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# Lipid and membrane interactions of neuropeptide Y

Larry R. McLean, Bruce M. Baron, Stephen H. Buck and John L. Krstenansky

*Merrell Dow Research Institute, Cincinnati, OH (U.S.A.)*

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**The interactions of neuropeptide Y with dimyristoylphosphatidylcholine and cell membranes were examined by several physical techniques to probe the potential role of its putative C-terminal amphipathic α-helix. Neuropeptide Y binding was demonstrated by a rapid release of entrapped 6-carboxyfluorescein and a rapid decrease in the turbidity of dimyristoylphosphatidylcholine liposomes. In addition, an increase in tyrosine fluorescence intensity and an increase in the anisotropy of diphenylhexatriene in dimyristoylphosphatidylcholine liposomes was observed. In isolated, aortic smooth muscle cell membranes, the anisotropy of diphenylhexatriene increased as a function of added neuropeptide Y. The concentration range (low μM) over which neuropeptide Y increases the polarization of diphenylhexatriene in cell membranes is similar to the range in which it inhibits isoproterenol-stimulated cAMP accumulation. This inhibition is not affected by pertussis toxin, nor does neuropeptide Y cause the release of preloaded [$^3$H]adenine from cells into the medium. These data suggest that neuropeptide Y contains an amphipathic α-helical region which interacts with lipids in much the same way as the amphipathic α-helical regions of the plasma apolipoproteins and that the inhibition of isoproterenol-stimulated cAMP accumulation at low μM concentrations of peptide may be the result of an alteration in the cell membrane bilayer structure.**

## Introduction

Neuropeptide Y, a 36 amino acid residue peptide first isolated from porcine brain, is widely distributed in the central and peripheral nervous systems where saturable high affinity binding sites have been observed [1]. It is found in many noradrenergic neurons innervating vascular smooth muscle and the heart, and is a modulator of noradrenergic neuroeffector junction function. In addition, the peptide is released from sympathetic neurons, supporting the hypothesis that neuropeptide Y is a neurotransmitter peptide. Recently, neuropeptide Y has been reported to inhibit cAMP formation in several tissues at nM concentrations [2–5] and this inhibition is blocked by pertussis toxin in heart and cultured neuronal cells [6]. At higher concentrations (low μM), a second inhibition phase, which may be GTP-independent, is also observed [3]. In addition, a general potentiation of vasoconstriction at concentrations approaching low μM has been reported [7].

The amino acid sequence of neuropeptide Y bears strong homology with peptide YY and the pancreatic polypeptides [8] and contains an intramolecularly-stabilized helical structure similar to that in avian pancreatic polypeptide [9,10] in which the C-terminal residues 14–30 form an amphipathic α-helix. By analogy with the importance of the amphipathic α-helix as a structural feature in several peptide hormones [11], this site may be critical to the binding of the peptide to receptors. In the present report, several physical techniques are used to characterize the interaction of neuropeptide Y with lipids. The possible role of the putative amphipathic α-helical region of the peptide is discussed in light of its effect on cAMP accumulation in vascular smooth muscle cells in the low μM concentration range.

## Methods

Porcine neuropeptide Y was synthesized by solid-phase techniques on *p*-methylbenzhydrylamine resin [9]. Purity was assessed by analytical high performance liquid chromatrography, amino acid analysis, and fast-atom bombardment mass spectrometry. The sequence of the porcine peptide differs from the human by Leu$^{17}$, which is Met in the human peptide. Apolipoprotein C-III was purified from human plasma [12].

For measurements of peptide-induced leakage of the contents of liposomes, dimyristoylphosphatidylcholine liposomes were probe sonicated in a saturated solution of 6-carboxyfluorescein, then separated from free fluo-

Correspondence: L.R. McLean, Merrell Dow Research Institute, 2110 East Galbraith Road, Cincinnati, OH 45215, U.S.A.

rophore by gel filtration chromatography. The fluorescence (excitation 490 nm, emission 515 nm) of 2 ml of liposomes (50 μg/ml) was measured at 24°C for 5 min, then 50 μg of neuropeptide Y or apolipoprotein C-III were added and the fluorescence was measured for an additional 15–20 min. Then, 0.1 ml of Triton N-101 (10%) was added to burst the liposomes and establish the fluorescence for 100% leakage.

For liposome clearing [13], the absorbance (400 nm) of 2 ml of dimyristoylphosphatidylcholine liposomes was recorded continuously at 24°C for 5 min. Then, 0.1 mg of peptide was added with stirring and recording was resumed for 60 min. Fluorescence spectra were recorded on samples from the clearing experiments after 24 h of incubation at 24° C.

For fluorescence polarization, 1,6-diphenyl-1,3,5-hexatriene was added to either dimyristoylphosphatidylcholine liposomes or rat aortic smooth muscle cell membranes (48 000 × $g$ pellet) in a 1 : 200 molar ratio (probe: PC) and the samples were incubated for 1 h at 24° C. The ratio of the parallel to horizontal emission signals was measured with the excitation polarizer oriented vertically ($R_V$) and horizontally ($R_H$) on an SLM 4800 in the T-format, where the anisotropy is given by $r = (R_V/R_H - 1)/(R_V/R_H + 2)$. Temperatures were regulated ± 0.2 C° externally, with a water bath.

For measurements of the neuropeptide Y-mediated attenuation of isoproterenol-stimulated cAMP accumulation, the growth medium from dissociated rat aortic smooth muscle cells grown in culture [14] was removed, and the cells were incubated for 2–4 h with [$^3$H]adenine in Krebs-Hepes buffer (140 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM Hepes (pH 7.35)). The cells were then washed, and test compounds were added in the same buffer. After incubation for 10 min at 37°C with 10 μM isoproterenol, [$^3$H]cAMP was extracted and quantitated by ion-exchange chromatography [15].

## Results

The association of neuropeptide Y with dimyristoylphosphatidylcholine liposomes was compared with that of apolipoprotein C-III (which binds to lipids by virtue of its amphipathic α-helical regions) by following the rate of release of 6-carboxyfluorescein entrapped in the vesicles. Both peptides rapidly induce the release of 6-carboxyfluorescein from the liposomes (Fig. 1). Similarly, neuropeptide Y rapidly decreases the turbidity of dimyristoylphosphatidylcholine liposomes when added at 24° C. A rate parameter for this process was calculated from $1 - A_5/A_0$, where the absorbances were measured at 400 nm before ($A_0$), and 5 min after ($A_5$), addition of peptide. This rate, as a function of added lipid concentration, is shown in Fig. 2. Addition of lipid also increases the fluorescence emission intensity of the tyrosine residues in neuropeptide Y. This increase in fluorescence is nearly maximal at a weight ratio of 4 : 1 (molar ratio 26 : 1) lipid to peptide (Fig. 2) at which ratio the rate of liposome clearing is also nearly maximal.

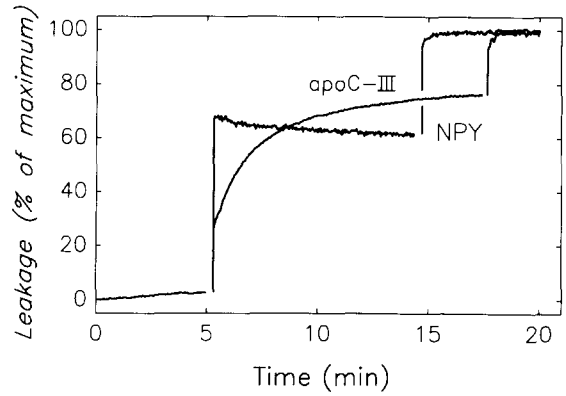


Fig. 1. Comparison of the effect of neuropeptide Y and apolipoprotein C-III on the rate of leakage of 6-carboxyfluorescein from dimyristoylphosphatidylcholine liposomes. The concentration of neuropeptide Y was 11.7 μM.

Neuropeptide Y increases the anisotropy of diphenylhexatriene in dimyristoylphosphatidylcholine liposomes and cell membranes at all temperatures from 12 to 50° C, corresponding to an increase in the rigidity of the acyl chains near the probe (data not shown). In liposomes, low μM concentrations are required to increase the anisotropy at 24°C, the phase transition temperature of the lipid (Fig. 3). At 37° C, the anisotropy is increased slightly, but significantly ($p < 0.05$, $n = 10$) from 0.065 ± 0.001 (mean ± S.E.) in the absence of peptide to 0.068 ± 0.001 in the presence of 1.1 μM neuropeptide Y. In aortic smooth muscle cell membranes, somewhat higher concentrations are required (Fig. 4).

In the absence of neuropeptide Y, isoproterenol stimulates cAMP accumulation in rat aortic smooth muscle cells approx. 15-fold. In the presence of low μM concentrations of neuropeptide Y, a dramatic inhibition of isoproterenol-stimulated cAMP accumulation is ob-

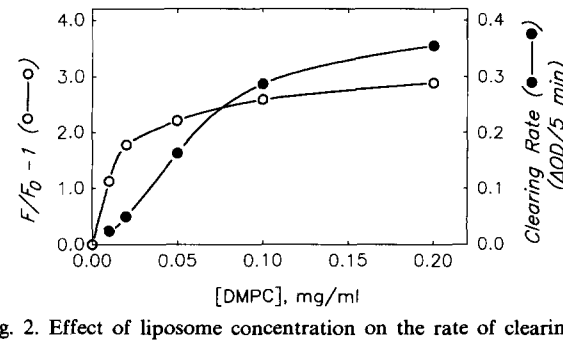


Fig. 2. Effect of liposome concentration on the rate of clearing and increase in fluorescence intensity (excitation 275 nm, emission 306 nm) of neuropeptide Y when mixed with dimyristoylphosphatidylcholine.

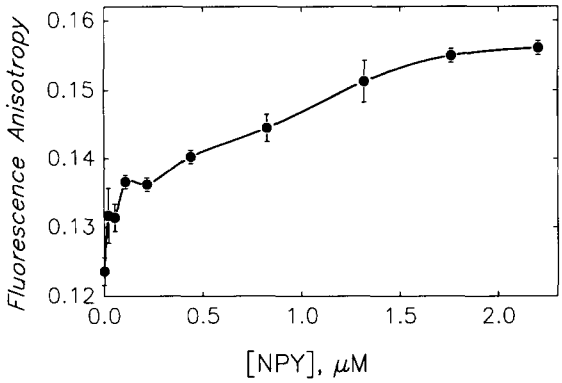


Fig. 3. Effect of neuropeptide Y on diphenylhexatriene anisotropy at 24° C in dimyristoylphosphatidylcholine liposomes as a function of neuropeptide Y concentration (mean ± S.E, $n = 3$–6 determinations).

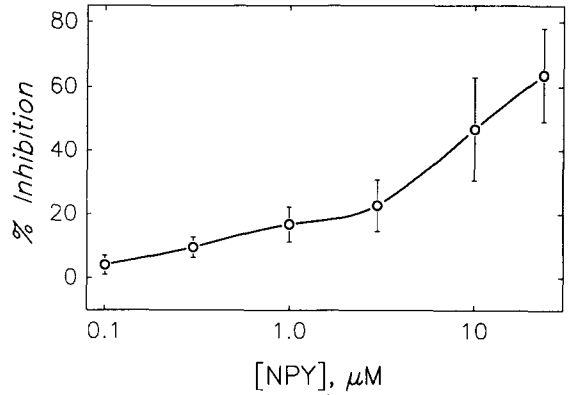


Fig. 5. Neuropeptide Y inhibition of isoproterenol-induced cAMP accumulation in smooth muscle cells (mean ± S.E, $n = 3$–6 determinations). Basal per cent conversion of cell-incorporated [$^3$H]adenine to [$^3$H]cAMP] was 0.06 ± 0.06%. In the presence of isoproterenol the incorporation was 1.27 ± 0.45%. The data are averaged from separate experiments on four cell lines.

served (Fig. 5) which exceeds the inhibition observed at nM concentrations of peptide in the four cell lines examined. In one cell line (WB-7), up to 30% inhibition at neuropeptide Y concentrations below 100 nM was observed, suggesting multiphasic effects of the peptide on cAMP accumulation [16]. The inhibition at low μM concentrations is not affected significantly by pertussis toxin in WB-7 cells: at 23.5 μM neuropeptide Y, the inhibition of cAMP accumulation in the absence of pertussis toxin is 72 ± 8% and in its presence (100 ng/ml)) is 63 ± 5% ($n = 3$). By contrast, at 100 nM neuropeptide Y, pertussis toxin decreased the inhibition from 38 ± 12% to 7 ± 5%. Previous work has demonstrated a similar degree of inhibition by nM concentrations of neuropeptide Y [2–4,6], that is sensitive to pertussis toxin in neuroblastoma [6] and cultured rat atrial cells [2]. The inhibition by μM concentrations of neuropeptide Y is not the result of a detergent-like effect of the peptide in that the release of [$^3$H]adenine from the cells is not increased in the presence of the peptide: in the absence of peptide 11.6 ± 0.6% of the label is recovered in the medium, while in the presence of 23.5 μM peptide 10.9 ± 2.0% is associated with the media.

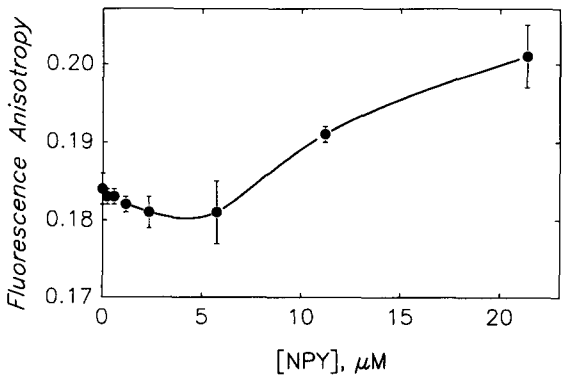


Fig. 4. Effect of neuropeptide Y concentration on diphenylhexatriene anisotropy in cell membranes. Polarization was measured at 37° C (mean ± S.E., $n = 3$–6 determinations).

## Discussion

Based on the primary sequence of neuropeptide Y and its CD spectrum, a C-terminal amphipathic α-helical region (residues 14–30) has been proposed [9]. The present data are consistent with the existence of such a region in neuropeptide Y which promotes lipid binding and may play a role in cellular interactions of the peptide. Neuropeptide Y consists of two opposing helices stabilized intramolecularly by hydrophobic interactions [9,10]. Upon binding to lipid, this intramolecularly stabilized helical structure may be disrupted, exposing the hydrophobic face of the amphipathic α-helix to the nonpolar regions of dimyristoylphosphatidylcholine and forming the characteristic clear micelles observed when amphipathic α-helical peptides bind to lipid. This would result in a burial of the tyrosine residues which lie at the edge of the hydrophobic face of the amphipathic α-helix, increasing the fluorescence of the peptide, since fluorescent residues at the edge of the helix are highly sensitive to the polarity of their environment [17].

The inhibition of isoproterenol-stimulated cAMP accumulation at low μM concentrations of neuropeptide Y is unaffected by pertussis toxin, suggesting that it is not mediated by inhibitory G-protein receptor-coupling. However, treatment with pertussis toxin may not result in complete ADP-ribosylation, leaving some intact $G_i$ protein which may still participate in the NPY-induced inhibition of cAMP accumulation. This activity at low μM concentrations must be differentiated from the inhibitory activity of neuropeptide Y at low nM concentrations, which is prevented by pertussis toxin and appears to be independent of lipid binding [18]. Low

nM concentrations of the peptide are not expected to have structural effects on the membrane bilayer. The dramatic effects of neuropeptide Y on lipid and cell membrane structure suggest that the inhibition which is observed at low $\mu$M concentrations of neuropeptide Y results from an interaction of the amphipathic $\alpha$-helical region of the peptide with the cell membrane bilayer. Since low $\mu$M concentrations of peptide might occur locally, near sites of neuropeptide Y release [19,20], this membrane perturbation may be of some physiological significance. Levitzki and co-workers have proposed that the rate of adenylate cyclase activation by the catecholamine-bound adrenergic receptor is diffusion controlled and depends on the 'fluidity' of the membrane [21]: increased 'fluidity' correlates with increased activity. A similar mechanism is possible for neuropeptide Y, in which a neuropeptide Y-mediated decrease in the 'fluidity' of the cell membrane, as measured by diphenylhexatriene polarization, is correlated with a decrease in $\beta$-adrenergic receptor-stimulated cAMP accumulation in the same concentration range. One possible mechanism to explain the inhibition of cAMP accumulation by neuropeptide Y is a decrease in the rate of association of the components of the adenylate cyclase stimulatory receptor/G-protein complex ($R\text{-}G_s$) within the cell membrane bilayer. Regardless of the details of the mechanism, the data suggest that inhibition of isoproterenol-stimulated cAMP accumulation at low $\mu$M concentrations of neuropeptide Y may be the result of alterations in the cell membrane bilayer structure.

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