Membrane Structure of Bombesin Studied by Infrared Spectroscopy. Prediction of Membrane Interactions of Gastrin-Releasing Peptide, Neuromedin B, and Neuromedin C

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Received June 26, 1987; Revised Manuscript Received July 31, 1987

ABSTRACT: Bombesin, in contact with flat phospholipid bilayer membranes, was shown to adopt a membrane structure similar to that of substance P, dynorphin-(1–13)-tridecapeptide, and adrenocorticotropin-(1–24)-tetracosapeptide. The C-terminal message segment, comprising 8–10 amino acid residues, is inserted into a relatively hydropohobic membrane compartment as an α-helical domain oriented perpendicularly on the membrane surface. The N-terminal, hydrophilic tetrapeptide segment remains in the aqueous compartment as a random coil. This was shown with IR and IR attenuated total reflection spectroscopy. Equilibrium thermodynamic estimations confirmed the observed membrane structure with respect to helix length, strength of hydrophobic membrane association, and orientation (caused by favorably oriented molecular amphiphilic and helix electric dipole moments). The membrane structure may explain why Trp-8 and His-12 are essential for biologic activity. Neuromedin B is predicted to be able to adopt a membrane structure similar to that of bombesin. However, gastrin-releasing peptide and neuromedin C are predicted not to behave in the same manner. The molecular mechanism of receptor subtype selection by bombesin-like peptides may prove to be similar to that observed earlier for opioid peptides and the neurokinins.

The tetradecapeptide bombesin was first isolated from the skin of the European frogs *Bombina bombina* and *Bombina variegata variegata* by Anastasi et al. (1971). It belongs to a family of structurally related peptides from amphibian skin such as alytesin (from *Alytes obstetricans*), ranatensin (from *Rana pipiens*), and litorin [from *Litoria* (*Hyla*) *aurea*]. These peptides share a broad spectrum of pharmacologic effects in avian and mammalian tissues (Erspamer, 1980). Bombesin has recently attracted much attention because of its growth-promoting properties in malignant (Cuttitta et al., 1985; Moody et al., 1985; Salomon & Perroteau, 1986) and normal tissues (Papp et al., 1987).

Bombesin-like immunoreactivity found in the mammalian gastrointestinal tract, lung, and central nervous system (Melchiorri, 1980) is probably caused by gastrin-releasing peptide (GRP;[1] McDonald et al., 1979), neuromedin B (Minamino et al., 1983; Okada et al., 1983), and neuromedin C (Minamino et al., 1984). Peptides of the bombesin–GRP–neuromedin family have similar C-terminal amino acid sequences (Table I), which are responsible for their related pharmacologic effects. The relatively hydrophobic C-terminal hepta- to nonapeptide segments contain the pharmacologic messages for triggering the responses. Trp-8 and His-12 are essential for biologic activity [for a review on structure–activity relationships, see Erspamer (1980)].

Cavatorta et al. (1986) find bombesin to exist as an ensemble of flexible conformers in solution. Upon interaction with electrically neutral and negatively charged lipid vesicles and micelles, it adopts a secondary structure that possibly resembles the receptor-triggering conformation. Circular dichroism indicates the formation of a partial α-helix. Fluorescence measurements of the peptide (Trp-8) in the presence of dimyristoylphosphatidylserine (DMPS) vesicles in the gel state (10 mM cacodylate buffer, pH 8.5, 20 °C) yielded an apparent associaton constant $K_a = 1.3 \times 10^5$ M^{-1} and a binding saturation of 1:57 peptide:lipid molecules. A blue shift of the fluorescence maximum was observed in the presence of both DMPS vesicles and lysolecithin micelles. This indicates that the Trp is inserted, presumably through a C-terminal α-helix, into the hydrophobic compartment of the membrane. A fluorescence–pH titration in water shows a clear change in fluorescence quantum yield at pH 6.5. The loss of quantum efficiency with decreasing pH is attributed to the ionizable group of His-12, which quenches the fluorescence of Trp-8 by a proton-transfer mechanism.

Table I: Bombesin and Related Mammalian Peptides

Bombesin	Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
Gastrin-Releasing Peptide (GRP)	Ala-Pro-Val-Ser-Val-Gly-Gly-Gly-Thr-Val-Leu-Ala-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
Neuromedin C	Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
Neuromedin B	Gly-Asn-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH_2

The described behavior of bombesin in the presence of lipid membranes is reminiscent of that of adrenocorticotropin-(1–24)-tetracosapeptide (Schwyzer et al., 1983a,b; Gremlich et al., 1983, 1984), dynorphin-(1–13)-tridecapeptide (Erne et al., 1985), and substance P (Schwyzer et al., 1986; Rolka et al., 1986; Erne et al., 1986). These neuropeptides insert their N-terminal or C-terminal message segments into the hydrophobic compartments of lipid membranes as perpendicularly oriented helical domains, whereas the remainder of the peptide remains exposed to the aqueous compartments in a more random fashion. The membrane structures of these peptides can be calculated with reasonable accuracy, giving the length of the inserted helix, its orientation on the membrane, and the

[1] Abbreviations: DMPS, dimyristoylphosphatidylserine; GRP, gastrin-releasing peptide; IR-ATR, infrared attenuated total reflection spectroscopy; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; Pyr, pyroglutamic acid; TFE, 2,2,2-trifluoroethanol.

0006-2960/87/0426-6316$01.50/0

hydrophobic association constant in agreement with the experimentally measured data (Schwyzer, 1986a,b; Schwyzer et al., 1986). This type of membrane structure is intimately connected with opioid and neurokinin receptor subtype selection by dynorphin and substance P peptides (Schwyzer, 1986c, 1987). We therefore investigated the conformation and orientation of bombesin as revealed by infrared studies in the membrane-mimicking solvent 2,2,2-trifluoroethanol and by infrared attenuated total reflection spectroscopy (IR-ATR) of flat multilayer phospholipid membranes. The results of these experiments and of the estimations indicated that bombesin adopts a membrane structure similar to substance P and inserts its C-terminal message segment into the membrane hydrophobic compartment as a perpendicularly oriented α-helical domain. A similar membrane structure is predicted for neuromedin B but not for neuromedin C or gastrin-releasing peptide.

Experimental Procedures

Materials. Bombesin was purchased from Bachem AG (Bubendorf, Switzerland). It was used as the acetate salt without further purification. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was from Avanti Polar Lipids Inc. (Birmingham, AL). 2,2,2-Trifluoroethanol (TFE) was of Uvasol grade from Merck (Darmstadt, FRG).

IR Spectra. IR spectra were recorded with a Perkin-Elmer PE983G ratio-recording grating spectrometer equipped with a Perkin-Elmer 3600 data station. Solution spectra were obtained in a CaF_2 cell (path length 50 μm) in the double-beam mode against air in the reference beam. The peptide spectra were then calculated by subtraction of the pure solvent spectra. IR-ATR spectra were obtained on a germanium internal reflection element with an incident angle of 45° (Harrick Scientific Corp., Ossining, NY). For measurements of orientation, linearly polarized IR radiation obtained with an AgBr grid polarizer was used. Vertical (vp, 0°) means vertical and parallel (pp, 90°) means parallel polarization with respect to the plane of the angle between the incident radiation and the internal reflection element. The lipid membranes were made from freshly prepared MeOH solutions of lipid and peptide, which were allowed to slowly evaporate on either side of the reflection plate. The peptide spectra were calculated by subtraction of the spectra of the pure lipid membranes. Samples were equilibrated with a current of N_2 saturated with H_2O and dried at 25 °C in a stream of dry gas before measurements. The spectra shown are averaged spectra of 16–64 scans; their reproducibility was checked with three different membrane preparations. Molecular orientation was determined by the dichroic ratio, $R = A_{pp}/A_{vp}$, where A_{pp} and A_{vp} are the absorbances measured in the parallel and vertical polarization modes, respectively (Fringeli & Günthard, 1981).

Estimated Membrane Structures. Thermodynamic estimation of the preferred conformation, orientation, and accumulation of bombesin and related peptides on neutral or anionic aqueous–hydrophobic interfaces was carried out as described for substance P (Schwyzer et al., 1986). The Gibbs free energy of hydrophobic association, $\Delta G°_a(m)$, through m residues at the C-terminal end was calculated from the free energies of transfer, $\Delta G°_{tr}(i)$, of the individual residues from their random-coil conformation in H_2O to their helical conformation in a hydrophobic phase, taking into account the loss of 2° of rotational and 1° of translational freedom of the bound peptide. However, according to Von Heijne and Blomberg (1979), it was assumed that only one H bond with water is broken by introducing the primary amide nitrogen of C-terminal methionine into the hydrophobic phase and that the energy needed to break H bonds of the methionine sulfur atom is proportional to its electronegativity. The Pauling electronegativities are 2.5 for sulfur, 3.0 for nitrogen, and 3.5 for oxygen. Because of electron delocalization in the peptide bond, an average electronegativity of 3.25 for peptide nitrogen and oxygen was assumed. The energy for breaking a hydrogen bond to N or O of the peptide bond is estimated as 10.5 kJ/mol (Von Heijne & Blomberg, 1979). Thus, the strength of the hydrogen bond between water and the thio sulfur becomes 8.1 kJ/mol.

The amphiphilic moment describes the segregation of polar and "unpolar" amino acid residues into hydrophilic and hydrophobic domains with respect to the helix center. An angle $\Phi = 0°$ indicates that the more hydrophilic end of the amphiphilic moment vector points along the helix axis toward the C-terminus. The scalar magnitude, A, is given in arbitrary units based on the free energies of transfer of the individual residues. The amphiphilic moment is therefore a measure for the orientation of a helix in a hydrophobic gradient or in a membrane–water interphase (Schwyzer, 1986a,b).

The helix electric dipole moment is caused by the ordering of the peptide bonds. Its magnitude is assumed to be 3.46 D per peptide unit; it points its negative end along the helix axis toward the C-terminus (Wada, 1976; Hol, 1985). As membranes have surface dipole moments pointing their negative end toward the aqueous phase, the helix dipole moment will tend to orient the helix axis with its C-terminus toward the hydrophobic layers.

Results

In spite of potentially interfering residues such as pyroglutamic acid (Pyr), glutamine, and asparagine, the IR spectra of bombesin showed a single maximum in KBr at 1660 cm^{-1} and in TFE at 1665 cm^{-1}. Shoulders on the amide I absorption band were hardly discernible. The signal in TFE was unusually sharp (half-bandwidth $w = 43$ cm^{-1}) as compared to the ones obtained for substance P ($w = 56$ cm^{-1}; Erne et al., 1986), physalaemin ($w = 55$ cm^{-1}; unpublished data), and eledoisin ($w = 63$ cm^{-1}; unpublished data). Position and shape of the amide I band of bombesin in TFE matches the absorption of a finite α-helix of 10 peptide groups (1664 cm^{-1} with a slight asymmetry on the low-frequency side of the maximum), Figure 1A, as predicted by Nevskaya and Chirgadze (1976). The amide II band, although of low diagnostic value, supports this model.

Figure 1B shows the polarized IR-ATR spectra obtained for bombesin on POPC multilayers after equilibration with H_2O vapor at a peptide to lipid ratio of 1:46. Although generally wider, the amide I absorption appears at 1667 cm^{-1}, just slightly higher than in TFE. The measured dichroic ratios are 1.30 and 1.08 for the amide I and amide II bands, respectively. They are significantly different from the value expected for an isotropic sample under the same conditions ($R_{iso} = 1.14$) and indicate a perpendicular orientation of the helix axis with respect to the plane of the bilayer membrane.

Figure 2A shows a schematic representation of the hydrogen-bonding system estimated for bombesin interacting with an aqueous–hydrophobic interface. Upon transfer of one amino acid residue after another starting at the more hydrophobic C-terminus ($m = 1$, $n = 14$) from its random-coil conformation in water to its α-helical conformation in the hydrophobic phase, energy was expended until Trp-8 had been transferred. An energy minimum, $\Delta G°_a(10) = -17.4$ kJ/mol, was reached with insertion of Gly-5, after which the free energy of association became unfavorable again. This energy minimum was only attained if a hydrogen bond between the

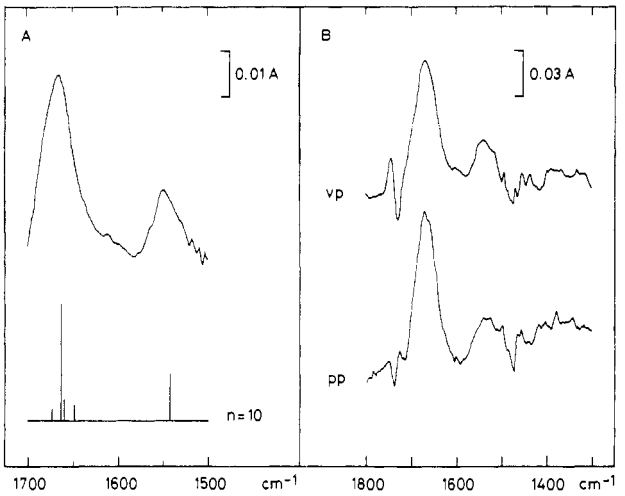

FIGURE 1: (A) IR amide I and amide II absorptions of bombesin in TFE (1 mM) and positions and absorbance ratios of the components of the amide signal as calculated for a finite α-helix with 10 peptide bonds (Nevskaya & Chirgadze, 1976). (B) IR-ATR spectra of bombesin obtained on POPC membranes with vertically (vp) and parallel (pp) polarized incident radiation. The lipid to peptide ratio was 46; the sample was allowed to equilibrate in H_2O vapor and was dried before measurement.

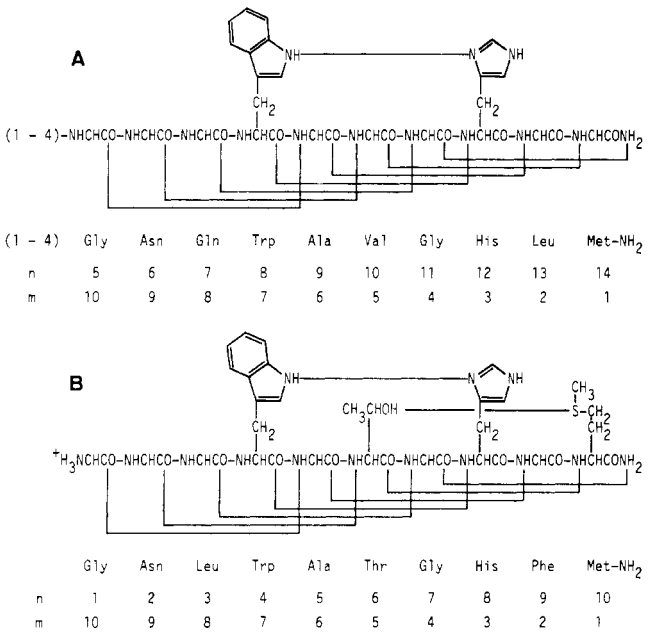

FIGURE 2: Schematic representation of the hydrogen-bond systems estimated for (A) bombesin and (B) neuromedin B interacting with an electrically neutral aqueous–hydrophobic interface, $K_d = 9 \times 10^{-4}$ and 1.4×10^{-4}, M, respectively. Except for Trp-8 and His-12 (A) and Trp-4, His-8, Thr-6, and Met-10 (B):, only the backbone atoms are shown. Horizontal lines indicate internal H bonds.

side chains of Trp-8 and His-12 was accounted for; otherwise, the association energy remained unfavorable throughout. The expected reduction of the pK value of His-12 by the H bond to Trp-8 was not considered; if taken into account, $\Delta G°_a(10)$ may be lower by almost 5 kJ/mol. The amphiphilic moment had an estimated scalar magnitude of $A = 300$ (arbitrary units; Schwyzer et al., 1986) and an angle with the helix axis of $\Phi = 172°$. The electric dipole moment of an α-helix with 10 peptide bonds amounts to $\mu = 34.6$ D.

In neuromedin B, residues 1–4 of bombesin are missing, and Gln-7, Val-10, and Leu-13 of bombesin are replaced by Leu, Thr, and Phe, respectively. This led to the calculated hydrogen-bonding system shown in Figure 2B. A salient feature is the H bond between the hydroxyl group of Thr-6 and the sulfur atom of Met-10 in hydrophobic surroundings. It was assumed to satisfy the H-bond requirements of the thio group (8.1 kJ/mol) but not of the oxygen atom (10.5 kJ/mol). The minimum of the free energy of association was reached at $m = 10$ with $\Delta G°_a = -22$ kJ/mol. The amphiphilic moment had $A = 132$ (arbitrary units) and $\Phi = 165°$. The scalar magnitude of the helix electric dipole moment was 34.6 D. For neuromedin C and for GRP, hydrophobic association through a C-terminal helix was too weak to become effective ($\Delta G°_a \geq +11$ kJ/mol).

Discussion

We found bombesin tetradecapeptide to exist as a partial α-helix in TFE and in contact with electrically neutral phospholipid membranes. TFE is known to favor ordered structures and to mimic membrane environment in the sense that it deprives solutes of the possibility to establish good hydrogen bonds with the solvent molecules. Therefore, intramolecular stabilization through a secondary structure is encouraged. The α-helix observed on flat POPC membranes is oriented normal to the membrane surface.

Thermodynamic estimations showed that bombesin can interact with an aqueous–hydrophobic interface by inserting its C-terminal message segment into the hydrophobic phase as a perpendicularly oriented α-helix domain and leaving its N-terminus exposed to the aqueous phase. The length of the inserted helix is compatible with the IR-ATR data. The calculated apparent molar dissociation constant is in the order of 10^{-3} M, in agreement with unpublished preliminary data obtained by the capacitance minimization technique (Schoch et al., 1979). In order to account for the electrostatic Boltzmann accumulation of bombesin on the vesicles used by Cavatorta et al. (1986), a Gouy–Chapman potential of about −120 mV may reasonably be assumed. The hydrophobic component of the reported dissociation constant (7.7×10^{-6} M) is then about 10^{-3} M, in agreement with our estimation. Furthermore, the molecular amphiphilic and helix dipole moments act in concert to orient the helix perpendicularly on a membrane surface, with the C-terminus pointing toward the hydrophobic layers.

The membrane structure of bombesin is thus very similar to that of substance P (Schwyzer et al., 1986; Rolka et al., 1986; Erne et al., 1986). Our results are supported by those of Cavatorta et al. (1986). The residues Trp-8 and His-12, which are essential for biologic activity, show a unique arrangement in the lipid-induced α-helix. The two side chains can be oriented to allow a perfect hydrogen bond between the indole NH group and the basic imidazole N atom. This is expected to stabilize the imidazole ring to such an extent that the pK value of His-12 must be lowered by a fair amount. This is certainly in favor of a good peptide–membrane interaction. Replacement of Trp-8 by D-Trp-8 in the intact helix would abolish the hydrogen bond to His-12 and prohibit the formation of the typical bombesin membrane structure by reducing strength of the hydrophobic interaction. This consideration may explain the observation that [D-Trp^8]bombesin is pharmacologically inactive (Erspamer, 1980). Thus, the observed membrane structure appears to be important for bombesin–receptor interactions.

Rivier and Brown (1978) report that [D-Ala^{11}]bombesin is equipotent with bombesin in its effects on thermoregulation of rats but that [L-Ala^{11}]bombesin is inactive. We have shown for [D-Leu^9]substance P that the enantiomeric amino acid residue is readily incorporated into an α-helix in hydrophobic environments (Erne et al., unpublished data). D-Leu and D-Ala replace homologous glycine residues in substance P and bom-

besin, respectively. We would therefore assume that [D-Ala[11]]bombesin may also have a helical structure on the membrane. This does not mean, however, that the helix is a receptor requirement of the thermoregulatory receptor. According to the model of membrane-assisted receptor subtype selection (Schwyzer, 1986c, 1987), a helix only serves to expose the peptide message in the same hydrophobic compartment as the receptor site in question. The interaction of the membrane-bound peptide with the receptor may involve a second conformational transition advantageous to membrane catalysis of receptor selection (Sargent & Schwyzer, 1986). Should the thermoregulatory bombesin receptor favor a hydrophobically bound peptide message, this may well be a transition to a β-turn structure as suggested by Rivier and Brown (1978). In case the receptor site is exposed to the aqueous membrane compartments, a β-turn requirement is quite normal.

Among the known mammalian bombesin-like peptides, only neuromedin B was predicted to be able to adopt a membrane structure similar to that of bombesin. Hydrophobic binding to the membrane may be slightly stronger, K_d about 10^{-4} M. The amphiphilic moment was less strong than in bombesin, but it pointed in the same direction and was reinforced by the helix electric dipole moment, thus assuring perpendicular orientation on the membrane surface. Replacement of Leu-3 in neuromedin B by His in GRP and neuromedin C reduced hydrophobic interaction so strongly as to make a bombesin-like membrane structure appear impossible. However, the net positive charges of these two neuropeptides may lead to their Boltzmann accumulation in the fixed charge layer of the membrane, with all its implications for receptor interactions and receptor selectivity (Sargent & Schwyzer, 1986; Schwyzer, 1986c, 1987).

We may conclude that the membrane structures of bombesin and perhaps of neuromedin B are in principle similar to those of adrenocorticotropin-(1–24)-tetracosapeptide, dynorphin-(1–13)-tridecapeptide, and substance P. The molecular mechanism of receptor subtype selection by the neuromedins and GRP may therefore be guided by the same principles governing opioid, melanocortin, and neurokinin receptor selection (Schwyzer, 1986b,c, 1987).

Registry No. Bombesin, 31362-50-2; neuromedin B, 87096-84-2; gastrin-releasing peptide, 80043-53-4; neuromedin C, 81608-30-2.

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