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Metal Ion Binding Affinities of Gastrin and CCK in Membrane Mimetic Environments

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Abstract: The fully active gastrin and CCK analogues [Nle^{15}]-gastrin-17 and [Nle,Thr]-CCK-9 were analysed for their Ca^{2+} and Tb^{3+} affinities in various membrane mimetic conditions. In TFE both gastrin and CCK exhibited high affinities for calcium and terbium. At saturation level identical metal ion/peptide ratios were determined with Ca^{2+} and Tb^{3+}, i.e. $R=3$ for gastrin and $R=1$ for CCK, confirming the very similar coordination properties of the two metal ions. The conformational effects of both metal ions were found to be very similar with a disordering effect in the case of gastrin and a conformational transition to β-turn type structure in the case of CCK. In order to mimic more properly physiological conditions, similar experiments were performed in the presence of phospholipid bilayers. No interaction of the peptides with the bilayers was observed even in the presence of mmolar Ca^{2+} concentrations. Induced lipid interaction via N-terminal lipo-derivatization of gastrin and CCK allowed to translocate quantitatively the two hormones into phospholipid bilayers and to examine the effect of extravesicular Ca^{2+} on the conformation of the peptide headgroups and on their display at the water/lipid interphase. The CCK moiety of the lipo-CCK inserted into phospholipid bilayers interacts with the lipid phase and addition of Ca^{2+} enhances the clustering of the peptide headgroups in a more β-sheet type conformation. Conversely, insertion of lipo-gastrin into the bilayers leads to full exposure of the gastrin headgroup to the bulk water in predominantly random coil structure. Again Ca^{2+} provokes aggregation. As the lipo-peptide/phospholipid system still represents only an artificial model, it remains hazardous to derive a biological relevance from these data. The significantly higher affinity of lanthanide ions than Ca^{2+} for the peptides could well play a role in the inhibitory activity of lanthanum on the signal transduction of the CCK family of hormones.

Keywords: Gastrin; CCK; lipo-derivatization; calcium; terbium; circular dichroism; phosphorescence; conformation; lipid bilayers

Abbreviations

CCK, cholecystokinin; [Thr,Nle]-CCK-9, [Thr^{28}, Nle^{31}]-CCK-(25–33); DMPC, dimyristoyl-phosphatidylcholine; DM-gastrin, dimyristoyl-mercaptoglycerol/N$^{\alpha}$-maleoyl-β-alanyl-[Nle^{15}]-gastrin-(2–17) adduct; IP_3, inositol 1,4,5-triphosphate; DM-CCK dimyristoyl-mercaptoglycerol/N$^{\alpha}$-maleoyl-β-alanyl-[Thr,Nle]-CCK-9 adduct; NMP, *N*-methylpyrrolidone; AcCN, acetonitrile; HOSu, *N*-hydroxysuccinimide; DCHA, dicyclohexylamine; AcOEt, ethyl acetate; TFE, trifluoroethanol; SUVs, small unilamellar vesicles; MOPS, 3-(*N*-morpholino)propanesulphonic acid.

INTRODUCTION

Gastrin and cholecystokinin (CCK) are gut/brain peptides with multiple functions in the gastrointestinal tract and in the brain (for recent review see [1]). Their receptors in the peripheral and central tissues

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are pharmacologically characterized [2–10] and the second messenger systems coupled to the CCK-A (sulphate-dependent) [11–14] and CCK-B/gastrin (sulphate-independent) receptor [15–18] well established. As known for G-protein-coupled receptors, occupancy of the CCK-A and CCK-B receptors by agonist ligands leads to activation of phospholipase C and, correspondingly, to hydrolysis of phosphatidylinositol 4,5-diphosphate, and generation of *sn*-1,2-diacylglycerol and IP_3 [19, 20]. The second messenger IP_3 is generally believed to be responsible for the release of calcium from the endosplasmatic reticulum, thereby raising intracellular free calcium levels [21]. The elevation of cytosolic calcium is associated with an increase in calcium influx and with calcium-induced calcium release from stores that appear to be close to the plasma membrane [22].

First indications about calcium fluxes accompanying the hormonal action of CCK and gastrin led to studies on the affinity of gastrin peptides for Ca^{2+} and Mg^{2+} in water and TFE [23–25]. In TFE as the membrane mimetic medium, the two fully active gastrin analogues [Nle^{15}]-gastrin-17 and [Nle^{15}]-gastrin-[5-17] showed high affinities for both Ca^{2+} and Mg^{2+}. The conformational changes induced by the metal ion coordination indicated that three metal ions per mole of hormone are bound with dissociation constants of $\leq 10^{-6}$ M^{-1} for K_1 and K_2 and of the order of 10^{-5} M^{-1} for K_3. Since the dichroic properties in the aromatic region were found to change cooperatively with the ion/hormone molar ratios, the C-terminal portion of the gastrin molecule which contains a tryptophan residue, was proposed as the main Ca^{2+} binding site. To date several additional peptide hormones have been found to exhibit high Ca^{2+} affinity in membrane-mimetic conditions [26–29], a fact which led to the proposal that the Ca^{2+}/peptide complexes are potential bioactive states of (neuro)hormones [30].

We have recently reported that lipo-derived gastrin and CCK retain receptor binding affinities comparable to those of the parent hormones despite the tight interdigitation of the di-fattyacyl-glycerol moieties, linked to the N-termini of the peptides, with the cell membrane bilayer [31, 32]. This double-tailed lipo-derivatization should prevent an escape of the ligands into the extracellular aqueous phase prior to receptor recognition. Correspondingly, a membrane-bound pathway of (neuro)hormones to their receptors is indeed possible and, therefore, a characterization of possible interactions between the tethered peptides and phospholipid bilayers should allow for insight into the first events occurring upon collision of hormones with cell membranes. Besides a differentiated mode of display of the peptide headgroups at the water/lipid interphase in terms of preferred conformations, the lipo-gastrin is inserted statistically into phospholipid bilayers, whereas lipo-CCK forms peptide domains of high Ca^{2+} affinity as determined by microcalorimetric measurements [33, 34]. In tissues the extracellular Ca^{2+} concentrations are in the mmolar range and even higher at the bilayer interphase via complexation of metal ions to the phospholipids [35]. Thus, a myriad of membrane surface equilibria and events are generally believed to involve bound Ca^{2+}, suggesting a possible role of the metal ion in the collisional event of bioactive peptides with the cell surface and possibly even in the receptor recognition process.

In the present study we have analysed in more detail the Ca^{2+} affinities of gastrin and CCK, and of their lipo-derivatives in different membrane mimetic conditions and the effect of Ca^{2+} binding on the conformational equilibria of the peptides. Thereby partial use was made of the lanthanide ion Tb^{+3} which is known to exhibit complexation properties similar to those of Ca^{2+} both regarding protein structures [36] and phospholipid bilayers [35].

MATERIALS AND METHODS

Materials

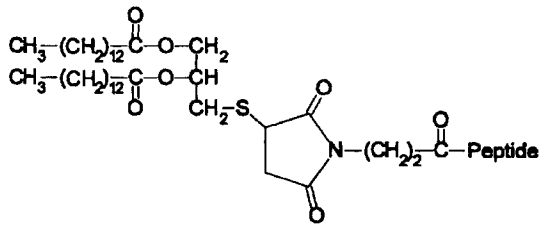
The peptides examined in the present work are listed in Table I. The synthesis of [Nle^{15}]-gastrin-17 [37], DM-gastrin [31], [Thr,Nle]-CCK-9 [38] and DM-CCK [34] have been described previously. Amino acid analysis of the acid hydrolysates (6 M HCl containing 2.5% thioglycolic acid; 110°C; 24 h) were carried out on a Biotronic analyser (LC 6001). DMPC and reagents for synthesis were purchased from Fluka (Buchs, Switzerland) and used without further purification. $TbCl_36H_2O$ was from Aldrich (Steinheim, Germany), $CaCl_22H_2O$ and $MnCl_22H_2O$ from Merck AG (Darmstadt, Germany), and 3-(*N*-morpholino)propanesulphonic acid (MOPS) from Sigma (München, Germany). For spectroscopic measurements TFE p.a. grade from Merck AG was used.

Synthesis of Peptides

[Nle^{15}]-gastrin-(9–17). The gastrin peptide was synthesized by coupling Z-Glu(O*t*Bu)-Glu(O*t*Bu)-OH [39] with H-Ala-Tyr(*t*Bu)-Gly-Trp-Nle-Asp(O*t*Bu)-Phe-NH_2 [37] via DCC/HOSu in DMF/NMP for two

Table I Gastrin and CCK Peptides Used in the Present Study

Pyr-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2 [Nle^{15}]-gastrin-(1–17)
H-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2 [Nle^{15}]-gastrin-(9–17)
H-Glu-Glu-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2 [Nle^{15}]-gastrin-(7–17)
H-Arg-Asp-Tyr(SO_3H)-Thr-Gly-Trp-Nle-Asp-Phe-NH_2 [Thr,Nle]-CCK-9



Peptide: Gly-Pro-Trp-Leu-$(Glu)_5$-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2 DM-gastrin
Arg-Asp-Tyr(SO_3H)-Thr-Gly-Trp-Nle-Asp-Phe-NH_2 DM-CCK-9

days. The fully protected nonapeptide derivative was isolated as homogeneous material in 45% yield by precipitation with MeOH. Subsequent hydrogenolysis over palladized charcoal in DMF/AcOH and exposure of the resulting derivative to TFA containing 20 equivalents of 2-methylindole led to the fully deprotected crude nonapeptide. This was purified by reversed-phase chromatography on Lichroprep RP 8 (Merck AG) using as eluent a linear gradient from 0.05 M ammonium acetate (pH 5.3)/2-propanol (95:5) to 0.05 M ammonium acetate (pH 5.3)/2-propanol/1-butanol (50:40:10) in 10 h. The title compound was obtained in 55% over the last two steps as homogeneous material according to HPLC [Nucleosil RP-8 (Macherey & Nagel, Dürren, Germany); eluent, acetonitrile/2% H_3PO_4, linear gradient from 15:85 to 67:33 in 30 min; flow rate, 1 ml/min; detection, UV at 210 nm; $R_t = 18.3$ min]; amino acid analysis of the acid hydrolysate, Glu 2.00 (2), Ala 1.02 (1), Tyr + Nle 1.95 (2), Gly 1.01 (1), Trp 1.00 (1), Asp 1.00 (1), Phe 0.99 (1); peptide content, 93% calculated for $M_r = 1127.5$; FAB-MS, 1128.6 [M + H].

[Nle^{15}]-gastrin-(7–17). H-[Glu(O*t*Bu)]$_4$-OH [39] was reacted with $(Boc)_2O$ [40] in DMF in the presence of 1 equivalent of triethylamine and worked up by evaporation of the solvent, washing of the AcOEt solution with 5% $KHSO_4$ and final isolation as DCHA salt; the yield was 50%; homogeneous on TLC. Boc-[Glu(O*t*Bu)]$_4$-OH obtained by acid washings of the related DCHA salt was coupled with H-Ala-Tyr(*t*Bu)-Gly-Trp-Nle-Asp(O*t*Bu)-Phe-NH_2 [37] via DCC/HOSu in DMF/NMP, and the reaction mixture was worked up as described above for [Nle^{15}]-gastrin-(9–17); the yield was 86% of the homogeneous product. Exposure of the fully protected undecapeptide to TFA containing 20 equivalents of 2-methylindole led to the crude product which was then purified by reversed-phase chromatography as described for [Nle^{15}]-gastrin-(9–17). The yield was 54%; homogeneous on HPLC [Nucleosil RP-8; eluent acetonitrile/2% H_3PO_4, linear gradient from 15:85 to 60.5:39.5 in 30 min; flow rate, 1 ml/min; detection, UV at 210 nm; $R_t = 20.7$ min]; amino acid analysis of the acid hydrolysate, Glu 3.95 (4), Ala 1.01 (1), Tyr + Nle 1.96 (2), Gly 0.99 (1), Trp 0.96 (1), Asp 1.00 (1), Phe 1.00 (1); peptide content, 81.2% calculated for $M_r = 1385.6$; FAB-MS, 1386.9 [M + H].

Methods

Sample Preparation. The peptide concentrations were determined by weight and peptide content and further controlled by near UV absorption measurements after filtration of the final solutions through a 0.45 μm polycarbonate filter (Millipore, Bedford, USA); all buffers and solvents were also Millipore-filtered. Aqueous solutions of [Nle^{15}]-gastrin-17 and [Thr,Nle]-CCK-9 were prepared by dissolving the peptide samples in 10 mM MOPS buffer (pH 7.0) for fluorescence measurements and in 10 mM MOPS, 100 mM NaCl (pH 7.0) for CD measurements. The solutions in TFE were prepared by dissolving the gastrin and CCK peptides in TFE/water (1:1) and diluting with TFE to reach a final water content of 2%. DM-gastrin and DM-CCK samples were soaked in buffer (10 mM MOPS, pH 7.0 for fluorescence measurements and 10 mM MOPS, 100 mM NaCl, pH 7.0 for CD measurements), vortexed and then sonicated for a short period of time with a Branson

titanium rod sonifier in a cooling bath until optically transparent vesicle preparations were obtained; these were filtered through a 0.45 μm polycarbonate filter (Millipore). Similarly, DMPC vesicles were prepared from phospholipid samples freshly lyophilized from 1-butanol. After sonication, the vesicle preparations were centrifuged in order to remove titanium dust contamination from the sonifier rod and were then incubated for 1 h at 30°C for annealing. For the preparation of fused lipo-peptide/DMPC vesicles lipid/peptide ratios of 50:1 were used, since this ratio was found to be optimal for a quantitative fusion of the lipo-peptide vesicle with phospholipid vesicles [33]. The lipopeptide and DMPC vesicles were mixed and vortexed in an ice-bath for a short period of time and then incubated for 1–3 h at 30°C to reach full equilibration of the systems. Tb^{3+} and Ca^{2+} stock solutions were prepared by dissolving $TbCl_36H_2O$ and $CaCl_22H_2O$ in 10 mM MOPS (pH 7.0) and 10 mM MOPS, 100 mM NaCl (pH 7.0), respectively. For metal ion titrations in 98% TFE the stock solutions of $TbCl_36H_2O$, $MnCl_22H_2O$ and $CaCl_22H_2O$ were prepared in 80% aqueous TFE.

Fluorescence Measurements. Fluorescence emission spectra were recorded on a Perkin Elmer LS 50B spectrofluorimeter equipped with a thermostatted cell holder and a data station for processing of the spectra. Quartz cells of 1 cm optical path were used and 5 nm excitation and emission bandwidths. The excitation wavelength was 280 nm and spectra were recorded in the 450–600 nm wavelength region using a time delay of 0.1 ms and a gate of 3 ms. Fluorescence emission was determined by area integration in the 535–555 nm range. The measurements were performed at 20°C and peptide concentrations were 10 μM for the gastrin and 20 μM for CCK peptides. They were corrected for the dilution. Titrations with Tb^{3+} were performed in 1/4 equivalent aliquots and the spectra were recorded 2 min after addition of the Tb^{3+} solution and subsequent mixing.

CD Measurements. CD spectra were recorded on a Jobin-Yvon dichrograph Mark IV equipped with a thermostatted cell holder and connected to a data station for signal averaging and processing of the spectra. The CD measurements were performed at 20°C at peptide concentrations in the range of 30–50 μM and at 30°C for the lipo-peptide/DMPC systems. Quartz cells of 0.1 cm (gastrin peptides) and 0.2 cm (CCK peptides) optical pathlength were used for recording the spectra in the far UV region; for the near UV 1 cm pathlength was used. The data averages are of 10 scans; the spectra are reported in ellipticity units per mole of amino acid residue in the far UV, $[\Theta]_R$, and in ellipticity units per mole of peptide in the near UV, $[\Theta]_M$. The diastereomeric mixture of the 3-[(3′Ξ)-3′-[(2*RS*)-1,2-dimyristoyl-3-thioglycerol]succinimidyl]propionyl moiety does not contribute to the optical activity, since negligible CD was detected in separate measurements for this group as a propionic acid derivative [31].

RESULTS AND DISCUSSION

Metal ion affinities of the gastrin and CCK peptides, listed in Table I, were determined by monitoring changes of the chiroptical properties in the far UV resulting from conformational transitions induced by metal ion complexation, and by measuring the energy transfer fluorescence of Tb^{3+} due to the presence of tryptophan residues in all peptide samples analysed in this study. Lanthanide ions are known to replace calcium without causing structural modifications in proteins since both ions exhibit a strong propensity for oxygen donor groups, very similar effective ionic radii, lack of a strong directionality in binding donor groups and an apparent variability in the coordination number [36].

Binding of Ca^{2+} and Tb^{3+} to gastrin and CCK Peptides in Aqueous Solution

From conformational studies on gastrin and CCK it is well established that these relatively short peptides do not adopt preferred ordered structures in the phosphate buffer, but assume defined conformations in aqueous organic media [41, 42]. In the present study MOPS buffer was used in order to avoid possible competition of the phosphate anions for metal ions. Despite the more detergent-like nature of this buffer, the CD spectra of [Nle^{15}]-gastrin-17 and [Thr,Nle]-CCK-9 in the far UV are very similar to those in the phosphate buffer and consistent with absence of ordered structure at significant extents. As already observed for the gastrin peptides in the phosphate buffer [23, 24] addition of Ca^{2+} at increasing concentrations to [Nle^{15}]-gastrin-17 and [Thr,Nle]-CCK-9 in MOPS produced only negligible CD changes in the far UV region, thus excluding metal ion binding at affinities detectable by this procedure. Identical results were obtained by replacing calcium with terbium and monitoring the energy transfer fluorescence of Tb^{3+}.

Binding of Ca^{2+} and Tb^{3+} to gastrin and CCK Peptides in Aqueous TFE

In titration experiments of gastrin peptides with Ca^{2+} and Mg^{2+} a conformational transition from ordered to predominantly random coil structure was observed [24, 25]. In order to determine whether Tb^{3+} can replace Ca^{2+} with very similar conformational effects, [Nle^{15}]-gastrin-17 was titrated in 98% TFE with Ca^{2+} and Tb^{3+} under identical conditions by monitoring in the far UV region the dichroic changes induced by metal ion complexation. As reported previously [24], binding of Ca^{2+} at an ion/peptide ratio of 3:1 leads to an almost complete loss of ordered structure. Conversely, the CD spectrum of gastrin at a Tb^{3+}/peptide ratio of 3:1 strongly resembles the CD spectra of shorter gastrin peptides, particularly of pentagastrin in TFE [41]. As shown in Figure 1, the spectrum clearly reveals persistence of ordered structure possibly of a 3_{10}-helix type, most probably located in the C-terminal portion of the molecule. A similar effect has already been observed by replacing Ca^{2+} with Mg^{2+} [25] and, thus, may not be related to the different charge of the terbium ion. The plots of the relative variation of the molar ellipticities of gastrin at 215 nm as a function of the metal ion/peptide ratios yielded titration curves from which both for Ca^{2+} and Tb^{3+} *R* values of 3 were extrapolated (see Table II). These results confirm the very similar coordination properties of calcium and terbium.

Tb^{3+} binding to gastrin was then monitored by measuring the energy transfer fluorescence in the 535–555 nm region as shown in Figure 2. Plotting the relative variation of the fluorescence against the Tb^{3+}/[Nle^{15}]-gastrin-17 ratios, only a maximum of two metal ions were found to bind per mole of gastrin (Figure 3). This result differs from those of the CD titration experiments. It can, however, be rationally explained by the fact that only two metal ions are coordinated to proper sites of the peptide molecule for an efficient energy transfer from the tryptophan

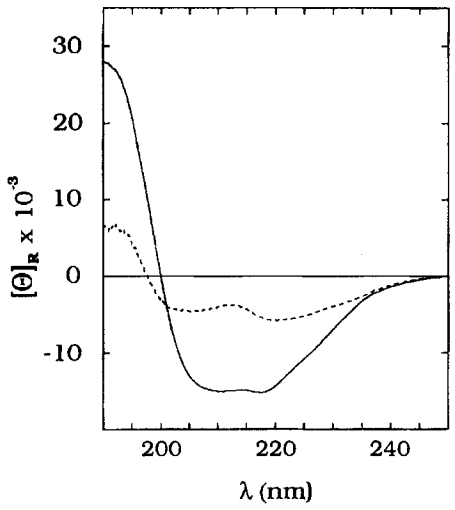


Figure 1 Far UV CD spectra of [Nle^{15}]-gastrin-17 in 98% TFE in absence (——) and in presence of 3 equiv. Tb^{3+} (- - - -).

sidechain chromophore to Tb^{3+}. That one or both of the tryptophan residues of gastrin are involved in the energy transfer process is confirmed by the parallel decrease of the intrinsic fluorescence of the tryptophan from which, again, a ratio of 2 for Tb^{3+}/[Nle^{15}]-gastrin-17 is derived. Presaturation of the gastrin peptide with calcium ions was without effect on the titration curve with Tb^{3+} confirming the expected higher binding affinity of this metal ion in comparison to calcium.

In order to locate the terbium binding sites more precisely, similar titration experiments were performed on N-truncated gastrin peptides. For [Nle^{15}]-gastrin-(9–17), which contains only two residues of the gastrin characteristic pentaglutamic acid sequence, an *R* value of 1 was determined, whereas upon elongation of the peptide with two additional glutamic acid residues, i.e. in the case of [Nle^{15}]-gastrin-(7–17), the identical *R* value of 2 was obtained as for the intact small gastrin sequence. This fact

Table II Affinities of Gastrin and CCK Peptides for Ca^{2+}, Mn^{2+} and Tb^{3+} in Different Media as Determined by CD and/or Tb^{3+} Energy Transfer Fluorescence

Peptide	10 mM MOPS, pH 7.0; Tb^{3+}	10 mM MOPS, 0.1 M NaCl; pH 7.0; Ca^{2+}; CD	98% TFE Tb^{3+}	98% TFE Ca^{2+}	98% TFE Mn^{2+}; CD
[Nle^{15}]-gastrin-17	0.0	0.0	2 (3 with CD)	3	n.d.
[Thr,Nle]-CCK-9	0.0	0.0	1 (1 with CD)	1	1
DM-gastrin (SUVs)	2	n.d.	n.d.	n.d.	n.d.
DM-CCK (SUVs)	1.75	n.d.	n.d.	n.d.	n.d.

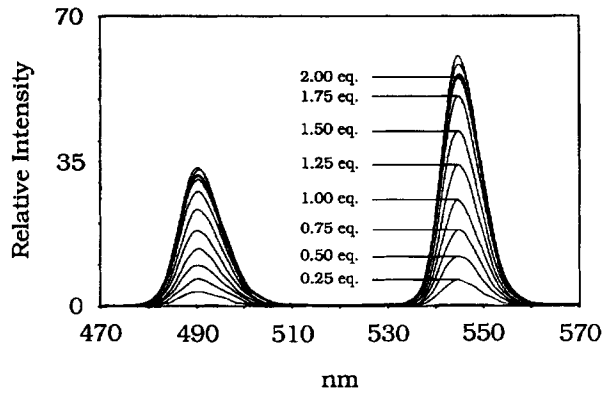


Figure 2 Energy transfer fluorescence spectra of Tb^{3+} at increasing metal ion/[Nle^{15}]-gastrin-17 molar ratios in 98% TFE.

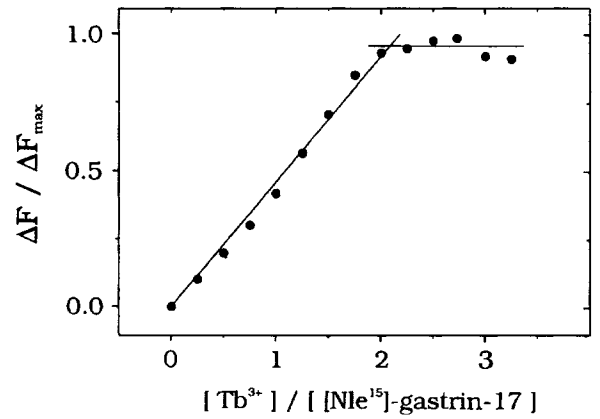


Figure 3 Relative variation of Tb^{3+} fluorescence measured by integration of the 535–555 nm band, as a function of the Tb^{3+}/[Nle^{15}]-gastrin-17 molar ratio R in 98% TFE.

may represent a fortuitous coincidence, but it is worth noting that the presence of four glutamic acid residues are required for the full onset of the gastrin characteristic conformation in aqueous TFE [43] and, more importantly, for full receptor binding affinity and gastric acid secretion potency [44].

The α-helix type CD spectrum of [Thr,Nle]-CCK-9 in 98% TFE (see Figure 4) is characterized by low intensity and despite its difficult interpretation, because of the presence of three aromatic residues in the relatively short sequence, it can be assigned to the presence of γ-turn and/or α-helix type ordered structure [42]. Addition of metal ions at increasing concentrations to [Thr,Nle]-CCK-9 in 98% TFE leads to a transition from one dominant conformational state to a second one as well evidenced by the isosbestic points at 202–203 and 216–217 nm obtained in both the Ca^{2+} and Tb^{3+} titration experiments shown in Figures 4(a) and (b). At metal ion/peptide ratios of 1 : 1 the CD spectra exhibit a strong negative maximum centred around 223–224 nm. The spectra are reminescent of β-turns, whereby the lack of a positive extremum around 210 nm could possibly result from the negative contributions of the tryptophan chromophore in this wavelength region. A very similar conformational transition is observed in the titration of the CCK peptide with Mn^{2+} (Figure 4(c)). Thereby complexation with this metal ion is apparently stabilizing to higher extents the β-turn structure as reflected by appearance of a positive extremum at 208 nm, confirming the above interpretation of the CD properties. Thus, coordination of metal ions to the CCK peptide leads to a conformational transition from one type to a second type ordered structure, and not to an unfolding

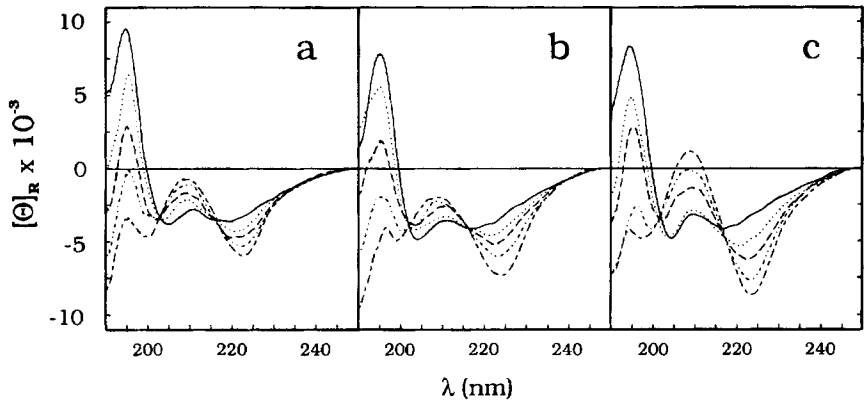


Figure 4 Far UV CD spectra of [Thr,Nle]-CCK-9 in 98% TFE at various metal ion/peptide molar ratios: $R=0$ (—), $R=0.25$ (. . . . .), $R=0.5$ (— ——), $R=0.75$ (– . .–), $R=1.0$ (– – –); (a) Ca^{2+}; (b) Tb^{3+}; (c) Mn^{2+}.

process as observed for the homologous gastrin peptide. At Ca^{2+}, Mn^{2+} and Tb^{3+}/[Thr,Nle]-CCK-9 ratios > 1 slight additional changes in the dichroic properties were observed, which could reflect interactions of the metal ions with the N-terminus where the presence of a salt bridge was postulated from ^{1}H-NMR data [45]. Plots of the relative variation of the ellipticity as a function of metal ion/CCK ratios allow for extrapolation of R values of 1 for Ca^{2+}, Mn^{2+} and Tb^{3+} (Table II). This R value of 1 was further confirmed by monitoring Tb^{3+} complexation via the energy transfer fluorescence measurements as shown in Figure 5.

In order to understand the different metal ion affinities in terms of R values of gastrin and CCK the structural models of [Nle^{15}]-gastrin-17 and [Thr,Nle]-CCK-9, as derived from ^{1}H-NMR data in aqueous organic solvents [45–47], were used to calculate the potential Ca^{2+} binding sites with the GRID program [48]. Both peptides show in their common C-terminal pentapeptide portion a high-affinity Ca^{2+} binding site involving the sidechain carboxyl function of the aspartic acid and the carbonyl group of glycine. For the gastrin molecule a second strong Ca^{2+} binding site was located in the pentaglutamic acid sequence and an additional low affinity binding site again in the C-terminal part of the molecule. This would fully agree with the experimental findings that gastrin can bind up to three metal ions, two with higher affinity and one with lower affinity as well assessed by Peggion *et al.* [24, 25]. Even in the case of CCK the computation predicts a second Ca^{2+} binding site in the C-terminal part of the molecule, but of very low affinity; therefore

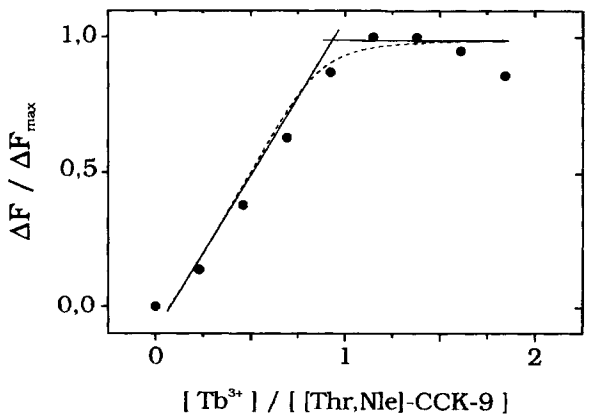


Figure 5 Relative variation of Tb^{3+} fluorescence measured by integration of the 535–555 nm band as a function of the Tb^{3+}/[Thr,Nle]-CCK-9 molar ratios in 98% TFE.

[Thr,Nle]-CCK-9 tends to form a 1 : 1 complex with the metal ion.

No reasonable explanation can be found for the different conformational effects of metal ion coordination to gastrin and CCK in view of their common C-terminal pentapeptide sequence, unless the different N-terminal extensions in the two peptides are affecting in differentiated mode the conformational state of the C-terminus. This would also agree with the observed different Ca^{2+} affinity of this common portion in the two molecules.

Binding of Ca^{2+} and Tb^{3+} to DM-gastrin and DM-CCK in Aqueous Buffer

Both DM-gastrin and DM-CCK aggregate spontaneously in aqueous solution to form relatively stable vesicles with the peptide headgroups more or less exposed at the water/lipid interphase [31, 34]. As the peptide headgroups of the inner layer should not be accessible to metal ions added to the vesicle preparations, Tb^{3+}/peptide molar ratios of maximally 1 : 1 for DM-gastrin and 0.5 : 1 for DM-CCK were expected by taking into account the R values determined in TFE. However, the R values determined by the fluorescence procedure were for both lipo-peptides significantly higher, i.e. 2 for DM-gastrin and 1.75 for DM-CCK (see Table II). These results can only be explained by the fact that the vesicles are leaking or that transbilayer flip-flop is taking place at high rates. Both processes are possible because of the high fluidity of these lipo-peptide vesicle preparations [33, 34]. Moreover, light-scattering experiments have clearly shown that DM-CCK SUVs are polydispersed and thus may also contain micelles [34]. Additionally the aspartic acid residues located in the N- or C-terminal portion of the CCK molecule could lead in this aggregated state to intermolecular ion complexation and, thus, account for the unexpectedly high Tb^{3+}/CCK peptide ratio of 1.75. Intermolecular Ca^{2+} complexation and, correspondingly, aggregation of aspartic acid-containing peptides in lipid bilayers has already been reported [49].

Regarding the conformational effects induced by coordination of Ca^{2+} to CCK it is again stabilizing secondary structure elements in the peptide headgroup as indicated by the increased intensity of the negative maxima in the CD spectrum of DM-CCK (Figure 6(a)). The opposite effect is observed in the case of DM-gastrin in full agreement with the data obtained in TFE. The CD spectrum of DM-gastrin in MOPS buffer (Figure 6(b)) resembles that monitored in Tris phosphate buffer [31] and supports the

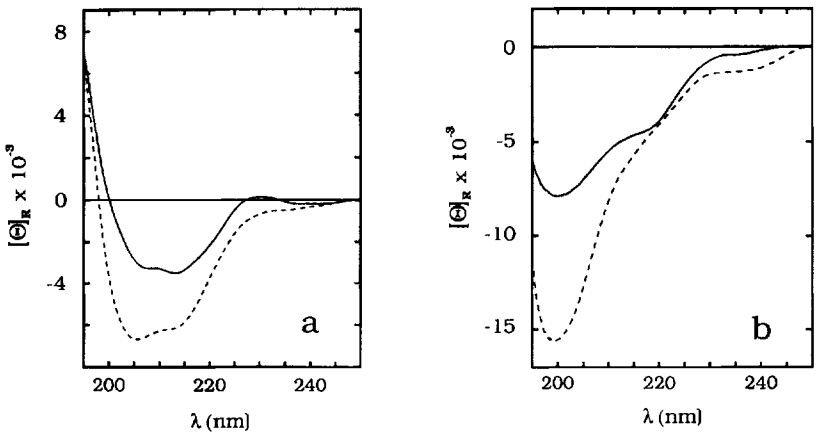


Figure 6 Far UV CD spectra of DM-CCK (a) and DM-gastrin (b) in 10 mM MOPS, 100 mM NaCl, pH 7.0 in absence (——) and presence (– – –) of 2 equiv. of Ca^{2+}.

persistence of some ordered structure of the gastrin headgroup in the DM-gastrin SUVs. Addition of Ca^{2+} leads to a strong increase of the negative maximum at 198 nm and a weakening of the shoulder around 215 nm, thus suggesting a shift to more random coil conformations.

Interaction of Ca^{2+} with DM-gastrin/DMPC and DM-CCK/DMPC Fused Vesicles

From microcalorimetric, fluorescence and CD measurements it was concluded that significant interactions of [Nle^{15}]-gastrin-17 and [Thr,Nle]-CCK-9 with zwitterionic phospholipid vesicles do not take place [32]. Interestingly, even in presence of metal ions this interaction could neither be induced nor enhanced to extents detectable by CD or Tb^{3+} energy transfer fluorescence measurements. This observation fully agrees with the lack of ionophoretic activity of [Nle^{15}]-gastrin-17 and [Nle,Thr]-CCK-9 which, however, was observed upon lipo-derivatization of the gastrin and CCK peptide and, thus, by forcing interactions with the lipid bilayers [50]. The ionophoretic activity of the lipo-peptides was comparable to those reported for insulin, glucagon and substance P [29], but significantly lower than those of known Ca^{2+} ionophores. It has recently been shown that interaction of peptides with lipid bilayers leads to strong perturbation of the fatty acid chain packing, a fact which markedly increases the rates of transbilayer flip-flop of lipid monomers [51]. Therefore the Ca^{2+} influxes into phospholipid vesicles provoked with DM-gastrin and DM-CCK could derive mainly from these relatively fast flip-flop processes of the bilayer lipids which are known to bind Ca^{2+} at the phosphatidylcholine headgroup. The lipo-peptides could also participate in the flip-flop movements and thus, translocate Ca^{2+} if the peptide headgroups are coordinated to Ca^{2+} as suggested by the Tb^{3+} and Ca^{2+} binding experiments with DM-gastrin and DM-CCK vesicle preparations.

Insertion of DM-gastrin into phospholipid vesicles leads to full exposure of the gastrin moiety to the bulk water in predominantly random coiled structure [33]. This effect was confirmed under the present experimental conditions too, where the resulting CD spectrum of DM-gastrin/DMPC (Figure 7(b)) clearly reflects the absence of ordered conformations in the peptide headgroups. Conversely, insertion of DM-CCK into DMPC bilayers is affecting the dichroic properties as well evidenced by a comparison of the related spectra in Figures 6(a) and 7(a). The shoulder at lower wavelengths is weakened and the negative maximum red-shifted from 213–214 to 216–217 nm. The overall CD spectrum is consistent with a more β-sheet type conformation of the peptide headgroup. Ca^{2+} and Tb^{3+} titration experiments were not performed with the merged vesicle systems because of the expectedly strong competition of the phosphatidylcholine headgroups for the metal ions. But, in order to simulate the conditions of the peptide hormones in the hypothetical cell membrane-bound state, the CD spectra of DM-gastrin/DMPC and DM-CCK/DMPC were recorded in the presence of 1 and 2 mM Ca^{2+} concentration. In both cases the effect of the Ca^{2+} ions on the dichroic properties of the peptide headgroups were weak in terms of the overall CD pattern, but the intensities of the spectra were reduced as shown in Figures 7(a) and (b). This hypochromic effect might result from peptide back-

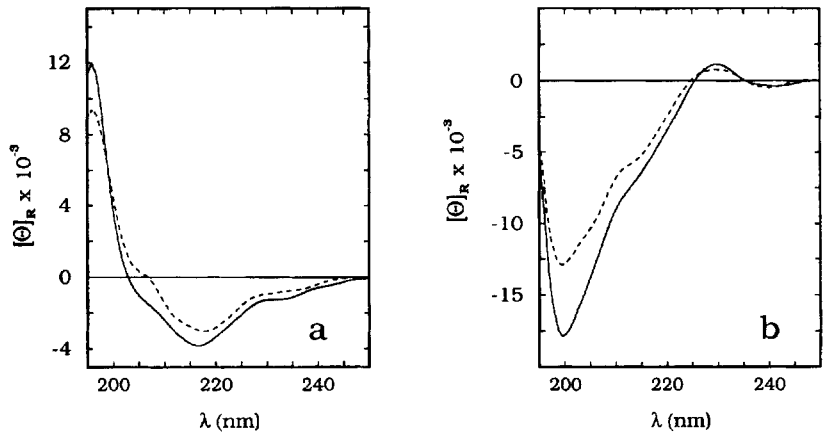


Figure 7 Far UV CD spectra of DM-CCK/DMPC (a) and DM-gastrin/DMPC (b) at peptide lipid molar ratios of 1 : 50 in 10 m M MOPS, 100 mM NaCl, pH 7.0, in absence (——) and presence (– – –) of 2 mM Ca^{2+}.

bone chromophore couplings due to aggregation phenomena induced by intermolecular metal ion complexation. Such clustering of the lipo-peptides into domains favoured by metal ions is reasonable and would confirm the experimental results of the microcalorimetric measurements at least in the case of DM-CCK [34].

CONCLUSIONS

A detailed study of the effects of extracellular calcium, lanthanum and manganese ions on the signal transduction of CCK [14] has clearly revealed an increased production of the second messenger IP_3 by calcium and strong inhibitory effects exerted by lanthanum and manganese. These inhibitory actions have been attributed to interference of lanthanum or manganese with the calcium-induced calcium release/influx possibly via a voltage-dependent calcium channel. The relatively small effect of the calcium-channel blockers verapamil, diltiazem and nifedipine could possibly suggest an alternative pathway more strictly related to the the results of the present study.

The lanthanide Tb^{3+} and even Mn^{2+} bind to CCK in low dielectric media at identical metal ion/peptide ratios as Ca^{2+}. Thereby very similar conformational changes of the peptide were induced in TFE. According to the hypothesis of Ananthanarayanan [30] the bioactive states of peptide hormones are the Ca^{2+}-bound forms which, upon interaction with the receptors, would deliver the metal ion to the binding pocket for the induction of local conformational changes in the receptor cleft as the first step in signal transduction. As Tb^{3+} was found to bind to the CCK class of hormones with remarkably higher affinity than Ca^{2+}, it is conceivable that in the presence of lanthanide ions in the extracellular phase or at the water/lipid interphase the peptides are complexed almost exclusively by this metal ion which could affect differently the topochemical environments of the receptor ligand-binding cleft. A similar effect could be exerted by Mn^{2+} ions which bind to CCK as Ca^{2+}. This explanation, however, is highly hypothetical, as in the present study it was clearly shown that data collected in low dielectric media such as TFE are not necessarily reflecting the environment in lipid bilayers. In fact, only lipo-derivatization of gastrin and CCK and thus, artificially enhanced lipid affinity allowed to translocate the two peptides to the lipid/water interphase of phospholipid vesicles, and even in this artificial model system the conformational effects of calcium on the peptide headgroups were rather weak. Our data are more consistent with the results of a conformational study on substance P in differently charged phospholipid milieus containing mmolar concentration of Ca^{2+} ions where neither lipid nor metal ion-induced conformational effects were detected [52]. Therefore the results of the present study do not support the generalized concept of Ca^{2+}-bound forms of hormones as the bioactive states [30] and confirm the strong sequence-dependent behaviour even of highly homologous bioactive peptides such as gastrin and CCK at the level of membrane interactions.

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