to analyze the

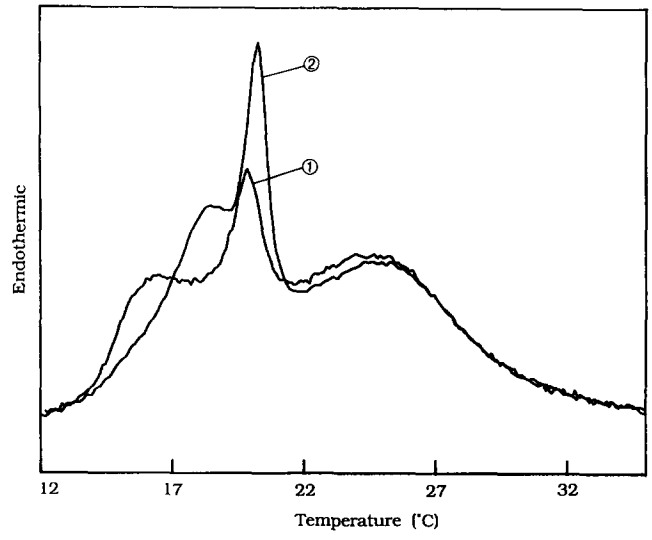


Fig. 4. hs-DSC endotherms of a 1:12 (molar ratio) mixture of the vortexed DM-CCK vesicle preparation and DMPC SUVs in 5 mM phosphate buffer containing 100 mM NaCl (pH 7.0); peptide concentration of $2.5 \cdot 10^{-4}$ M. Endotherm 1 corresponds to the first scan and endotherm 2 to the eighth scan; scan rate of 30 C°/h.

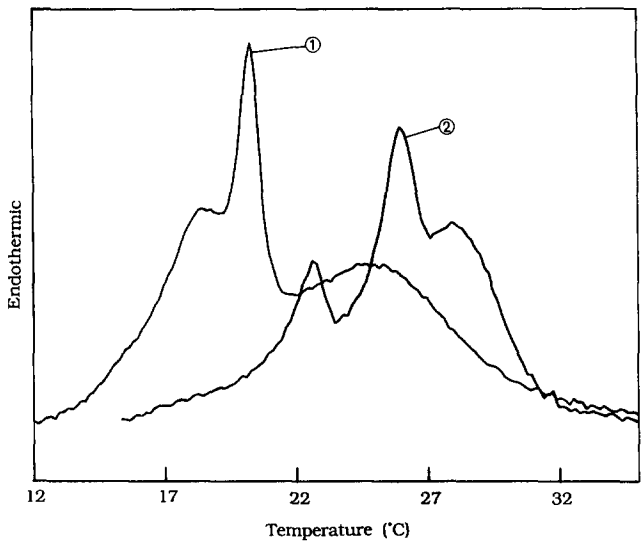


Fig. 5. hs-DSC endotherms of a 1:12 (mol) mixture of the vortexed DM-CCK vesicle preparation and DMPC SUVs in 5 mM phosphate buffer containing 100 mM NaCl (pH 7.0); $2.5 \cdot 10^{-4}$ M peptide concentration. Endotherm 1 represents the eighth scan of a cycle with 30 C°/h scan rate and endotherm 2 the scan after addition of calcium (25 mM $CaCl_2$).

effect of reduced collisional events. The pattern remained unchanged and again a net transfer was observed within the first scan. The process, in fact, is occurring too fast for this type of monitoring and therefore a final answer to the open question could not be obtained. Nevertheless the relative instability of the DM-CCK vesicles as discussed above should be in favor of a monomer transfer of DM-CCK through the bulk water phase.

Insertion of DM-CCK into DMPC vesicles with concomitant formation of more or less CCK-9-rich domains is accompanied by a strong blue shift of the fluorescence emission maximum from 355 nm to 342–343 nm (Table I). Further incubation at 30°C for 16 h leads to an additional slight blue shift to 338–340 nm. This blue shift of the tryptophan fluorescence reflects a remarkably more hydrophobic environment of this portion of the peptide molecule when inserted into the DMPC bilayer than in the DM-CCK vesicles. Thus embedment of DM-CCK into DMPC membranes apparently leads to an overall enhanced interaction of the C-terminus of the peptide head group with more lipophilic compartments of the bilayer. The observed shift compares well with that determined for pentagastrin, i.e., for Boc-Gly-Trp-Asp-Phe-NH_2, upon its interaction with phospholipids [26]. The additional blue shift measured in function of annealing time is consistent with the findings of the hs-DSC measurements. After net transfer of DM-CCK to DMPC SUVs has occurred, a rearrangement of the bilayers is taking place which is involving the peptide moiety, too, as further confirmed by the CD spectra (see below). Re-

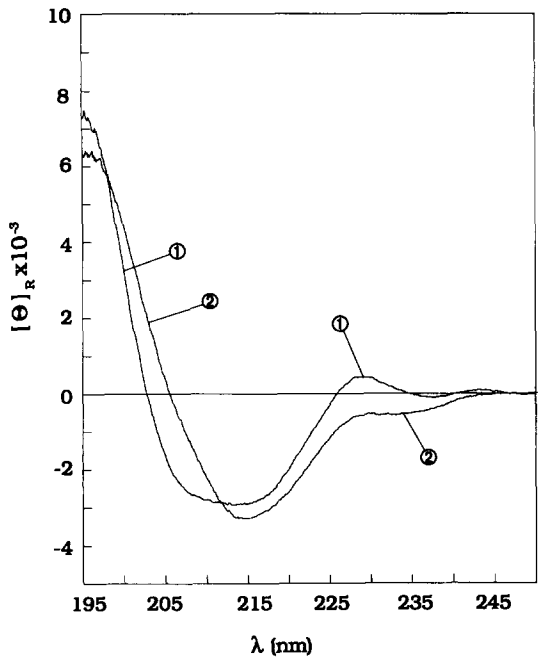


Fig. 6. CD spectra of a 1:25 (mol) mixture of a vortexed DM-CCK vesicle preparation and DMPC SUVs in 5 mM phosphate buffer containing 100 mM NaCl (pH 7.0) after 10 min (curve 1) and after 12 h annealing at 30°C; peptide concentration of $3.5 \cdot 10^{-4}$ M.

duced accessibility of the tryptophan residue upon insertion of DM-CCK from vortexed preparations into DMPC SUVs was also confirmed by fluorescence quenching experiments with NaI. As shown in Table I, the k_{sv} value of 1.2 determined for DM-CCK in the polydispersed vesicle state, is decreased to 0.85 and after additional annealing of the system for 16 h at 30°C to 0.80. Besides confirming the bilayer character of the DM-CCK domains, these k_{sv} values indicate an enhanced interaction of the peptide C-terminus with inner compartments of the bilayer as the accessibility of the tryptophan residue on the outer layer of the SUVs is reduced by about 30%.

The CD spectra of DM-CCK transferred to DMPC bilayers (DM-CCK/DMPC molar ratio 1:25) were recorded upon incubation for 30 min and after annealing of the system for 12 h at 30°C (Fig. 6). Despite the relatively strong positive CD at low wavelengths if compared to that of the DM-CCK spectrum, the similarity of the negative CD pattern recorded for DM-CCK vesicles and DM-CCK/DMPC merged vesicles agrees with the formation of CCK clusters. In fact, the CD spectrum is suggesting β-type structures as possibly resulting from peptide-peptide interactions at the C-termini. According to the fluorescence data this portion of the peptide moiety seems to be inserted into more hydrophobic compartments of the bilayer. Such insertion would require a chain reversal and this could occur with Thr-Gly in positions $i+1$ and $i+2$ of a β-turn whereby the highly charged head group Arg-Asp-Tyr(SO3H) would then remain fully exposed to the water contact. Annealing of the system is apparently strengthening the peptide-peptide interactions in the β-sheet type aggregates as indicated by the red-shifted negative CD maximum to 214 nm and the crossover point to 202 nm. This would also be conform with the observed further decreased accessibility of the tryptophan residue as monitored both by the blue-shifted fluorescence emission maximum and the slight decrease of the k_{sv} from 0.85 to 0.80.

Discussion

N-terminal derivatization of gastrin with di-fatty-acyl-glycerol moieties has been shown to provoke spontaneous aggregation into stable middle size unilamellar vesicles both upon sonication or extrusion of the lipogastrins in buffer [7]. By comparing the aggregational properties of DM-gastrin and DM-CCK the most striking observation is the pronounced effect of the peptide head group. In fact, by replacing the sequence Gly-Pro-Trp-Leu-$(Glu)_5$-Ala-Tyr of gastrin-(2–17) with Arg-Asp-Tyr(SO_3H)-Thr of CCK-9, but retaining the identical C-terminus of the peptide, i.e., Gly-Trp-Nle-Asp-Phe-NH_2, substantial differences in the packing of the fatty-acid chains are observed. Although both lipopeptides aggregate into vesicles at the liquid state, the DM-CCK vesicles were found to be reamarkably less stable than those of DM-gastrin.

The low stability of the DM-CCK bilayers is also reflected by the fast and net transfer of DM-CCK to DMPC SUVs even at temperatures below the T_c of the phospholipid. Thereby particularly surprising was the observation that the lipo-CCK compound tends to form peptide rich domains on the DMPC bilayer which are exhibiting a higher Ca^{2+} ion affinity than the phospholipid itself. This phenomenon could well be implicated in the physiological action of CCK, as this hormone is known to stimulate calcium release from reserves on cell membranes [27]. Conversely, transfer of the homologous DM-gastrin to DPPC bilayers is occurring at remarkably lower rates whereby only statistical insertion of the lipopeptide into the phospholipid bilayer was observed without formation of clusters [8]. Gastrin has been shown to bind Ca^{2+} in TFE [28,29]; however the lack of gastrin domains in DPPC bilayers did not allow us to confirm this possibly physiologically significant property in the membrane-bound state by the hs-DSC technique.

Conformational analysis on membrane-bound peptide hormones is severely hampered by the presence at equilibrium of lipid-bound species and of monomers in the aqueous phase at ratios difficult to assess quantitatively. Correspondingly, spectroscopic measurements are monitoring average conformational preferences of both types of species and thus, unambiguous interpre-

tation of spectroscopic data are difficult. Double-tailed lipopeptide derivatives should allow to bypass this problem as the distribution equilibrium should be in great favor of the membrane-bound state. The main drawback of the latter approach is the predetermined site of lipid anchorage. Therefore, in our model studies on the peptides of the gastrin family derivatization was performed at the N-terminus, known to be not involved in the receptor recognition process. DM-gastrin was found to exhibit a 7-fold reduced binding affinity for its receptors on AR4–2J cells if compared to that of the parent hormone [7]. But since this reduced receptor affinity is sensibly affected by the length of the fatty acids it has been attributed to reduced two-dimensional migration rates of the lipopeptide monomers in the natural bilayers as induced by the di-fattyacyl moieties, and not to the N-terminal derivatization. This is further confirmed by the observation that DM-CCK exhibits a binding affinity for CCK-receptors on rat pancreatic acini which is lowered by a similar factor, i.e., 6-fold (the biological properties of DM-CCK will be reported in detail elsewhere). Nevertheless, taking into account possible effects of the predetermined orientation, a comparison of the mode of interaction of lipo-derivatized gastrin and CCK with the phospholipid bilayer clearly revealed distinct behaviours of the two homologous hormones. Both in the pure DM-gastrin bilayer and in the DM-gastrin/DPPC mixed vesicles the gastrin moiety remains widely exposed to the water phase on the bilayer surface in mainly random coil structures as suggested by the CD measurements [7,8]. Conversely, the peptide portion of DM-CCK is apparently bended via a chain reversal centered on Thr-Gly to allow insertion of the more hydrophobic C-terminus into lipophilic compartments of the DM-CCK and DM-CCK/DMPC vesicles. This would compare well with the preferred conformation of the CCK-9 analogue as determined by ^{1}H-NMR techniques in the cryomixture dimethylsulfoxide/water [30,31]. In this solvent the CCK-peptide structure consists of a γ-turn centered on Thr and separated by Gly from an α-helix in the C-terminus, whereas the N-terminus is salt bridged but flexible.

Insertion of the C-terminus of the CCK moiety into the lipid bilayer with formation of β-sheet type structures between neighboring molecules, as suggested by the CD spectra, would account for the observed weak packing of the fatty acid chains in the DM-CCK vesicles, but also for the tendency to form CCK rich domains in DM-CCK/DMPC merged vesicles. Moreover, interaction of the C-terminal portion of the CCK molecule with hydrophobic compartments of the bilayer fully agrees with previous studies [26,32,33] which clearly demonstrated insertion of pentagastrin, i.e., of Boc-Gly-Trp-Met-Asp-Phe-NH_2, into phospholipid bilayers. However, a similar bending of the gastrin molecule with insertion of the C-terminus into the bilayer was not observed in the identical model system.

In conclusion, the significantly different behavior of the lipogastrin and -CCK derivatives in their mode of display on the water lipid interphase, of insertion into bilayers and interaction with inner compartments of the membrane confirms that already at the level of the collisional event with cell membranes sequence-dependent characteristic properties are initiating the differentiation process for a selective receptor recognition of the two homologous peptide hormones.

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