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REVIEW ARTICLE

On the Mechanism of Hormone Recognition and Binding by the CCK-B/Gastrin Receptor

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Abstract: Lipidation with long-chain di-fattyacyl-glycerol moieties was used to anchor gastrin and CCK peptides irreversibly to lipid bilayers. Intervesicular lipopeptide transfer to model phospholipid bilayers is fast and quantitative, leading to a different mode of insertion of lipo-gastrin and lipo-CCK in lipid bilayers. Lipo-gastrin remains exposed to the bulk solvent in a predominantly random coil structure as a consequence of electrostatic repulsion, whereas lipo-CCK exhibits a pronounced tendency to form peptide domains with insertion of its C-terminus into more hydrophobic compartments of the bilayer. Thereby Ca^{2+} at physiological concentrations favours this aggregational phenomenon. Since both lipo-peptides were found to retain almost full receptor affinity despite their irreversible anchorage to the bilayer, a membrane-bound pathway in the receptor recognition and binding process is indeed possible. According to the data collected in this study, CCK might possibly use this pathway, whereas accumulation of gastrin on the cell membrane with prefolding of the ligand at the water/lipid interface is hardly conceivable. Nevertheless the observed receptor interaction of the deliberately membrane-anchored gastrin offers interesting constraints for computational docking experiments on a modelled CCK-B/gastrin receptor by additionally taking into account information derived from mutagenesis studies. Despite the limitations of such modelling experiments, the resulting picture of the gastrin/receptor complex allowed the visualization and rationalization of the experimental results of the extensive structure–function studies performed previously on this family of gastrointestinal hormones.

Keywords: peptide lipidation; vesicles; lipid interaction; conformation; receptor binding; ligand receptor docking

INTRODUCTION

Recognition and binding of a hormone by the membrane-bound receptor is a bimolecular interaction for which an entropic cost derives that is a consequence of the degrees of freedom of motion lost when the two molecules are rigidly constrained within a complex. If the binding process involves capturing a flexible molecule like a peptide, whose internal rotations about single bonds must also be restricted, the consequence is an additional adverse entropic penality that has to be compensated by the enthalpy of binding to the receptor.

Extensive studies have been performed on the enthalpy/entropy compensation in the binding of agonists, partial agonists and antagonists to β-adrenergic receptors [1–3]. It has been clearly shown that agonists bind with large enthalpies and entropies, whereas antagonists are generally observed to bind with much less negative enthalpies, but also much less negative entropies than agonists. These facts strongly support binding in a tight complex in

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the case of agonists with loss of translational and rotational degrees of freedom, whereas the small enthalpy changes of antagonists seem to be consistent with the formation of a 'loose complex'. In the latter case mainly hydrophobic interactions should provide the mechanism for the entropy-driven binding by the release of ordered water from polar groups and hydrophobic surfaces that become buried from the solvent.

In order to account better for the thermodynamics of the binding process of both agonists and antagonists to membrane-bound receptors, the intermediate formation of membrane-bound states of the ligands has been proposed by Schwyzer [4–6]. An accumulation of peptide ligands on the membrane surface followed by two-dimensional diffusion to the receptor is statistically more favoured than their direct collision with the surface-exposed binding sites of the receptor. Thereby the major driving force for this partitioning process between the extracellular aqueous phase and the lipid bilayer is widely assumed to the hydrophobic effect, although convincing evidence has been accumulated from experimental studies on peptide–membrane interactions that the process is more complicated owing to the fact that membrane bilayers are anisotropic and chemical heterogeneous interfacial phases [7–9]. In fact, it has been shown that the 'classical' entropy-driven hydrophobic partitioning [10] may not be operative in some, if not all, bilayer systems where the enthalpy-driven partitioning, i.e. the 'non-classical' hydrophobic effect if frequently dominant [11–13]. Thus, solute–bilayer interactions and the response of the bilayer to partitioning contribute significantly to the overall thermodynamics of the process.

As a consequence the electrostatic and hydrophobic gradients of the lipid/water interface may lead to preorientation and prefolding of the adsorbed peptide ligands. Thereby strong electrostatic interactions between lipid headgroups and captured ligands could offset the enthalpic cost of the conformational transition. Moreover, the adverse entropy changes associated with transferring the flexible ligand from solution to the membrane-bound state could be compensated partly by the increase in entropy due to the known perturbation of the lipid bilayer [14] and partly by the classical hydrophobic effect [10] resulting from the release of ligand-bound 'high-energy' water into the bulk water phase. This accumulation of peptide ligands at the cell-membrane surface would greatly increase the local ligand concentration, and this possibly in cell membrane domains. It would also induce a prefolding of the ligand into a bioactive-like conformational state that facilitates recognition and binding by the mechanism 'conformational selection' [15] instead of the 'classical' mechanism of induced fit [16]

This hypothesis of a hormone/receptor recognition process via an intermediate membrane-bound state, although thermodynamically attractive, has recently been biased by the increased knowledge about the chemical structure of membrane-bound receptors. The majority of these are composed of seven transmembrane segments connected at the cytoplasmic and extracellular face of the lipid bilayer by more or less extended loops that may preclude a lateral access of a membrane-associated ligand at the water/lipid interface. For this reason the author has undertaken a study of the hypothetical membrane-bound pathway, shown in Figure 1, by using gastrin (HG) and cholecystokinin (CCK) as model peptide hormones, and a lipophilic derivatization of these peptides to force their capture by membrane bilayers without the possibility of escape into the extracellular water phase. By such deliberate anchorage of the hormones in the bilayer their interaction with the receptor should be limited to a two-dimensional diffusion at the bilayer surface and to a lateral penetration of the receptor to reach the binding cleft.

Nature exploits various types of lipidation to anchor more or less tightly biomolecules to cell membranes (for recent review see [17]). It uses fattyacylation of amino and thiol functions, prenylation of cysteine residues, but also double-tailed phospholipid derivatives as in the case of the glycosylphosphatidylinositol anchor (GPI). Studies of the association of mono-fattyacylated or monoprenylated peptides and proteins with lipid bilayers have shown that the binding is very rapidly reversible, even when it is 'strong' in the thermodynamic sense [18–23], and that additional electrostatic interactions are required to reach apparent dissociation constants of $10^{-7}\,\mathrm{M}^{-1}$ [21,24]. With two proximal acyl and/or isoprenyl substituents, i.e. with the 'dual anchor' motifs, the rapid reversibility of membrane-binding is suppressed and peptides and proteins remain trapped for all practical purposes irreversibly in the bilayer [25]. Similarly, GPI-anchored proteins are 'firmly' membrane-associated in the sense that they cannot by extracted into the aqueous phase without grossly disrupting the membrane [26, 27]. Correspondingly, for the purpose of our study a GPI-like membrane-anchor was designed with di-fattyacyl-glycerol as the lipid moiety

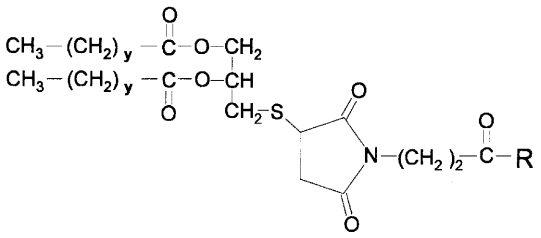
to ensure as much irreversible capture as possible of the lipo-peptides by plasma membranes [28].

For a facile and selective grafting of this di-fattyacyl-moiety to peptides a thiol-functionalized glycerol derivative was selected that allows the smooth and well established thiol-maleinimide reaction to be exploited. The resulting anchor, shown in Figure 2), consists of the double-tailed lipophilic moiety which is spaced from the peptide by the more hydrophilic thiosuccinimido portion and a short ethylene group which should suffice for full flexibility in the linker group. Since the chirality of the glycerol group affects only marginally the packing of bilayers [29], (2*R,S*)-1,2-di-fattyacyl-3-mercaptoglycerol derivatives were used throughout this study for lipidation of the peptides. Recently, alternative double-tailed peptide lipidations have been reported, e.g. acylation of peptides with 1,2-di-fattyacyl-glyceric acid pentafluorophenyl ester [30] or esterification of C-terminal carboxyl functions with 1,3-di-fattyacyl-2-bromoacetyl-glycerol [31]. Although both methods enable better mimicking of natural lipidation than the di-alkyl-amides [32] or glutamic acid bis-alkyl-amide used in other studies [33, 34], a spacer between lipid and peptide moiety as present in GPI and in our lipidation procedure is missing.

Synthesis and Physical Properties of Lipo-Gastrin and Lipo-CCK Peptides

From extensive structure–function studies on gastrin and CCK it is well established that N-terminal modifications of these peptide hormones only marginally affect their bioactivity profiles, mainly since the intact C-terminal tetrapeptide moiety represents the message site of this family of gastrointestinal hormones (for a recent comprehensive review see [35]). Consequently, the double-tailed lipid moieties were grafted to the N-termini of the peptides via reaction of (2*R,S*)-1,2-di-fattyacyl-3-mercaptoglycerol derivatives with the N^{α}-maleoyl-β-alanyl-gastrin and N^{α}-maleoyl-β-alanyl-CCK peptides to produce the lipo-compounds listed in Figure 2 [36–38].

For phosphatidylcholine it is known that the critical micellar concentration (CMC) decreases exponentially in function of the acyl chain length with CMC values of 1.4×10^{-3}, 2.7×10^{-4}, 5×10^{-6} and 4.7×10^{-10} M for diheptanoyl-, dioctanoyl-, didecanoyl- and dipalmitoylphosphatidylcholine, respectively [10]. Although the nature of the hydrophilic headgroup is known to affect the CMC of amphiphiles, the values determined for lipo-tripeptides and -tetrapeptides were found to be in the order of magnitude of the phospholipids [34]. We therefore



	R	y
DM-CCK	-Arg-Asp-Tyr(SO_3H)-Thr-Gly-Trp-Nle-Asp-Phe-NH_2	12
DP-HG-[2-17]	-Gly-Pro-Trp-Leu-$(Glu)_5$-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2	14
DM-HG-[2-17]	-Gly-Pro-Trp-Leu-$(Glu)_5$-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2	12
DM-HG-[7-17]	-$(Glu)_4$-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2	12
DM-HG-[9-17]	-$(Glu)_2$-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH_2	12
DPr-HG-[14-17]	-Trp-Nle-Asp-Phe-NH_2	1
DH-HG-[14-17]	-Trp-Nle-Asp-Phe-NH_2	4
DO-HG-[14-17]	-Trp-Nle-Asp-Phe-NH_2	6
DD-HG-[14-17]	-Trp-Nle-Asp-Phe-NH_2	8
DM-HG-[14-17]	-Trp-Nle-Asp-Phe-NH_2	12
DP-HG-[14-17]	-Trp-Nle-Asp-Phe-NH_2	14

Figure 2 Chemical structure of the lipidated gastrin and CCK peptides used in the present study.

expected that, for example, the CMC values of the lipo-tetragastrins with increasing fatty acid chains (Figure 2) would be in the range of 10^{-2}–10^{-3} M for DPr-HG-[14–17] and DH-HG-[14–17], 10^{-3}–10^{-4} M for DO-HG-[14–17], 10^{-5}–10^{-6} M for DD-HG-[14–17], 10^{-7}–10^{-8} M for DM-HG-[14–17] and approximately 10^{-10} M for DP-HG-[14–17]. By CD measurements on this series of compounds at 5×10^{-5} M concentration three distinct types of spectra were recorded related to DPr-HG-[14–17] and DH-HG-[14–17], DO-HG-[14–17] and DD-HG-[14–17], and to DM-HG-[14–17] and DP-HG-[14–17], respectively, (Figure 3). An unambiguous interpretation of the spectra in terms of conformational states of the peptide headgroups is impossible because of the presence of two strong chromophores in the short tetrapeptide amide sequence. But the three CD patterns differ significantly and thus may well be correlated to the expected three aggregational states, i.e. prevalently monomeric for DPr-HG-[14–17] and DH-HG-[14–17], micellar for DO-HG-[14–17] and DD-HG-[14–17], and vesicle type for DM-HG-[14–17] and DP-HG-[14–17]. Taking into account a possibly perturbing effect of the relatively large peptide headgroup on the fatty acid packaging as confirmed by the fluid state of the DM-HG-[14–17] vesicles (see below), these aggregational states would fully agree with findings on phosphatidylcholine derivatives where the C9-compound represents the last in the series capable of forming vesicles, whereas C8-phosphatidylcholine has already aggregated into micelles [39].

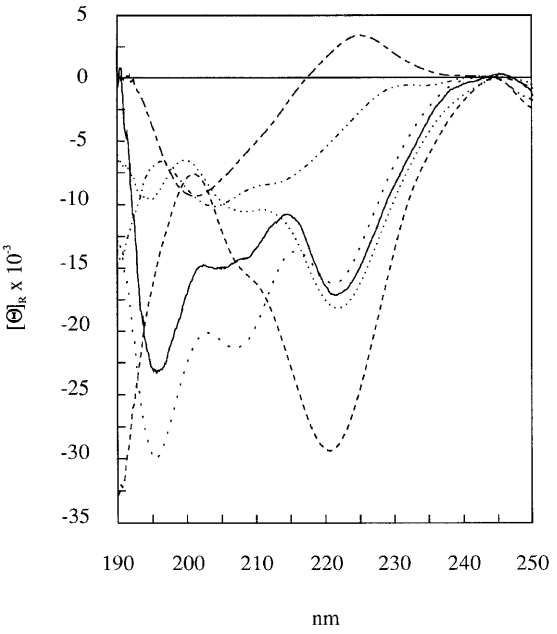


Figure 3 CD spectra of DPr-HG-[14–17] (– – –), DH-HG-[14–17] (·····), DO-HG-[14–17] (·····),DD-HG-[14–17] (– – –), DM-HG-[14–17] (—) and DP-HG-[14–17] (-----) in 10 mM MOPS buffer, 100 mM NaCl (pH 7.0).

In recent years the self-assembling properties of various synthetic double-tailed lipids with carbohydrates, poly-α-amino acids, amino acids and peptides as headgroups have been studied and the amphiphiles containing long alkyl chains were found to assemble into bilayer structures characterized by phase transitions temperatures (T_m) that depend upon the chemical structure of the headgroup [30, 32–34, 40–43]. In full agreement with these data the higher homologues of the double-tailed lipo-peptides examined in our studies aggregate spontaneously in aqueous solution to form vesicles; e.g. extrusion of DM-HG-[2–17] and DM-HG-[4–17] generates a monodispersed population of vesicles of the diameter size of the filter used and of a surprisingly high stability as determined by light scattering experiments [36, 38]. By freeze-fracture electron microscopy only unilamellar spherical vesicles were detected (Figure 4). This monodispersity of both lipopeptide systems may possibly result from a fixed curvature of the vesicles induced by the bulkiness of the peptide headgroup. This could also account for the unilamellar character because of its large hydration shell. However, such explanation is not supported by the findings with the DM-CCK system which even after extrusion rearranges into a polydispersed population of vesicles (Figure 5) confirming the strong effect of the chemical nature of the peptide headgroup on the fatty acid packing [37].

Differential scanning calorimetry measurements revealed no phase transition between 3 and 65 °C for DP-HG-[2–17], DM-HG-[2–17], DM-HG-[14–17] and DM-CCK, suggesting that these vesicle systems are in the fluid phase despite the double-tailed C16- and C14-lipidation [38, 44]. Strong reduction of the peptide size to the tetragastrin was expected to increase the fatty acid packing, but the liquid state of the DM-HG-[14–17] vesicles at 20 °C was fully confirmed by the FTIR-ATR spectrum. It shows the CH_2 stretching bands at 2922.5 and 2852.3 cm^{-1} exactly as DMPC vesicles in the fluid phase (30 °C); conversely, in the ripple phase of DMPC (18 °C) these bands are shifted to 2918.4 and 2850 cm^{-1}, respectively [38].

Lipid Transfer between Lipo-Peptide and Phosphatidylcholine Vesicles

The mechanism of intervesicular lipid transfer via monomer diffusion through the bulk solution im-

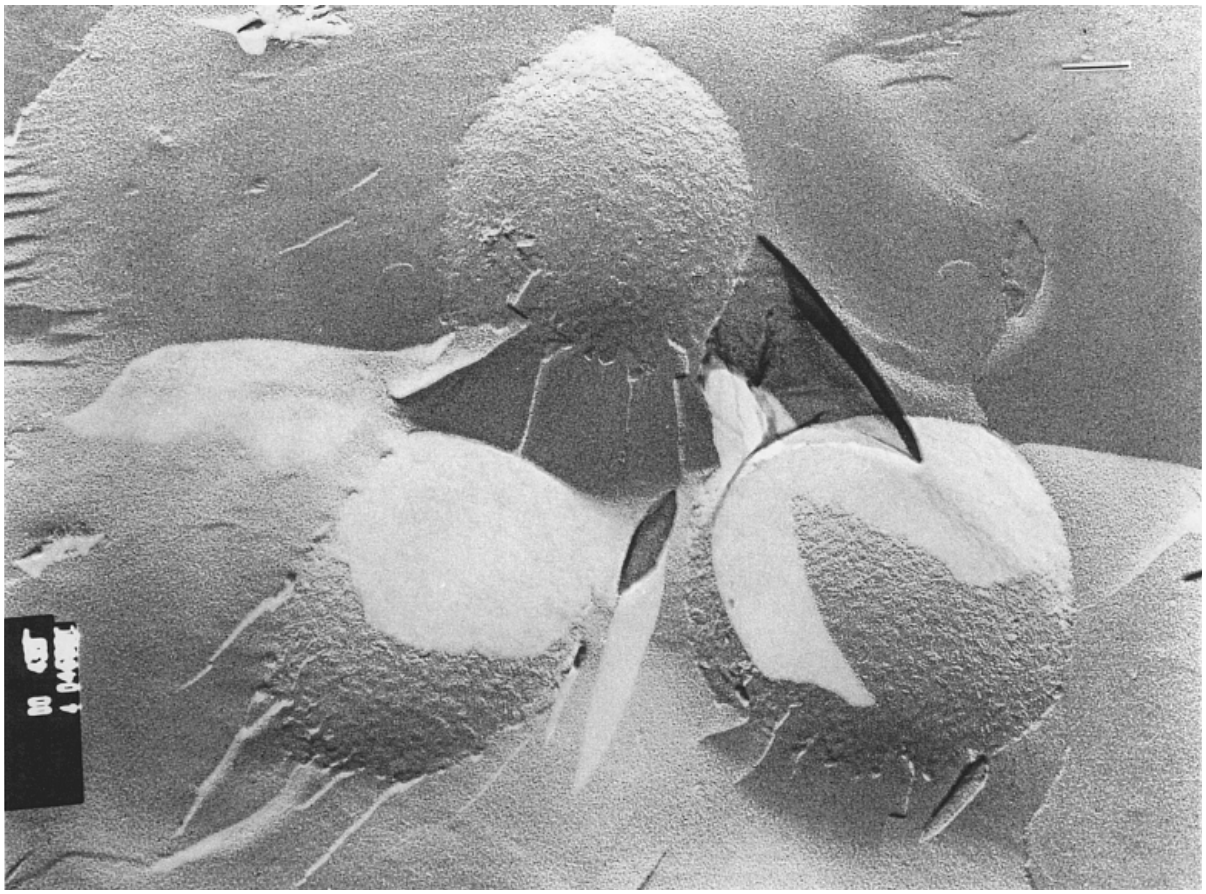


Figure 4 Freeze-fracture electronmicrograph of DM-HG-[2–17] vesicles ($R_H = 270$ nm); the bar corresponds to 117 nm. The peptide concentration was 1.5 mM in 5 mM phosphate buffer, 100 mM NaCl (pH 7.0); sonicated sample.

plies a transfer rate that depends upon both the molecular structure and the characteristics of the donor and acceptor vesicles [45]; thereby the relatively fluidity of the donor vesicles is apparently much more important than that of the acceptor vesicles [46]. The low T_m values of the lipo-gastrin and lipo-CCK peptides were expected to greatly facilitate their merging into phosphatidylcholine vesicles as model cell membranes. In fact, carboxyfluorescein leakage experiments from DP- and DM-gastrin vesicles upon addition of dipalmitoyl- (DPPC) or dimyristoylphosphatidylcholine (DMPC) vesicles at increasing lipid/lipo-peptide ratios indicated that at temperatures above the T_m values of DMPC and DPPC a maximum ratio of 100:1 is required for total dissolution of the lipo-peptide vesicles [44]. Similarly, endotherms obtained upon mixing DPPC or DMPC vesicles with lipo-gastrin and with lipo-CCK vesicles confirmed a fast and quantitative lipo-peptide transfer to the phospholipid bilayer [37, 44]. The rates of intervesicular lipid transfer are known to depend exponentially on the acyl chain length [47, 48] irrespective of the degree of fluidity of the donor and acceptor vesicle [49]. This was fully confirmed by comparing the time-dependency of the endotherms recorded for the merging of DP- and DM-gastrin vesicles with DPPC small unilamellar vesicles (SUVs). Moreover, a significant effect of the chemical nature of the peptide headgroup on the mode of merging the lipids was observed. In fact, DM-gastrin is apparently inserted into the DMPC bilayer in a statistical manner, while DM-CCK is forming differently enriched DM-CCK domains [37, 44]. As shown in Figure 6, generation of domains in this merging process was fully confirmed by the addition of Ca^{2+} that resulted in a parallel, but more intense shift of the T_m values of the DM-CCK rich domains than that recorded for the DMPC domain. Binding of Ca^{2+} to lipids reduces the electrostatic charge of the headgroups, thus depleting them of water and increasing the packing density in the gel phase which in turn leads to a rise in the transition

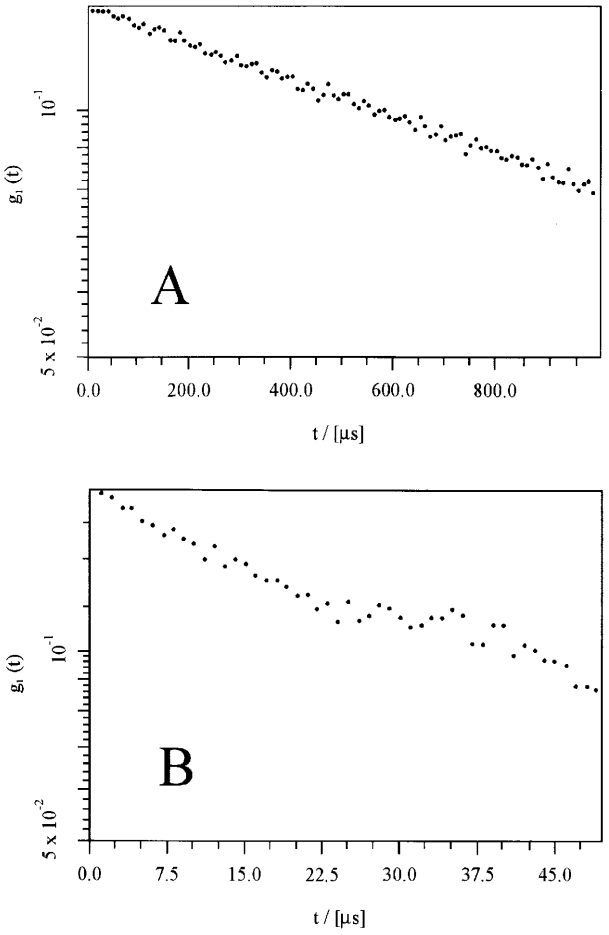


Figure 5 Normalized autocorrelation functions $g_1(t)$ obtained for the extruded samples of (A) DM-HG-[2–17] (32 μM) and (B) DM-CCK (32 μM) in 5 mM phosphate buffer, 100 mM NaCl (pH 7.0) and plotted as $\log[g_1(t)]$ *vs.* (*t*).

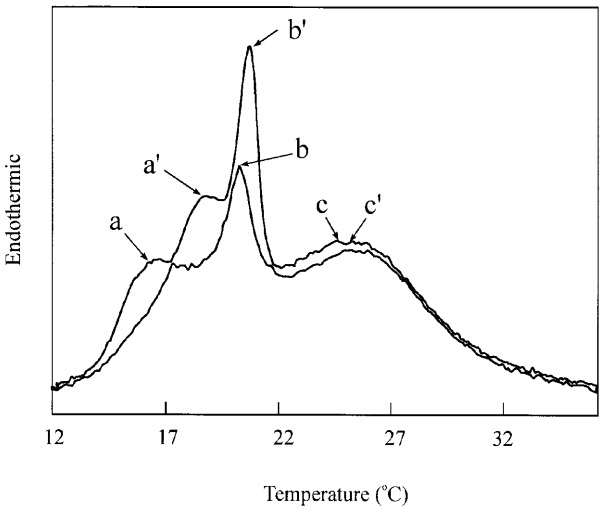


Figure 6 Effect of Ca^{2+} ions on the endotherm of merged DM-CCK/DMPC vesicles (1:12 molar ratio) in 5 mM phosphate buffer, 100 mM NaCl (pH 7.0); 0.25 mM peptide concentration: a, b, c, peaks before and a′, b′, c′ peaks after addition of calcium (25 mM $CaCl_2$).

temperature. The differentiated effect of the metal ion on the various domains strongly suggested a higher affinity of the CCK-peptide headgroup for Ca^{2+} than the phosphatidylcholine group.

Occupancy of G-protein-coupled receptors is known to activate phospholipase C and, correspondingly, to induce hydrolysis of phosphatidylinositol 4,5-diphosphate and production of *sn*-1,2-diacylglycerol and inositoltrisphosphate (IP_3) [50]. The second messenger IP_3 is generally believed to be responisble for the release of calcium from endosplasmic reticulum, thereby raising intracellular free calcium levels [51]. 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 [52]. A detailed study of the effects of extracellular calcium, lanthanum and manganese ions on the signal transduction of CCK [53] 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 affinity of the peptide hormones for Ca^{2+} as observed in the system of DM-CCK/DMPC merged vesicles. We have, therefore, analysed in more detail this system by measuring the rates of Ca^{2+} influxes into DMPC SUVs induced by the lipo-peptides [54]. As shown in Figure 7, underivatized gastrin and CCK were unable to induce metal ion influxes into DMPC vesicles although capable of binding Ca^{2+} in TFE [55–57]. This observation fully agrees with the results of microcalorimetric and CD measurements which excluded major interactions of these peptides with DMPC bilayers [58, 59]. Conversely, by forcing an interaction of DM-HG-[2–17] and DM-CCK with the lipid vesicles ion fluxes were induced with full equilibrium of the system only after more than 1 h. The rate is similar to that observed for other peptide hormones, e.g. insulin, glucagon and substance P

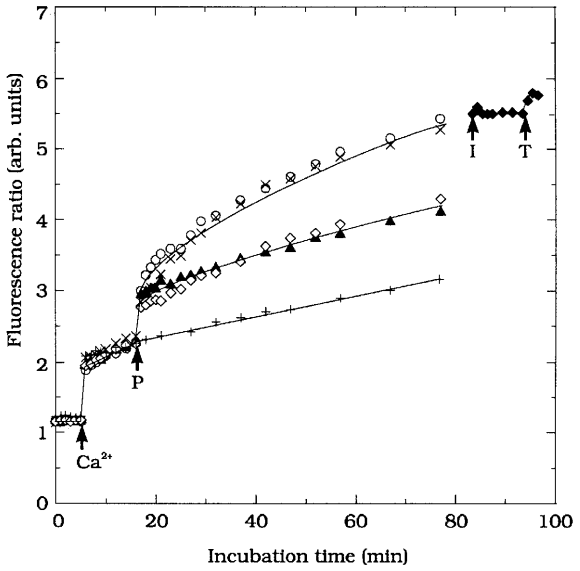


Figure 7 Time-dependence of the fluorescence ratio on incubation of DMPC SUVs with entrapped fura-2 after addition of the peptides (P): [Thr, Nle]-CCK-9 (◇-◇), [Nle^{15}]-gastrin (▲-▲), DM-CCK (×-×) and DM-HG-(2–17) (○-○); the buffer is used as blank (+-+). The fluorescence increase induced by addition of Ca^{2+} is due to traces of fura-2 in the suspension medium. After addition of the ionophore Br-A23187 (I) immediate equilibration of the system is observed with only a slight further increase of fluorescence upon disrupture of the vesicles with triton ×-100 (T).

[60], but significantly lower than that of known Ca^{2+} ionophores, e.g. Br-A23187 (see Figure 7).

It has recently been shown that interaction of peptides with lipid bilayers leads to perturbation of the fatty acid chain packing, a fact which markedly increases the rates of transbilayer flip-flop of lipid monomers [61]. In view of these findings the Ca^{2+} influxes into phopholid 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 phosphatidyl-choline headgroup. Moreover, even the lipo-peptides could participate in the flip-flop movements and translocate Ca^{2+} if the peptide headgroups are coordinated to the metal ion as suggested by the Tb^{3+} and Ca^{2+} binding experiments with DM-gastrin and DM-CCK vesicle preparations [57]. Both the rate of ion fluxes and the most probable mechanism exclude the observed effects being physiologically significant. This was further confirmed by the finding that similar calcium influxes were obtained in CCK- and gastrin-unrelated cells such as fibro-blasts too, and are simply a consequence of lipida-tion of the peptides [54].

Conformational States of the Membrane-Anchored Lipo-Peptides

Fluorescence quenching data and CD spectra of DM-HG-[2–17] embedded in DPPC and DMPC bilayers indicate full exposure of the gastrin headgroup to the bulk solvent in predominantly random coil structure [44]. The large peptide headgroup, although anchored to the lipid bilayer, remains desorbed from the lipid/water interface most prob-ably as a consequence of a strong electrostatic repulsion of the cluster of negative charges in the pentaglutamic acid sequence of gastrin. Conversely, for DM-CCK anchored in DMPC a significant blue shift of the fluorescence emission maximum and a reduced accessibility of the tryptophan residue to iodide quenching indicate insertion of the C-termi-nus of the peptide headgroup into the bilayer [37]. Moreover, the related CD spectrum is reminiscent of β-type structure and, thus, in full agreement with an aggregation of the peptide headgroups into clusters as observed in the differential scanning calorimetry experiments. In analogy to the conformation pro-posed for the CCK headgroup in DM-CCK vesicles on the basis of spectroscopic data and molecular dynamics simulations in the biphasic CCl_4/water system [59], the molecule apparently retains its overall structure, even upon insertion into lipid bilayers, with exposure of the highly charged sequence portion Arg-Asp-Tyr(SO_3H) to the bulk water and immersion of the C-terminus into the more hydrophobic compartment of the bilayer.

The high Ca^{2+} affinity observed for several pep-tide hormones in membrane-mimetic conditions led Ananthanarayanan [62] to propose the Ca^{2+}/pep-tide complexes as potential bioactive states of hormones. These would deliver the metal ion to the binding pocket of the receptors for the induction of local conformational changes in the binding cleft as first step in the signal transduction pathway.

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 [58, 59]. Interestingly, even in the presence of metal ions this interaction could neither be induced nor enhanced to extents detectable by CD or Tb^{3+} energy transfer fluorescence measure-ments [57]. In order to stimulate the high calcium concentrations at the cell membrane/water inter-face and, thus, to analyse the effect of this metal ion on the conformation of peptide hormones in a hypothetical 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+} concentrations. In both cases only weak effects on the dichroic properties of the peptide headgroups were detected in terms of the overall CD pattern, but the intensities of the spectra were sensibly reduced [57]. This hypochromic effect could result from peptide backbone chromophore couplings due to aggregation phenomena induced by intermolecular metal ion complexation. Such clustering of the lipopeptides into domains favoured by metal ions is reasonable and has been reported to occur [63]. The findings of this study clearly indicate that low dielectric media like TFE do not necessarily reflect the environment of lipid bilayers. In fact, only lipoderivatization of gastrin and CCK and, thus artificially enhanced lipid affinity, allowed the translocation of the two peptides to the lipid/water interphase of phospholipid vesicles, and even in this artificial model system the conformational effects of calcium ions 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 milieux containing mmolar concentrations of Ca^{2+} ions where neither lipid- nor metal ion-induced conformational effects were detected [64]. Therefore, the results of the present study do not support the generalized concept of Ca^{2+}-bound forms of hormones as the bioactive states [62].

A Membrane-Bound Pathway in the Hormone Receptor Binding Process is indeed Possible

Studies on thermodynamics and kinetics of interbilayer transfer of phospholipids and their desorption into the bulk water have shown that the rate of desorption decreased exponentially with increasing chain length and that the increment in the standard free energy of lipid desorption corresponds to approximately 1.2 kcal/mol by addition of one methylene group to each acyl chain of the amphiphiles [42, 48, 65]. This corresponds to a decrease of the rate of desorption of roughly 4.2-fold for each added 'effective carbon' [48]. It was further demonstrated that the rate of desorption of lipids and lipidated peptides is only slightly affected by the structure and molecular weight of the headgroup [18, 42]. Correspondingly, if desorption of the lipogastrin and lipo-CCK peptides is required for binding to the receptor, the related IC_{50} values were expected to increase exponentially with the chain length of the lipid moiety.

In order to analyse this aspect the tetragastrin peptide was chosen as it represents the shortest gastrin sequence possessing high affinity for the CCK-B/gastrin receptor [66]. A series of double-tailed tetragastrin lipo-derivatives with increasing fatty acid chain length was synthesized as shown in Figure 2, and their binding affinities [38] were determined using the CCK-B/gastrin receptor cloned in COS 7 cells [67].

Derivatization of tetragastrin to the 1,2-dipropionyl-3-mercapto-glycerol/maleoyl-β-Ala-Trp-Nle-Asp-Phe-NH_2 produced a slight increase in binding affinity with an IC_{50} of 3×10^{-9} M *vs.* 12×10^{-9} M for H-Trp-Met-Asp-Phe-NH_2. This is in full agreement with previous findings related to the extension of the tetragastrin to the pentagastrin sequence H-Gly-Trp-Met-Asp-Phe-NH_2 or to the analogue Boc-β-Ala-Trp-Met-Asp-Phe-NH_2 (Peptavlon, ICI, Manchester) [68] and may therefore not be directly related to the built-in short diacyl-moiety. By further increasing the size of the fatty acids in the lipo-tetragastrin peptides the receptor binding affinities decrease exponentially, but unexpectedly in a sharp sigmoidal manner with a transition midpoint at the level of the dioctanoly derivative to be reached then with the didecanoyl compound a 200-fold increased IC_{50} plateau value. In fact, the binding affinity remains constant within the limits of error of the assays up to the dipalmitoyl-tetragastrin derivative.

According to the model experiments discussed above, upon addition of the double-tailed lipopeptides to the cell membrane preparations a fast partitioning of these amphiphiles between bilayer and bulk water has to take place in a manner independent of their aggregational state, if sufficient incubation time is allowed for full equilibration of the system. Thereby the standard free energy of transfer of the lipids from water to the membranes ($\Delta G^{\circ}_{w \rightarrow m}$) becomes more favourable by *ca.* 1.2 kcal/mol per pair of methylene groups added to the difattyacyl-glycerol moiety [48, 65, 69]. Recognition of these ligands by the receptor has then to occur either via a membrane-bound pathway with or without concomitant desorption of the lipo-peptides from the membrane or via prior desorption to allow for direct collision from the extracellular aqueous phase. The exponentially increasing IC_{50} values from the C3- up to the C10-derivative support a desorption event; the data, however, do not differentiate whether this desorption is taking place prior or concomitantly to the binding event. Further

elongation to the dimyristoyl- and finally to the dipalmitoyl-peptide is not reflected by changes in the binding affinities, although the desorption rate of the didecanoyl-peptide should be orders of magnitude faster than that of the dipalmitoyl-derivative. The free energy of binding of the ligand to the receptor does apparently not compensate anymore for the unfavourable free energy of desorption. But, since binding is still observed, even if with a 200-fold decreased affinity, it has to occur at least for the large size fatty acid-chain derivatives via a membrane-bound pathway. In order to account for the expectedly different rates of two-dimensional migration of the bilayer-associated lipo-peptides the systems were allowed to reach full equilibration. In fact, in the membrane-bound mechanism the lipophilic character of the anchorage finger signifantly affects the rate of two-dimensional diffusion in the bilayer as is well shown by comparing the binding affinities of DP-HG-[2–17] and DM-HG-[2–17] [36] with that of the parent peptide, and of DM-CCK with [Thr,Nle]-CCK-9 prior to the full equilibration of the system [59]. When a sufficiently long incubation time was applied, the binding affinities of the lipopeptides were almost identical with those of the unmodified ligands.

That full binding affinity, as determined by displacement of radio-labelled tracers, not necessarily equals to full functional binding, was demonstrated in the case of DM-CCK where the poor amplification between receptor binding and amylase secretion had to be attributed to inadequate occupancy of low-affinity CCK-A receptors in a manner similar to partial agonists [59]. Conversely, in the case of DM-gastrin only a slightly reduced potency was recorded [44], thus indicating that even anchorage of the hormone in the bilayer allows for optimal interaction with the receptor.

Docking of Lipo-Gastrin on a CCK-B/Gastrin Receptor Model

The findings that both lipo-gastrin and lipo-CCK retain their ability to bind to the receptor in a functional manner despite their anchorage at the N-termini in the lipid bilayer adjacent to the receptor molecule, offers an interesting restriction in the mode of access of these ligands and, thus, attractive experimental restraints for docking experiments on modelled receptor molecules. Such restraints should allow for less fortuitous computational docking experiments if additional data from mutagenesis studies are available as is the case for the CCK-B/gastrin receptor [70–77].

Once it had been established that the di-myristoyl-moiety was ensuring irreversible capture of peptides by cell membranes, gastrin peptides of increasing chain length were derivatized with this lipophilic moiety in order to identify the minimum peptide size required for maximum binding affinity in the attempt to uncover additional useful constraints for docking experiments. By comparing the binding affinities of lipo-gastrin peptides of increasing peptide size with those of the underivatized peptides, the differences observed in function of the peptide chain length were found to be remarkably enhanced by the lipo-derivatization [38]. Only upon elongation of the peptide chain to the gastrin sequence 2–17, i.e. with DM-HG-[2–17], this difference is almost annealed suggesting that a critical length of the peptide headgroup is required for the core gastrin moiety, i.e. the C-terminal portion, to reach the binding cleft of the receptor despite the anchorage of the lipid-tail in the membrane bilayer.

Three-dimensional models of G-protein-coupled receptors have been constructed using both the experimental structure of bacteriorhodopsin [78] and of bovine rhodopsin [79] as template. Our model of the CCK-B/gastrin receptor was constructed on the basis of the helical arrangement proposed by Schertler *et al.* [79] and by Baldwin [80] for G-protein-coupled receptors and taking into account experimental data from mutagenesis experiments [72]. In this model [38] shown in Figure 8, the amino acid residues identified as possibly responsible for the high binding affinity of the CCK-B/gastrin receptor for the gastrin ligand, i.e. Arg-57, Ser-131, Ser-219 and His-376, were located on the inward pointing faces of the helices and in the transmembrane part adjacent to the extracellular space, as this portion of the receptor should act as the potential ligand-binding site. By examining this CCK-B/gastrin receptor model it is evident that the cleft between helix 1 and 2 represents the less crowded part of the extracellular region, since the disulfide bridge between Cys-127 and Cys-205 and the rather large extracellular loop betwen helices 4 and 5 are strongly occluding other approaching sites. Taking into account that the lipid-tail of DM-HG-[2–17] has to be constrained in an anchored position outside the seven-helix bundle and inserted into a fictive membrane bilayer, an approaching mode between helices 1 and 2 had to be chosen as putative access site. Moreover, the 16-membered peptide moiety of the lipo-gastrin was presented to

the receptor in an extended random structure, since from CD measurements performed on DM-gastrin inserted into phosphatidylcholine vesicles a folding of the peptide moiety on the bilayer surface into ordered type structures could be excluded [44]. With this starting system molecular dynamics simulations and energy minimizations were then performed to dock DM-gastrin onto the CCK-B/gastrin receptor (Figures 9 and 10).

From previous conformational studies in various membrane and receptor mimetic environments a hairpin-like structure was proposed for gastrin as potential bioactive conformation consisting of an N-terminal α-helix, a chain reversal at the sequence Ala-Tyr-Gly-Trp followed by γ-turns toward the C-terminus [81, 82]. Similarly, CD and NMR analysis of (Thr,Nle)-CCK-9 in dimethylsulfoxide/water revealed as the preferred conformation a γ-turn centred on Thr and separated by Gly from an α-helix extending toward the C-terminus [83, 84]. In the modelled receptor-bound state of gastrin a β-turn-like kink at the Ala-Tyr sequence of the ligand is retained as well as the tendency for a γ-turn type ordered structure in the C-terminus. This is itself inserted deeply into the helix bundle between helices 3, 4, 5, 6 and 7 (Figure 11) and strongly restricted in its conformational space by a potential hydrogen bonding network that involves the 'pore-forming' residues of these five helices and particularly, the side chains of Ser-219, Ser-131 and His-376, i.e. three of the four residues identified by mutagenesis studies as critical interacting sites with the gastrin ligand. The phenolic side-chain function of Tyr-12 of the DM-gastrin peptide is protruding in this model from the extracellular surface of the receptor/peptide complex and becomes solvent-accessible while the aromatic ring is tightly packed against residues of the second extracellular loop connecting helices 4 and 5. In this putative bound state of the gastrin molecule the Tyr-12 OH is located near the residues Arg-201, Gln-204, Trp-209 and Arg-215. The Arg residues could represent potential interaction candidates for the sulfated gastrin forms. In fact, sulfation of Tyr-12 is known to enhance the binding affinity of gastrin for CCK-B/gastrin receptor [36, 85, 86].

Possible hydrogen bonding interactions can also be envisioned between the peptide backbone of the sequence portion Glu-6 to Glu-9 of the ligand and the extracellular part of the receptor. The Glu-6 side chain is within interaction distance to the third extracellular loop, and Glu-7 and Glu-9 are located near the first extracellular loop of the receptor. This could explain the weak, but experimentally well-documented, contribution of this portion of the molecule to the binding affinity [87–89]. Recent mutagenesis [90] on the sequence 204–209 of the CCK-B/gastrin receptor fully confirms a Glu-9/His-207 interaction.

The α-helix spanning the pentaglutamic acid sequence of gastrin in aqueous trifluoroethanol dissolves into a more extended structure in the modelled receptor-bound state with one γ-turn centred on Glu-10 and with hydrogen bonding contacts to the receptor surface. The lack of onset of α-helical structure could derive from the artificial stretching of the molecule imparted by the N-terminal anchorage of the lipid-tail in the membrane. Therefore, the anchorage was cut and the peptide tail was allowed to find, in an unconstrained conformational space, its optimal interaction with the receptor via an energy minimization and additional molecular dynamics calculations. Only minimal rearrangements were observed with retention of hydrogen bonding to the receptor surface. This observation fully supports previous reports about the too-strong α-helix-inducing effect of trifluoroethanol when searching for potential bioactive structures of peptides in protein- or membrane-mimetic environments [91, 92].

Besides Arg-57 on top of helix 1 of the receptor, for which no potential interaction with the ligand could be detected, the other amino acid residues identified by mutagenesis studies as putative gastrin-specific recognition sites were found to represent potential interacting partners with the C-terminal portion of gastrin in the modelled gastrin/receptor complex.

Even taking into account the strong limitations of the model system presented in this study [38], it offers an interesting tool for rationalizing the experimental results of the extensive structure–function studies performed previously on gastrin and CCK [35, 93] and to delineate more precisely putative ligand binding sites on the extracellular face of the receptor. But further validation is required by docking the other lipo-gastrin peptides in an attempt to find a correlation between mode of binding and binding affinity. Since gastrin and CCK are both recognized by the CCK-B receptor with almost identical affinity, their common message part, i.e. the C-terminal tetrapeptide amide, has to assume a very similar conformation in the receptor-bound state. Interesting in this context is the observation that the two aromatic side chains of the C-terminal portion of the docked gastrin are pointing in the

same direction with the phenyl ring almost perpendicular to the indole side chain. This array of the two aromatic side chains as well as the orientation of the Nle-15 and Asp-16 side chains toward the other face of the structure is very similar to that found in preferred conformational states of these two peptides in aqueous organic media [82, 83]. An identical 'gastrin-active' conformation was deduced using conformational computational analysis [94], whereas for the 'CCK-active' conformation an opposite orientation of the aromatic rings was postulated taking into account the structure and high CCK-A receptor affinity of indole derivatives of benzodiazepines [95].

CONCLUSIONS

By using the lipo-derivatives of gastrin and CCK it was confirmed that a membrane-bound pathway is indeed possible. However, it could also be shown that for the inderivatized peptides the mechanism may strongly depend upon the overall chemical structure of the ligand. In fact, despite the common C-terminal peptide sequence, the homologous hormones gastrin and CCK were found to exhibit a significantly different behaviour in terms of interaction with lipid bilayers. While CCK is partially inserted into the lipid bilayer upon its lipidation, the gastrin molecule behaves like electrostatically repelled from the charged surface. Although phospholipid bilayers represent only a crude model of natural membranes, a preadsorption of this negatively charge molecule on the cell membrane seems largely unfavoured. Correspondingly, the two hormones are expected to be behaving differently even in the early step of their collision with target cells. It remains to be verified whether this different lipid affinity contributes to enhancing the selectivity of the recognition process of the two hormones *in vivo*, since *in vitro* experiments reveal significant cross-reactivities with the CCK-A and CCK-B/gastrin receptors.

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