Examination of the Role of the Amphipathic α-Helix in the Interaction of Neuropeptide Y and Active Cyclic Analogues with Cell Membrane Receptors and Dimyristoylphosphatidylcholine

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ABSTRACT: To test the potential importance of the putative C-terminal amphipathic α-helical region of neuropeptide Y (NPY) in receptor binding, the interactions of porcine NPY and several peptide analogues with lipid and cell membrane receptors were compared. Cyclic analogues were designed to constrain the N- and C-terminal regions of the peptide and to retain the folded conformation of NPY predicted from its sequence analogy with pancreatic polypeptide and its similar spectral behavior. The three cyclic peptides were [Cys^2, 8-aminooctanoic acid$^{5-24}$, D-Cys^{27}]-NPY (C2-NPY), [Cys^5, 8-aminooctanoic acid$^{7-20}$, D-Cys^{24}]-NPY (C5-NPY), and [D-Cys^7, 8-aminooctanoic acid$^{8-17}$, Cys^{20}]-NPY (C7-NPY). All of the peptides bind with high affinity to pig spleen membranes, but only NPY and [Glu^{16}, Ser^{18}, Ala^{22}, Leu^{28,31}]-NPY (ESALL-NPY) bind quantitatively to dimyristoylphosphatidylcholine (DMPC) liposomes. C7-NPY and NPY_{20-36} bind with moderate affinity to liposomes, but only NPY and C7-NPY bind with high affinity to mouse brain receptors. Thus, lipid binding and receptor binding are not correlated in this series of peptides, and binding to the pig spleen receptor appears to require only the C-terminal region of the peptide. Simple lipid binding, as in NPY_{20-36}, is insufficient for binding to the mouse brain receptor, suggesting that the N-terminal region of the peptide is required for high-affinity binding to this receptor. Data from fluorescence, differential scanning calorimetry, and liposome clearing experiments suggest that, although the interaction of NPY with lipid is consistent with formation of an amphipathic α-helix, a simple amphipathic α-helical model for the interaction with the high-affinity NPY receptor is insufficient to explain the data. Rather, the data suggest that the amphipathic α-helix in NPY plays a significant role in stabilizing the spatial relationship between the N- and C-terminal residues of the peptide when bound to its receptor.

Neuropeptide Y (NPY),[1] a 36 amino acid residue peptide first isolated from porcine brain (Tatemoto, 1982), is widely distributed in the central and peripheral nervous systems [see Potter (1988) and Allen and Bloom (1986), for recent reviews]. It has been identified within specific peptidergic neurons of the central and autonomic nervous system. In the central nervous system, NPY stimulates food intake, produces cardiovascular depression, and inhibits the release of luteininzing hormone. In the periphery, it is a potent vasoconstrictor and presynaptic inhibitor of neurotransmission. Reversible, saturable high-affinity binding sites in membranes from the central nervous system and peripheral tissues have been demonstrated for NPY, and the peptide is released from sympathetic neurons. These observations support the proposal that NPY is a neurotransmitter peptide.

Neuropeptide Y belongs to a family of peptides, with strong sequence homology, which includes peptide YY and the pancreatic polypeptides (Allen et al., 1983). The porcine peptide differs from the human peptide by only one amino acid, Leu^{17}, which is replaced by Met in the human peptide. Avian pancreatic polypeptide contains an intramolecularly stabilized helical structure (Blundell et al., 1981). Spectral evidence shows that NPY may have a similar intramolecularly stabilized structure (Krstenansky & Buck, 1987). Molecular modeling supports this structural assignment (Allen et al., 1987; MacKerrell, 1988; Krstenansky et al., 1989). Inspection of a helical wheel diagram (Schiffer & Edmundson, 1967) of neuropeptide Y reveals that the C-terminal residues 14–30 may be represented as an amphipathic α-helix. This site may be critical to the binding of the peptide to receptors, on the basis of the importance of the amphipathic α-helix as a structural feature of several peptide hormones (Kaiser & Kezdy, 1984).

It has been suggested that the ability of a peptide hormone to form an amphipathic α-helix and bind to phospholipids may be related to the biological potency of the peptide (Gysin & Schwyzer, 1983; Epand et al., 1985). To test the general applicability of this hypothesis to NPY, three cyclic peptide analogues of porcine neuropeptide Y were synthesized that retained the overall structure of NPY in terms of its folded conformation but which contained different lengths of the putative amphipathic α-helical region. The peptides contain 8-aminooctanoic acid in place of selected residues of neuropeptide Y and a D-Cys–Cys disulfide bond to conformationally restrict the NPY in a folded helical structure (Krstenansky et al., 1989). Neuropeptide Y was modeled on the crystal structure of avian pancreatic polypeptide by mutating nonhomologous residues in a molecular-modeling program. The cyclic analogues were then designed by choosing a D-Cys–Cys disulfide cross-linking site in which distortion of the folded helix was minimized. In the present paper, the interactions of these active, conformationally restricted cyclic analogues with lipids are compared with those of porcine neuropeptide Y. In addition, a peptide with enhanced α-helical tendency based on

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[1] Abbreviations: NPY, porcine neuropeptide Y; ESALL-NPY, [Glu^{16}, Ser^{18}, Ala^{22}, Leu^{28,31}]-NPY; C2-NPY, [Cys^2, 8-aminooctanoic acid$^{5-24}$, D-Cys^{27}]-NPY; C5-NPY, [Cys^5, 8-aminooctanoic acid$^{7-20}$, D-Cys^{24}]-NPY; C7-NPY, [D-Cys^7, 8-aminooctanoic acid$^{8-17}$, Cys^{20}]-NPY; DMPC, dimyristoylphosphatidylcholine.

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Chou and Fasman (1978) prediction rules and a peptide that contained the amphipathic α-helical region of NPY were examined. The interaction of the peptides with lipid is compared to their binding affinities for mouse brain and pig spleen receptors to test the relationship between receptor binding and the potential role of the amphipathic α-helical region of NPY.

Experimental Procedures

Peptide Synthesis. Peptides were synthesized on a 0.5-mmol scale by solid-phase techniques with an Applied Biosystems Inc. Model 430-A peptide synthesizer on *p*-methylbenzhydrylamine resin by protocols supplied by the manufacturer. All residues were double-coupled as the preformed symmetrical anhydrides of the N^{α}-*t*-Boc amino acid derivatives except for Asn, Arg, and Gln, which were double-coupled with DCC/HOBT. Details of the side-chain protection, deprotection, resin cleavage, and peptide purification have been reported (Krstenansky & Buck, 1987). Purity of the peptides was assessed by analytical high-performance lipid chromatography, amino acid analysis, and fast-atom bombardment mass spectrometry.

Receptor Binding. ^{125}I-Labeled Bolton-Hunter–neuropeptide Y (Amersham Corp.) binding was carried out in pig spleen and mouse brain crude membranes by a modification of the method of Lundberg et al. (1988). Membranes from frozen spleen were prepared as described previously for tachykinin peptide binding studies (Buck et al., 1984). An aliquot of membrane preparation (approximately 15 mg of tissue) was incubated at room temperature for 2 h in buffer (130 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 20 mM HEPES, pH 7.4) containing one of the peptides at concentrations from 0.1 to 100 nM, 4 mg/mL bovine serum albumin, 0.04 mg/mL bacitracin, 4 μg/mL leupeptin, and 4 μg/mL chymostatin. ^{125}I-Labeled neuropeptide Y was included at a concentration of 0.1 nM, and nonspecific binding was determined by including 1 μM porcine neuropeptide Y. Samples were rapidly filtered over Whatman GF/C filters presoaked overnight in 0.5% histone (type II-AS, Sigma) and washed two times with ice-cold, 130 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 20 mM HEPES buffer (pH 7.4). IC_{50} values for the analogues were calculated from 6- to 10-point competition curves.

Liposome Binding. The fraction of peptide bound to liposomes was measured by centrifugal filtration. Liposomes were prepared by dissolving 5 mg of DMPC (Avanti Polar Lipids, Birmingham, AL) in chloroform, drying the lipid under N_2 on the walls of a test tube, lyophilizing for 30–60 min, and incubating in 1 mL of 10 mM Tris-HCl, pH 7.40, buffer for 1 h at 30 °C with occasional vortexing. Dry peptide was added to obtain various ratios of peptide/lipid in the mixture. The mixture was centrifuged in a Centricon 30 microconcentrator (Amicon; soaked in ethanol overnight) until the filtrate and infranate volumes were approximately equal (5–10 min in a table-top centrifuge). Then aliquots of the filtrate and the infranate were sampled and the peptide concentrations determined by BCA (Pierce) analysis. The recovery of peptide was generally 85–95%. The fraction of bound peptide is given by $B = (C_i - C_f)V_i/(C_iV_i + C_fV_f)$, where C_i is the concentration of peptide in the infranate (retained by the filter), C_f is the peptide concentration in the filtrate, and V_i and V_f are the respective volumes of infranate and filtrate.

Liposome Clearing. The effects of the peptides on the integrity of DMPC liposomes were measured as described previously (McLean & Hagaman, 1988). Liposomes were diluted to concentrations of 0.02–0.20 mg/mL of DMPC in 2 mL of buffer. Then the absorbance of the samples at 400 nm was recorded continuously on a Beckman DU-7 spectrophotometer at 24.0 ± 0.2 °C. After complete temperature equilibration, 0.1 mg of peptide (25 μg of a 4 mg/mL solution in 6 M guanidine hydrochloride or dry peptide) was added with stirring, and recording was resumed for 60 min.

Table I: Sequences of Neuropeptide Y and Analogues[a]

	1 10 20 30 36
Neuropeptide Y	YPSKPDNPGE DAPAEDLARY YSALRHYINL ITRQRY#
NPY_{20-36}	Y YSALRHYINL ITRQRY#
ESALL-NPY	YPSKPDNPGE DAPAEELSRY YAALRHYLNL LTRQRY#
C2-NPY	YCSK----------RHcINL ITRQRY#
C5-NPY	YPSKCD----------YSAcRHYINL ITRQRY#
C7-NPY	YPSKPDc----------ARC YSALRHYINL ITRQRY#

[a] The numbers correspond to the position in the sequence of NPY. The dashed line represents a single 8-aminooctanoic acid residue. Underlined residues are those which differ from the porcine neuropeptide Y sequence. Abbreviations: A, alanine; C, cysteine; c, D-cysteine; D, aspartic acid; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; Y, tyrosine.

Differential Scanning Calorimetry. DSC was performed on a Microcal MC-2 differential scanning calorimeter at a scan rate of 20 °C/h. Sample concentrations were 0.1–0.4 mg/mL. The differential voltage signal from the themopiles, the temperature of the heat sink, and the time were recorded at 0.02 °C intervals on an IBM PC-AT computer. The data were converted to cal/(g·°C) after dividing by the scan rate and the weight of sample. Enthalpies were calculated by numerical integration; the phase transition temperatures (T_m) correspond to the temperatures at which the heat capacities reach maximum values during the transitions. Where two transitions overlapped, the underlying transitions were decomposed into symmetrical peaks with software provided by Microcal.

Spectra. Spectra of the samples from the clearing experiments were recorded after 24 h of incubation at 24 °C. Fluorescence spectra were recorded on an SLM 4800 spectrofluorometer under a N_2 atmosphere at 25 °C. Emission spectra were recorded with an excitation wavelength of 275 nm. Circular dichroic (CD) spectra of samples in 1-mm circular cuvettes were recorded at 25 °C from 300 to 190 nm on a Jasco J-500A spectropolarimeter with 2-nm slit width. The CD spectrum of buffer was subtracted from the CD spectrum of the sample after each scan. A total of nine scans were averaged. The data were transferred to a computer, and the data from 250 to 200 nm at 1-nm intervals were fit by nonlinear regression analysis with the reference spectra of Bolotina et al. (1980).

Results

The sequences and trivial names for the synthetic peptides are given in Table I. For the cyclic peptides, the names are derived from the position of the first Cys residue in the sequence. The amphipathic helical region of NPY and the three cyclic analogues are shown at the bottom of Figure 1 in helical wheel diagrams. The presentation of the sequences in these diagrams is for purposes of comparison and not to suggest a particular structural motif, particularly with regard to the cyclic peptides. In NPY, a distinct band of hydrophobic residues on one face of the helix is evident. The extent of the hydrophobic region decreases with the shorter cyclic analogues. In the top panels of Figure 1, ribbon diagrams of the peptide backbones are superimposed on the peptide structures. The

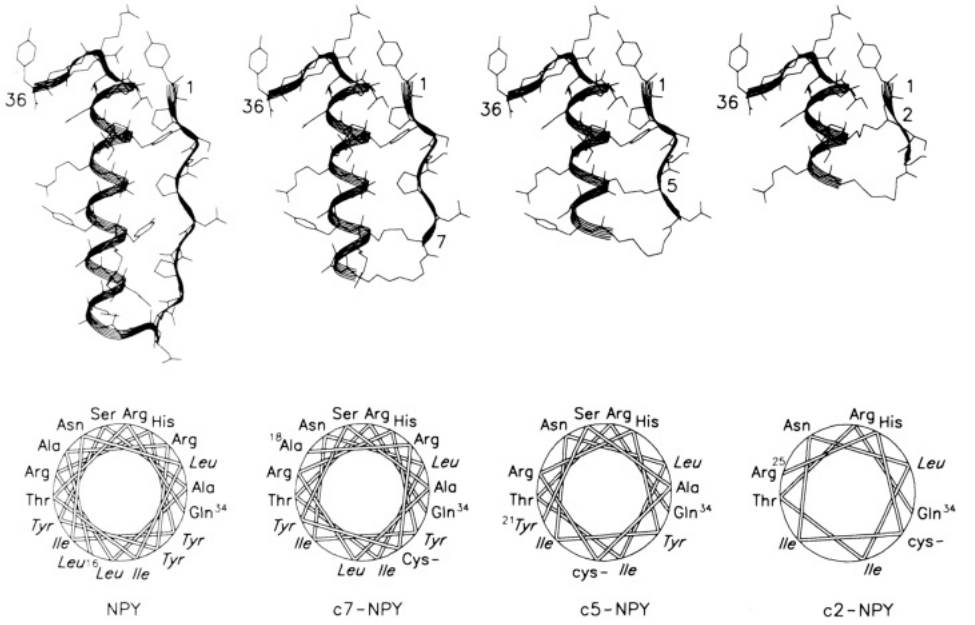

FIGURE 1: Proposed structures of neuropeptide Y and analogues. In the upper panel are ribbon diagrams of NPY and the three cyclic analogues superimposed over the carbon backbone of the peptides. Below each ribbon diagram is an amphipathic α-helical wheel (Schiffer & Edmundson, 1967) representation of the putative amphipathic region of each peptide, which corresponds to the α-helical region in the ribbon diagram. Hydrophobic residues are indicated in italics.

putative amphipathic α-helical regions (residues 19–32) are indicated by the helical ribbons, and the more linear helical portion of the ribbon represents the N-terminal polyproline type II helical region (residues 1–13). The structural assignments are based on a model constructed from the crystallographically determined structure of avian pancreatic polypeptide (Krstenansky et al., 1989). The two helical regions are connected by a central turn region (residues 14–18) which is replaced by 8-aminooctanoic acid in the cyclic peptides (Krstenansky et al., 1989). The two helical regions are arranged so that their lipophilic faces are juxtaposed. Exposure of the hydrophobic residues in the amphipathic helical region requires either an unfolding or a twisting away of the hydrophobic helical faces from each other. In the cyclic analogues, this juxtaposition is stabilized by a disulfide bond near the N- and C-terminal residues of the peptides that limits the extent and degree of exposure of the hydrophobic faces.

The interactions of the NPY analogues with specific neuropeptide Y receptors on pig spleen membrane receptors and mouse brain membrane receptors were determined by competitive binding of the peptides, at equilibrium, in the presence of ^{125}I-labeled neuropeptide Y (Figure 2). The IC_{50}, defined as the concentration that displaced half of the ^{125}I-labeled neuropeptide Y from the receptor, was determined graphically from three to five separate determinations for each peptide. The IC_{50} values for each of the peptide analogues are in Table II. In the pig spleen, all of the peptides bind in the low nanomolar range. For the cyclic analogues, the order of potency is C7-NPY > C5-NPY > C2-NPY. Since the NPY_{20-36} peptide also binds well at this receptor, the binding of the cyclic analogues most likely reflects the presence of the C-terminal residues. By contrast, in the mouse brain, only NPY and C7-NPY bind in the low nanomolar range.

The fraction of peptide that binds to DMPC after incubation at 24 °C for 24 h is also given in Table II for two different peptide/lipid ratios. The receptor-binding activity is not correlated with the liposome-binding activity for either the mouse brain or the pig spleen membranes. Both NPY and ESALL-NPY bind tightly to liposomes (Table II), but NPY_{20-36}, which binds somewhat better than C7-NPY to liposomes, does not bind to the mouse brain receptors. For the pig spleen receptors, even C2-NPY and C5-NPY, which

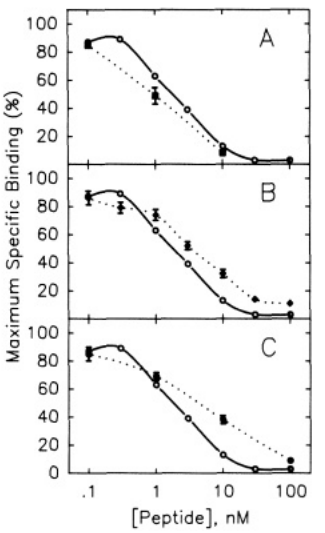

FIGURE 2: Binding of NPY and cyclic analogues to pig spleen membranes. In each panel (O—O) is NPY for purposes of comparison: (A) (■···■) C7-NPY; (B) (◆···◆) C5-NPY; (C) (●···●) C2-NPY. The error bars represent the SEM for three to five experiments. Where bars are not shown, the error is within the size of the symbol.

Table II: Binding of NPY and Related Peptide Analogues to Cell Membrane Receptors and to DMPC Liposomes[a]

peptide	mouse brain membranes IC_{50} (nM)	pig spleen membranes IC_{50} (nM)	liposomes (% bound) 1:1	liposomes (% bound) 1:4
neuropeptide Y	3.6 ± 0.3[b]	1.5 ± 0.5	86 ± 1	93 ± 1
ESALL-NPY	ND	1.6 ± 0.4	70 ± 5	94 ± 3
NPY_{20-36}	266 ± 80[b]	2.2 ± 0.7	38 ± 9	38 ± 6
C2-NPY	7400 ± 4000	21 ± 12	5 ± 1	4 ± 2
C5-NPY	150 ± 28[b]	2.7 ± 0.4	6 ± 2	10 ± 1
C7-NPY	2.3 ± 0.7[b]	1.1 ± 0.3	17 ± 2	34 ± 3

[a] Each value is the mean ± SEM of three to five experiments. For liposome binding the ratios are peptide/lipid (w/w). ND, not determined. [b] Data for mouse brain are from Krstenansky et al. (1989).

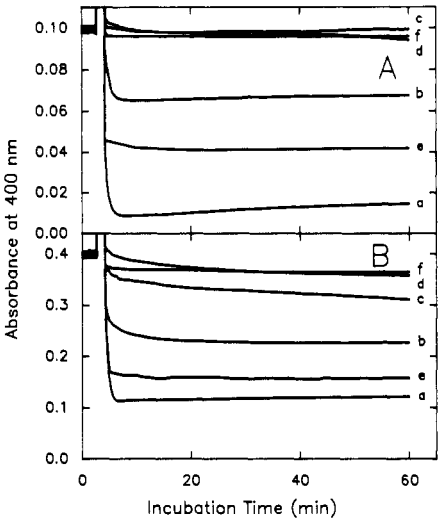

FIGURE 3: Clearing of DMPC bilayers by neuropeptide Y and analogues. Peptide (0.1 mg) was added to 0.1 mg (A) or 0.4 mg (B) of DMPC liposomes in 2 mL of buffer at 24 °C. The absorbance was measured at 400 nm for 60 min. (a) NPY; (b) ESALL-NPY; (c) C2-NPY; (d) C5-NPY; (e) C7-NPY; (f) NPY_{20-36}.

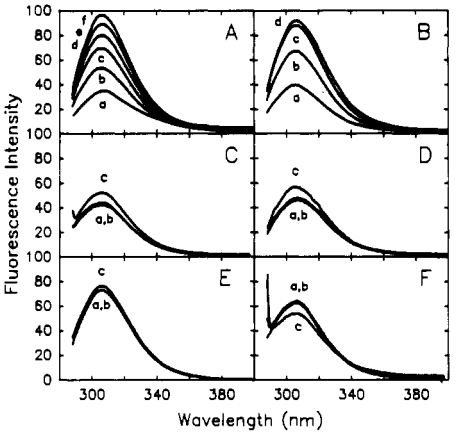

FIGURE 4: Fluorescence spectra of mixtures of neuropeptide Y and analogues with DMPC. Peptide (0.1 mg) was added to various concentrations of DMPC liposomes in 2 mL of buffer and incubated at 24 °C for 24 h. The ratios correspond to peptide/DMPC (w/w) (A) NPY: (a) peptide alone; (b) 1:0.5; (c) 1:1; (d) 1:2; (e) 1:3; (f) 1:4. (B) ESALL-NPY: (a) peptide alone; (b) 1:1; (c) 1:2; (d) 1:4. (C) NPY_{20-36}, (D) C7-NPY, (E) C5-NPY, (F) C2-NPY: (a) peptide alone; (b) 1:1; (c) 1:4.

bind very little to liposomes, bind at low nanomolar concentrations to the receptor.

The decrease in the absorbance at 400 nm of DMPC liposomes following addition of neuropeptide Y and the peptide analogues is shown in Figure 3. Neuropeptide Y rapidly clears DMPC liposomes at 24 °C (line a). However, the truncated 20–36 residue portion of NPY, which binds quite effectively to liposomes, has little or no effect on the turbidity of the liposomes (line f). The C7-NPY analogue, by contrast, is nearly as effective as NPY in clearing liposomes (line e); ESALL-NPY is somewhat less effective (line b). The lack of effect of C2-NPY and C5-NPY on liposome turbidity is consistent with their minimal binding to liposomes.

The fluorescence of each of the peptides arises entirely from tyrosine since the peptides do not contain any tryptophan or phenylalanine. For NPY, three tyrosine residues lie within the hydrophobic face of the putative amphipathic α-helical region of the peptide. As expected for tyrosine, the fluorescence emission maximum of the peptide alone is at 306 nm and does not change after addition of DMPC liposomes

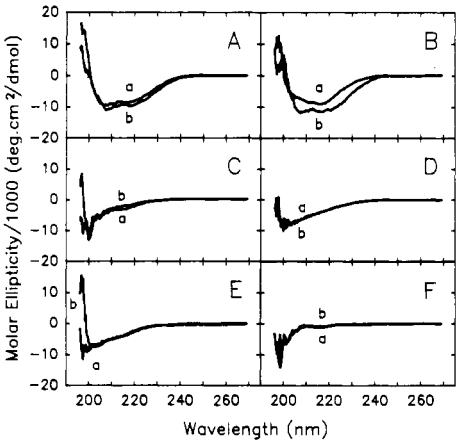

FIGURE 5: Circular dichroic spectra of neuropeptide Y and analogues in DMPC micelles. CD spectra of micelles prepared as described in the legend to Figure 4 were measured at 24 °C. For each panel, (a) is peptide alone and (b) is 1:4 peptide/DMPC (w/w). (A) NPY; (B) ESALL-NPY; (C) NPY_{20-36}; (D) C7-NPY; (E) C5-NPY; (F) C2-NPY.

Table III: Secondary Structural Components of Peptides in Buffer and Dimyristoylphosphatidylcholine[a]

sample	α	β	turn	random
NPY	23	18	17	41
+DMPC (1:1)	27	16	13	44
+DMPC (1:4)	27	17	16	40
ESALL-NPY	17	25	15	42
+DMPC (1:1)	29	17	15	39
+DMPC (1:4)	34	20	19	28
NPY_{20-36}	1	10	16	73
+DMPC (1:1)	0	14	15	71
+DMPC (1:4)	0	13	15	73
C7-NPY	10	14	17	59
+DMPC (1:1)	12	15	19	54
+DMPC (1:4)	10	14	17	59
C5-NPY	8	15	18	59
+DMPC (1:1)	9	16	19	55
+DMPC (1:4)	10	16	19	56

[a] CD spectra were measured on each sample after 24 h of incubation at 24 °C. The ratios are peptide/lipid (w/w). The numbers are the mean of the percent of each structure. The errors for the estimates of each structure are <2% of the mean values.

(Figure 4). The intensities of the fluorescence of NPY and ESALL-NPY are markedly increased with increasing lipid concentrations up to a peptide/lipid weight ratio of 1:4. Only a small increase in intensity is observed with NPY_{20-36}, C7-NPY, or C5-NPY. No change is observed with C2-NPY at a 1:1 ratio, and a small decrease in intensity is observed at 1:4.

The CD spectra of the peptides alone and after incubation for 24 h with DMPC liposomes are shown in Figure 5. Association of NPY and ESALL-NPY with phosphatidylcholine increases the intensity of the negative bands at 208 and 222 nm which are characteristic of α-helical peptides. DMPC has an insignificant effect on the CD of any of the cyclic peptides or on NPY_{20-36}. The CD data for the peptides in the presence and absence of DMPC were analyzed for four secondary structural components (Table III). The α-helical content of the neuropeptide Y peptide alone is somewhat smaller than that previously published (Krstenansky & Buck, 1987) owing to the lower ionic strength of the buffer used here. Spectra similar to those of the NPY_{20-36} analogue have been obtained for NPY_{18-36} (Boublik et al., 1989). The content of α-helix in NPY is increased to a small extent in lipid-containing samples. The increase in α-helix content for ESALL-NPY is greater than that for NPY and is at the expense of a decrease in the random and β-structures of the

Table IV: Phase Transitions in Mixtures of Neuropeptide Y and Related Peptides with Dimyristoylphosphatidylcholine[a]

sample	pretransition T_m (°C)	pretransition ΔH (kcal/mol)	transition 1 T_m (°C)	transition 1 ΔH (kcal/mol)	transition 2 T_m (°C)	transition 2 ΔH (kcal/mol)
DMPC	14.8	1.57	24.0	6.64		
+C2-NPY (1:1)	14.6	0.87	24.0	4.53		
+C2-NPY (1:4)	14.6	1.24	23.9	6.36		
+C5-NPY (1:1)	none[b]		23.8	4.78		
+C5-NPY (1:4)	13.6	0.85	23.9	6.07		
+C7-NPY (1:1)	none		23.5	0.26		
+C7-NPY (1:4), scan 1	14.8	0.86	23.9	2.55	24.5	3.16
+C7-NPY (1:4), scan 2	13.2	0.74	23.9	3.20	24.8	2.40
+NPY (1:1)	none		none			
+NPY (1:4), scan 1	none		22.9	0.75	26.0	1.18
+NPY (1:4), scan 2	none		23.2	1.50	26.1	2.16
+ESALL-NPY (1:1)	none		none			
+ESALL-NPY (1:4)	none		none			
+NPY_{20-36} (1:1)	none		none			
+NPY_{20-36} (1:4), scan 1	12.5	0.45	23.0	2.20	23.9	0.67
+NPY_{20-36} (1:4), scan 2	12.2	0.80	22.9	3.16	23.9	0.60

[a] Phase transition temperatures (T_m) correspond to the midpoint of the transition. The ratios in parentheses are peptide/lipid (w/w). [b] Transition is too small to accurately measure its temperature since $\Delta H < 0.2$ kcal/mol.

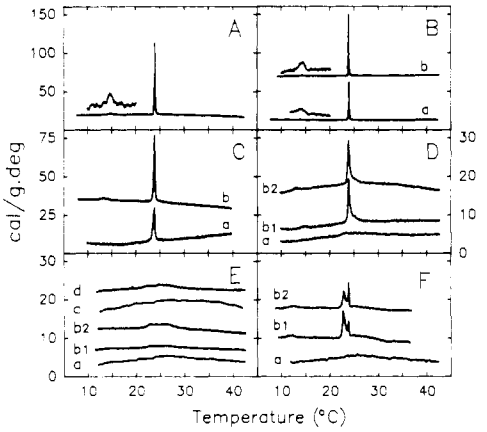

FIGURE 6: DSC scans of neuropeptide Y and analogues in DMPC micelles. DMPC and micelles prepared as described in the legend to Figure 4 were scanned at a rate of 20 °C/h. The insets are the pretransition regions scaled up 10×. For each panel, (a) 1:1, (b) 1:4 (peptide:DMPC, w/w). (A) DMPC; (B) C2-NPY; (C) C5-NPY; (D) C7-NPY, (b1) 1:4 scan 1, (b2) 1:4 scan 2; (E) NPY, (b1) 1:4 scan 1, (b2) 1:4 scan 2, (c) ESALL-NPY (1:1), (d) ESALL-NPY (1:4); (F) NPY_{20-36}, (b1) 1:4 scan 1, (b2) 1:4 scan 2.

peptide. No significant change in secondary structural components is observed for the other peptides. For C2-NPY, the α-helical content is nearly zero, so the data cannot be fit very well with the Bolotina et al. (1980) parameters.

DMPC alone has a sharp gel to liquid-crystalline (main) phase transition centered at 24.0 °C (Figure 6) with an enthalpy of 6.64 kcal/mol [cf. Phillips (1972)]. Addition of either C2-NPY or C5-NPY has only a minimal effect on the enthalpy or temperature of this transition, even at equal peptide/lipid weight ratios (Figure 6 and Table IV). The pretransition near 15 °C in DMPC is also relatively unaffected by C2-NPY. However, with C5-NPY at a 1:1 weight ratio, the pretransition is broadened so that its enthalpy and temperature could not be determined. C7-NPY broadens the main transition considerably more than either of the smaller cyclic analogues. At a peptide/lipid weight ratio of 1:4, two peaks in the main transition are evident; about half of the enthalpy of the main transition is centered near 24 °C, and about half is 1 °C higher. At equal peptide/lipid weight ratios, C7-NPY broadens the main transition, reducing its enthalpy to nearly zero. Second scans of C2-NPY, C5-NPY, or C7-NPY at 1:1 are identical. However, a second scan of C7-NPY at 1:4 results in an increase in the enthalpy of the 24 °C transition at the expense of the 25 °C transition. These data suggest that C7-NPY binds reversibly to the lipid at low peptide/lipid ratios.

NPY and ESALL-NPY considerably broaden the DMPC main transition even at 1:4 peptide/lipid ratios. With the ESALL-NPY analogue, no transition can be measured and multiple scans are identical. With NPY, two small, broad transitions are evident at a 1:4 ratio. Since the total enthalpy of the transitions increases during a second scan, NPY appears to bind reversibly at this ratio. NPY_{20-36} reduces the enthalpy of the pretransition by about half and shifts it to a somewhat lower temperature. In addition, multiple peaks appear in the main transition: the small shoulder centered at 24 °C probably corresponds to unreacted lipid. In a second scan of this peptide, the total enthalpy of these two transitions is increased, but a redistribution of the enthalpy into the "free lipid" transition is not observed.

Discussion

Although we have proposed that NPY contains an amphipathic α-helical region on the basis of CD and its primary sequence (Krstenansky & Buck, 1987), no data on the physical properties of NPY and its interaction with lipid have been reported. Our approach to the role of the amphipathic α-helix in this peptide was to synthesize cyclic, conformationally restricted analogues of NPY, which contain various lengths of the amphipathic α-helical region of the peptide. One of this series, C7-NPY, binds to mouse brain receptors and to pig spleen receptors with potency equivalent to that of NPY. The shorter analogues bind only to pig spleen receptors, as does also a putative amphipathic α-helical region (residues 20–36) of NPY. To assess the role of the amphipathic α-helical region in NPY, the receptor-binding activities of the various peptides were compared with their effects on a model membrane system. Although liposomes provide a model for studies of interactions between peptides and membranes or receptors in more complex biological systems, some care in interpretation must be taken since the molecular basis of the relationship between potency and the physical properties of simple lipid systems is not well understood. However, liposomes provide an excellent model for testing the role of lipid binding in the interaction of peptides with the cell membrane. Generally, lipid–peptide interactions for peptide hormones and neuropeptides have been restricted to linear analogues. Here we have investigated the interactions of NPY and its putative

amphipathic α-helical region with lipid, in addition to showing several interesting features of the lipid interactions of cyclic peptides that contain hydrophobic residues in a particular spatial arrangement in the secondary structure. The discussion will first focus on the mode of interaction of the cyclic peptides with lipid and then compare their interaction with that of the linear analogues. Next, the role of the amphipathic α-helix in lipid binding and the receptor interaction will be considered, and finally a model for the interaction of NPY with its receptors on the cell membrane will be proposed.

Since the conformationally restricted analogues of NPY were expected to less readily unfold upon interaction with lipid, these peptides were expected to have less effect on DMPC than the linear analogues. This conformational restriction differentiates the present peptides from the linear analogues described by Beck et al. (1989) in that the disulfide bond partially restricts the cyclic analogues to a folded conformation. For the shorter cyclic peptides, C2-NPY and C5-NPY, <10% of the peptide binds to liposomes even in the presence of excess lipid. In addition, only a small increase in fluorescence intensity in the presence of lipid is observed with C7–NPY, and no change is observed with C2–NPY or C5–NPY. However, in contrast to the shorter cyclic analogues, C7–NPY rapidly decreases the turbidity of DMPC liposomes at 24 °C and significantly reduces the enthalpy of the phase transitions of DMPC. This interaction of C7-NPY with DMPC appears to be reversible since the distribution of the peaks observed in the main phase transition in the presence of the peptide favors the 24 °C transition in a second scan. Since C7–NPY and NPY_{20-36} have similar effects on phase transitions in DMPC and contain a similar amount of amphipathic α-helix, the data suggest that the effect on the phase transition requires the amphipathic α-helical region of the peptides.

One might suppose that the two opposing helices of neuropeptide Y unfold upon binding to liposomes, exposing their hydrophobic faces to the lipid and burying the tyrosine residues which are at the edge of the hydrophobic face of the amphipathic α-helix. Since the fluorescence of a residue in such an interfacial position is highly sensitive to the local environment and to interaction with lipid (McLean et al., 1988, 1989), this burial of tyrosines would increase the fluorescence intensity. On the basis of this model, the peptide must partially unfold to interact rapidly with liposomes. Conformational restriction in the cyclic analogue prevents the complete unfolding of the peptide to a more linear structure. Thus, relatively ineffective lipid-binding interactions are expected. However, some unfolding of the cyclic peptide at the N- and C-terminal ends may be possible, which exposes a limited number of hydrophobic residues to the lipid. This would explain the small effects of C2-NPY and C5-NPY on the phase transitions of DMPC. For C7-NPY, the interaction with lipid is considerably more dramatic, consistent with its larger amphipathic α-helical region.

Active analogues of peptide hormones have been designed on the basis of the amphipathic α-helix model (Moe et al., 1983; Taylor et al., 1983) in which the ability of a peptide hormone to form an amphipathic α-helix and bind to phospholipids has been related to the biological potency of the peptide (Gysin & Schwyzer, 1983; Epand et al., 1985). A correlation between the ability to form an amphipathic α-helix and binding to specific receptors has been made for several peptides (Gysin & Schwyzer, 1983; Hammonds et al., 1982; Krstenansky et al., 1986). Some evidene also exists for an important role for the putative NPY amphipathic α-helical region (residues 19–36) in receptor binding (Martel et al., 1986; Rioux et al., 1986) and in its surface activity (Minakata et al., 1989). Decreases in the turbidity of liposomes on addition of peptides do not necessarily indicate the presence of an amphipathic α-helix (Epand et al., 1983). However, NPY fits several criteria for a lipid-binding amphipathic α-helical peptide (Morrisett et al., 1977) including tight binding to liposomes, disruption of the vesicular structure of liposomes resulting in a decrease in turbidity of lipid suspensions, an increase in the α-helicity and fluorescence intensity of the peptide upon interaction with lipid, and a decrease in the enthalpy of DMPC in peptide/DMPC mixtures. In addition, secondary structural predictions (Chou & Fasman, 1978) and a helical wheel representation of residues 18–36 are consistent with the amphipathic α-helix model. The peptide ESALL-NPY, which was designed as an amphipathic analogue, shows similar lipid-binding characteristics as neuropeptide Y, and the α-helix content of the peptide is more enhanced on binding to lipid, as expected from Chou and Fasman (1978) predictions for the amphipathic α-helical region of the peptide, in which the substitution of Glu, Ser, Ala, and Leu increases the α-helical probability from 1.06 to 1.10 and decreases the β-sheet probability from 1.07 to 1.03. Such lipid–peptide interactions are consistent with an amphipathic α-helical hypothesis for the receptor-binding potency of neuropeptide Y. However, in the active conformationally restricted cyclic analogues of neuropeptide Y with high receptor-binding affinity, the portion of the amphipathic α-helix that remains is prevented from interacting in a normal fashion due to the presence of the disulfide bridge. Thus, a simple amphipathic α-helix model for the biological potency of neuropeptide Y is insufficient to explain its interactions with receptors.

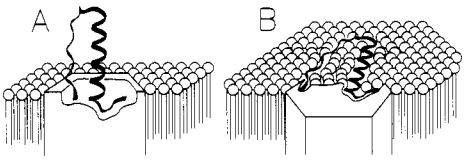

FIGURE 7: Representations of two possible modes of interaction of neuropeptide Y with its cell membrane receptor. For the lipids, the circle represents the head group and the straight lines represent the acyl chains. In panel A, the view is a section through the membrane bilayer, and the peptide is shown interacting with the protein receptor by attachment of its N- and C-terminal regions, without interaction of the polyproline or amphipathic α-helical regions. In panel B, the view is looking down on the cell membrane. In this model, the hydrophobic residues in the polyproline and α-helical regions of the peptide interact with the lipids of the membrane and the N- and C-terminal regions of the peptide bind to the receptor protein.

Two limiting models for the binding of NPY to its receptors may be proposed (Figure 7). In the first model (A), lipid binding plays no role, but a defined spatial relationship between the N- and C-terminal residues of the peptide is required. In the second model (B), the peptide binds to the receptor protein and to the lipid, thereby increasing the binding interaction energy. The binding of the cyclic analogues and NPY_{20-36} to pig spleen receptors is consistent with a molecular model in which only the C-terminal end of the peptide interacts with the receptor. A critical role for the C-terminal amide has been demonstrated in several recent papers (Potter, 1988, for review). This interaction is similar to that shown in Figure 7A, in which the amphipathic α-helix does not bind to membrane lipid, except that only the C-terminal region of the peptide binds to the receptor. By contrast, binding to the mouse brain receptor appears to require residues in the N-terminal region of the peptide, since NPY_{20-36}, which contains the C-terminal region of C7-NPY, does not bind to this receptor. However, the N- and C-terminal residues are not sufficient for receptor

binding, since the affinities of C2-NPY and C5-NPY are much reduced compared with that of C7-NPY. The attenuation of binding potency in these analogues may result from the loss of residues involved in direct receptor interaction or from a preference for a conformation dissimilar to the receptor-bound conformation which reduces the ability of the N- and C-terminal residues to interact with the receptor.

At high peptide/lipid ratios, C7-NPY, NPY, and ESALL-NPY are unusually effective in disrupting lipid structure, so that lipid–peptide interactions cannot be entirely ruled out in receptor binding. However, NPY_{20-36} and C7-NPY bind with equal affinities to lipid, ruling out a simple lipid-binding hypothesis for the increased receptor-binding potency of C7-NPY for mouse brain receptors. The strong lipid interaction of both C7-NPY and NPY may be a consequence of exposure of the hydrophobic faces of both the N- and C-terminal regions of the peptides to lipid. The structural motif that allows such interaction may also serve to stabilize the interaction of the hydrophobic faces of the amphipathic α-helix and the polyproline helix in solution, thus conferring a proper spatial relationship between the N and C termini of the peptide and promoting binding to the receptor as shown in model A (Figure 7). The data do not support a model, such as B (Figure 7), in which lipid binding stablizes the receptor-bound conformation. Rather, the data are consistent with a model in which the spatial relationship between the N and C termini of NPY is stabilized by an intramolecular association of the hydrophobic faces of the polyproline helix and the amphipathic α-helix of the peptide.

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