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Substitution of D-Trp^{32} in NPY Destabilizes the Binding Transition State to the Y1 Receptor Site in SK-N-MC Cell Membranes*

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The retention rate of the spin label 3-isothiocyanto methyl-2,2,5,5-tetramethyl-1-pyrrolidinyl oxyl spin label (proxyl) attached to the porcine N-acetyl-NPY peptide and the porcine N-acetyl-D-Trp^{32}-NPY peptide at Lys^4 was investigated using SK-N-MC neuroblastoma cell membranes containing the Y1 receptor. The release rate of the spin labeled peptides was monitored by electron spin resonance and the K_D was determined by a direct radiolabeled NPY displacement binding assay. The analyses show that for the porcine [Ac-Tyr^{1}N$^{\varepsilon 4}$-proxyl]-NPY, the K_D was 8×10^{-10} M and k_{off} was 2.7×10^{-4} sec^{-1} yielding a value for k_{on} of 3.3×10^5 sec^{-1} M^{-1}. The [Ac-Tyr^1, N$^{\varepsilon 4}$-proxyl,-D-Trp^{32}]-NPY antagonist ligand had a value of K_D equal to 1.35×10^{-7} M and k_{off} was 1.7×10^{-4} sec^{-1} leading to a value for k_{on} of 1.2×10^3 sec^{-1} M^{-1}. The difference in the k_{on} rates of two orders of magnitude is interpreted as demonstrating the N-acetyl-N$^{\varepsilon 4}$ proxyl-D-Trp^{32}-NPY ligand binding transition state to be of higher energy then for the unmodified NPY amino acid sequence.

KEY WORDS: NPY binding; on and off rates, NPY agonist and antagonist binding; activation energy difference in receptor binding.

INTRODUCTION

Neuropeptide Y (NPY) is a 36 amino acid peptide that is widely distributed in both the central nervous system (CNS) and the peripheral nervous system (PNS). Among the many biological activities in the CNS that have been attributed to this peptide are stimulation of appetite activity (1), regulation of circadian rhythm activity (2), modulation of body temperature, and induction of catalepsy (3), and effects on memory processing (4). In the PNS the NPY ligand in conjunction with noradrenalin is reported to function as a regulator of vascular tone (5). The isolation of Neuropeptide Y from porcine brain was reported by Tatemoto (6,7) who noted that it bore a close primary structure analogy to avian pancreatic polypeptide (APP) (6) and to porcine pancreatic polypeptide. NPY has a primary structure homology to peptide YY (PYY) of about 69% (8) and to porcine polypeptide of about 50% homology (9). Since 1982 a number of NPY's from other species have been isolated and sequenced, and a large number of NPY analogs have been synthesized and evaluated with respect to biological activity and receptor binding behavior (10).

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* Dedicated to Professor Eduardo Soto on the occasion of his retirement.

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The secondary and tertiary structures of NPY have been investigated using circular dichroism and NMR, and a 3D model has been proposed from these studies (11,12,13). The x-ray structure of the homologous peptide APP, has been reported (14); however, to date neither the parent peptide nor any analogs have been crystallized. This may, in part, be attributed to a more flexible N-terminal structure than the N-terminal structure of APP (15). There are three known NPY receptor subtypes which have been designated as Y_1, Y_2, and Y_3 (16). The human and rat Y receptor genes have been cloned and sequenced (17,18,19) and permit the characterization of the Y_1 receptor as a G protein coupled receptor that mediates the inhibition of cyclic AMP in some cell types and is coupled to the elevation of intracellular calcium in other cell types. The length of time that a neuropeptide is retained by its G-protein coupled receptor component is an essential parameter in the elucidation of the time sequence in G-protein signal transduction. Neuropeptide Y and its receptor linked G protein signal transduction system can be used to accurately determine the residence time of the ligand to its receptor using spin labeled ligands. The report by Balasubramaniam et al. (20) that D-Trp^{32}-NPY was a competitive antagonist of NPY suggested a series of experiments to measure the retention time of the NPY ligand and the D-Trp^{32} NPY ligand, by the Y_1 receptor in the SK-N-MC cell membrane. The SK-N-MC cell line is reported to selectively express the number of Y_1 receptors as between ≈ 85,000 (21) and 200,000 receptors per cell, with a K_D ≈ 2nM, almost to the exclusion of the other Y type receptors, (22,23). Our search of the literature has not found any report for the on or off rates of NPY binding to its receptor. Using the epr technique to measure the off rate allows for immediate monitoring of the amount of ligand bound and free when the starting point begins with 100 percent bound ligand. We have synthesized spin labeled porcine NPY and porcine D-Trp^{32} NPY in order to determine their respective on/off binding properties to the NPY receptor in the SK-N-MC cell membrane.

The amino acid sequence for the porcine NPY is shown below and differs from human NPY only in the substitution of a leucine residue for a methionine residue at position 17.

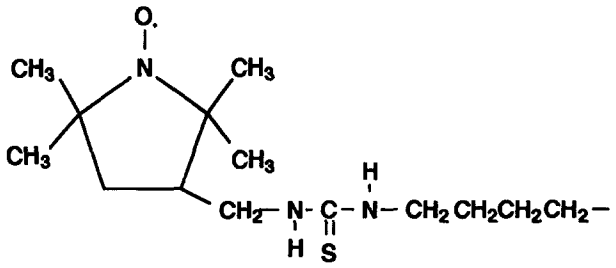
SEQUENCE for porcine N-Acetyl NPY

Ac-Y-P-S-K-P-D-N-P-G-E-D-A-P-A-E-D-L-A-R-Y-Y-S-A-L-R-H-Y-I-N-L-I-T-R-Q-R-Y-NH_2

SEQUENCE for porcine N-Acetyl-D-Trp^{32}-NPY

Ac-Y-P-S-K-P-D-N-P-G-E-D-A-P-A-E-D-L-A-R-Y-Y-S-A-L-R-H-Y-I-N-L-I-D**W**-R-Q-R-Y-NH_2

The proxyl spin label was attached to the lysine residue at position four in the peptide chain using the isothiocyanate derivative of the proxyl compound, Structure 1.



Structure 1.

We report the first determinations for the on/off rate of spin labeled NPY and D^{32}Trp NPY with respective binding constants similar to the binding constants for the untagged NPY's.

EXPERIMENTAL PROCEDURE

Synthesis of NPY and D*Trp^{32} NPY.* Typical peptide synthesis was carried out using Rink amide resin (0.25mmol, 0.5 g) and the stepwise coupling of Fmoc amino acids using an Applied Biosystems model 431A peptide synthesizer.

*Acetylation of NPY and [*D*Trp^{32}]NPY and Cleavage from the Resin.* The peptide, while still attached to the resin (0.30g), was treated with 10 ml of NMP, 1.0 ml of pyridine and 1 ml of acetic anhydride. The suspension was shaken at room temperature for 30 minutes and then drained. After an initial washing with NMP and then with dicholoromethane, the N-acetylated peptide attached to the resin was treated with 0.5 ml of thioanisole, 0.5 ml of EDT and 10 ml of 95% trifluoroacetic acid for 3 hours. After filtering, the residual resin was subjected to an additional wash with 1.0 ml of 95% TFA, and the combined washings and filtrate concentrated in vacuo to yield an oil which was then triturated with diethyl ether to give 0.14 g of crude peptide.

Attachment of the Spin Label 3-Isothiocyanto Methyl-2,2,5,5-Tetramethyl-1-Pyrrolidinyl Oxyl to the Peptide at Lys-4. Crude peptide (0.020 g) was dissolved in 1.0 ml of an H_2O/CH_3CN (1:1 v/v) mixture and this solution was then adjusted to pH 9.0 with saturated $NaHCO_3$ solution. A solution of 5.7 mg of the spin label dissolved in 0.1ml of NMP was added to the peptide solution. The progress of the reaction was monitored periodically, and after 4 hours an additional 6.0 mg of the spin label reagent was added to the reaction. The reaction appeared to be complete after 6.5 hours. The mixture was subjected to HPLC on a Vydac C_{18} reversed phase preparative column and eluted with a H_2O/CH_3CN gradient containing 0.1% TFA. Amino acid analysis and electrospray mass spectroscopy on each of the purified peptides were in excellent agreement with the predicted values.

Preparation of Cells and Cell Membranes. Human neuroblastoma cells, SK-N-MC, obtained from American Type Culture Collection (Cat. No. HTP10) were grown in Dulbecco's modified Eagle medium (Gibco) containing 10% fetal bovine serum and 100 units per ml/100μg per ml, penicillin/streptomycin (Gibco). After confluence the cells were fed and incubated at 37°C for an additional 24 hrs. The growth medium was removed, and the cells are then rinsed in a buffer consisting of: 25 mM Tris, pH7.4, 6 mM $MgCl_2$, 250μg/ml bacitracin, 250 μg/ml aprotinin, 250 μg/ml leupeptin, 250 μg/ml Peflabloc, (Pen-

tapharm AG). The cells were lifted from the flasks with Dulbecco's phosphate buffered saline solution (PBS) containing 0.02% EDTA. Cells were pelleted and homogenized using a polytron and the broken cell membranes were centrifuged at 18,000 rpm for 10 min. at 4°C. The pelleted membranes were resuspended in the TRIS buffer and frozen at −70°C.

Binding Assay Protocol. Dulbecco-PBS (Gibco) pH 7.4 containing 0.5 g/l bacitracin and 1 g/l BSA was used in the preparation of all assay components. Each assay tube consisted of the following: 100 µl of varied concentrations of [Ac-Tyr[1]N[ε4]-proxyl]-NPY or [Ac-Tyr[1], N[ε4]-proxyl,-D-Trp[32]]-NPY or buffer, 100 µl of a solution of porcine ^{125}I-PYY (30pmoles/tube) and 50 µl of SK-N-MC membrane preparation containing 50 µl/tube of protein for a total volume of 250 µl per tube. Nonspecific binding was determined by the addition of excess cold neuropeptide Y (300 nM). After the addition of all the reagents, the tubes were shaken while incubating for sixty minutes at room temperature. The assay was terminated by filtering the suspension through a Whitman GF/C glass filter pad previously saturated with 0.1% polyethylene imine in 10 mM Tris, pH 7.5 containing 0.1% BSA. The filters were punched from the filter mat and counted for 1 min.

In the binding protocol the radioiodinated NPY ligand concentration was well below the value of K_D. A concentration of 1 nM is required to saturate all of the receptors. In this binding assay the radioligand concentration is such that no more than 10 percent of the radioligand is bound, insuring that the radioligand concentration is not rate limiting. The assay is carried out under equilibrium conditions in which the membranes, radioligand, and different concentrations of cold ligand are maintained at constant temperature for 1 hr and then the amount of bound radiolabel is determined as a function of cold ligand concentration. A plot of percent bound radiolabel against the concentration of cold ligand yields the IC_{50} and this is taken as the value of K_i which is equal to the binding constant K_D, as derived by Cheng and Prusoff, (24).

Spin Labeling Methods. The [Ac-Tyr[1]N[ε4]-proxyl]-NPY, and the [Ac-Tyr[1],N[ε4]-proxyl,-D-Trp[32]]-NPY were dissolved in 1–2 drops of DMSO and then diluted with Dulbecco's modified Eagle Medium (GIBCO BRL') to 6 µM and 20 µM, respectively, the final concentration depending on the signal to noise ratio of the probe's epr signal.

Fifty mg of frozen membrane prepared from SK-N-MC cells was centrifuged at 4000 rpm for 5 to 10 seconds in an Eppendorf centrifuge. One ml of ice cold spin label solution was added to the membrane pellet which was resuspended and incubated on ice for 15 minutes. The suspension was centrifuged for 10 seconds, the spin probe solution decanted and 1.0 ml of ice cold phosphate buffered saline (PBS, GIBCO, BRL, with calcium) was added and the membranes resuspended by light vortexing. The suspension was centrifuged and cold rinsed two more times before being resuspended in 60–80 µl of PBS and loaded at room temperature into the flat epr cell (≈100 µl volume) for epr measurement.

Two to three T-150 flasks containing 20–30 × 10[6] SK-N-MC cell monolayers were used for each epr measurement. Two mM KCN/iodoacetamide in Dulbecco's medium containing 10% fetal calf serum was added to the monolayers to inhibit oxidative metabolism and the cells kept on ice for 2 hours. The medium was decanted, the monolayers rinsed three times with cold PBS and the cells scraped using a Costar cell scraper. The cells were centrifuged and resuspended in 1.0 ml of the spin label medium (ice cold). After 15 minutes incubation on ice, the cells were centrifuged and washed three times with cold PBS. The cells were resuspended in 200 µl and 100 µl was loaded into the flat cells for epr measurement.

EPR Measurement. The membrane pellet is transferred to an epr flat cell (W6-808-Q, Wilmad, Buena, NJ) for data collection. The epr protocol requires that the data be collected as 4×1-minute-sweep spectra, the acquisitions being separated to provide a time evolution of the spectral features. Typically, the evolution of the spectra is complete in an hour. The data are collected in a signal averager (Model NS-900, TRACOR/Northern, Middleton, WI) at 37°C, 5 mW incident microwave power, and 0.08 mT field modulation, 9.174 GHz microwave frequency. The data are reduced to five parameters using previously developed software that determines the g- and a-values and the three linewidths appropriate to each spectrum.

The linewidth values are converted to relative concentrations by establishing the ratios of bound-to-free labeled ligand. The linewidths of the free ligand at 37°C (0.097, 0.095 and 0.109 mT) were determined by obtaining the spectrum of dissolved spin labeled ligand in buffer and computer simulating the epr signal. The linewidths of the bound ligand (0.155, 0.130, 0.256 mT) were determined by trial experiments where membrane samples were exposed to labeled ligand, and then their epr spectra were obtained in the shortest possible time (~2 minutes after warming the sample to 37°C). The linewidths of the bound label and the free label did not differ sufficiently to prevent the use of Lorentzian lineshapes to approximate the lineshape of a sample containing both bound and free label.

To use the Lorentzian lineshape approximation, we need to calculate the observed lineshape of the spectral resonance that is the sum of two different lineshapes. The component lineshapes are assumed to be Lorentzians and functions of the variable x, which is zero at the center of the resonance. If we assume that the free label has linewidth, Γ_1, and maximum intensity equal to one, and that the bound label has linewidth, Γ_2, and maximum intensity equal to α, then the composite lineshape will have a maximum equal to $1 + \alpha$ at $x = 0$. The linewidth of the sum of these two lineshapes can be approximated by finding the point where the composite lineshape is at half maximum. To find that value, the following expression should be solved for x:

$$\frac{1+\alpha}{2} = \frac{\Gamma_1^2}{\Gamma_1^2 + x^2} + \frac{\alpha\Gamma_2^2}{\Gamma_2^2 + x^2} \tag{1}$$

If we make the approximation that the solution of Eqn. 1 is the linewidth of the composite lineshape, Γ_3, then

$$\alpha = \frac{\left(\Gamma_1^2 - \Gamma_3^2\right)}{\left(\Gamma_3^2 - \Gamma_2^2\right)} \cdot \frac{\left(\Gamma_2^2 + \Gamma_3^2\right)}{\left(\Gamma_1^2 + \Gamma_3^2\right)} \tag{2}$$

The value of $\langle\alpha\rangle$ is calculated as the average from that derived from the three linewidths. The per cent bound label, B, is given by

$$B = \frac{100 <\alpha>}{1 + <\alpha>} \tag{3}$$

By computer simulating trial lineshapes and then fitting these simulations, we have determined the error inherent in this data reduction method (in the absence of noise). The standard error of B (over its entire range from zero to 100) is 1.5%, with the maximum error (4.1%) occurring at B equal to 66%.

RESULTS AND DISCUSSION

The ability of Neuropeptide Y to elicit its diverse physiological effects derives from its actions as a ligand for its G protein coupled receptor. The biochemical con-

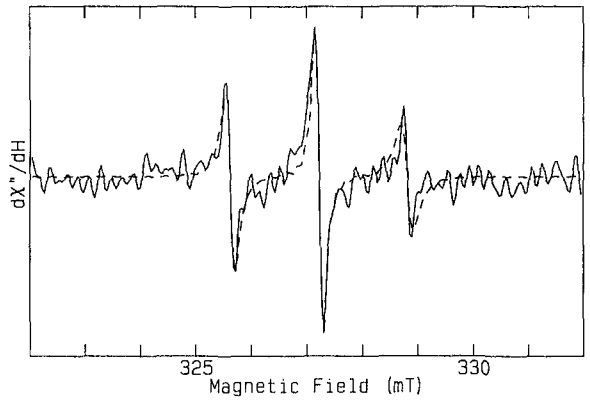


Fig. 1. Epr spectrum of unbound proxyl-NPY at 37°C. These data (solid line) are typical for the unbound spin labeled peptide (see Fig 2B) and are superimposed by a computer simulation (dashed line), which is based on five parameters: the a- and g-values and the three Lorentzian linewidths.

sequence of this ligand receptor binding is the inhibition of adenylate cyclase since the G protein coupled to this receptor is a G_i subtype (10). The receptor itself is known to be a seven helix bundle but the locus of the binding site and the forces involved in the binding are yet to be elucidated. No significant change in the binding constant was found when a proxyl spin label was covalently attached to the ε-amino group of the lysine residue at position 4 in the NPY peptide. Since the determination of the binding constant is not done by the determination of the equilibrium concentrations of the species involved, but rather by the displacement of a bound radioactive PYY ligand, the constant that is determined is therefore equivalent to an inhibition constant. The relationship of K_I to I_{50} for a system that appears to obey Michaelis-Menten or Langmuir adsorption was reported in 1973 by Cheng and Prusoff(24). Their derivation shows that for bisubstrate competitive or noncompetitive inhibition, $I_{50} = K_i$ provided that the affinity of the inhibitor for the free receptor is the same as that for the receptor ligand complex. The constant for [Ac-Tyr^{1}N$^{\varepsilon 4}$-proxyl]-NPY, was determined to be 8.0×10^{-10} M [0.8 nM]. In contrast to this finding, the substitution of a D-tryptophan residue at position 32 in the NPY sequence containing the spin label, resulted in a constant of 1.35×10^{-7} M [135 nM].

Although the derivation for the calculation of the binding constant is based upon a kinetic argument, the constant is inherently an expression of an equilibrium condition. One approach to the derivation of the law of mass action is based on a kinetic argument and, therefore, it is considered valid to develop the following expression that relates the binding constant to the on and off rate constants of the ligands.

$$K_D = K_i = \frac{k_{off}}{k_{on}} = \frac{[R][L]}{[RL]}$$

and the value for k_{on} can be calculated from the relationship

$$k_{on} = \frac{k_{off}}{K_i}$$

where K_i is taken as equivalent to the binding constant K_D.

Figure 1 shows the epr spectrum of unbound NPY containing the spin label. The cells were incubated at 4°C for 15 minutes, cold rinsed and centrifuged three times, and then transferred to the epr flat cell and placed in the spectrometer at 37°C for spectral acquisition. If the ligand did not dissociate at 4°C then the appropriate onset for the dissociation of the ligand would be close to 18 minutes, in agreement with our extrapolated values of 12 and 19 minutes. The spin-labeled neuropeptides were added to SK-N-MC cell membrane preparations, Fig. 2, to determine the ratio of bound to free spin-labeled neuropeptide. The percent bound neuropeptide Y was then fit by a linear regression to establish the rate of release. The above procedure was repeated for the D-Trp peptide (representative data shown in Fig. 3). Fig. 2 and 3 reveal that the effects of ligand binding on the epr linewidth are subtle and difficult to see because of the spectral noise. Nevertheless, the graphs in Fig. 4 demonstrate that these effects are measurable and reproducible. Analysis of the epr data showed that the label was slowly released at different rates for the two ligand molecules. The data indicates that the antagonist neuropeptide with a D-tryptophan residue at position 32 was released at a rate of 1.0 per cent per minute ($k_{off} = 1.7 \times 10^{-4}$ sec^{-1}) from the receptor while the unmodified neuropeptide Y was released at the rate of 1.6 per cent per minute ($k_{off} = 2.7 \times 10^{-4}$ sec^{-1}). The R values for these two regression lines are modest: 0.94 for the D-trp^{32} containing peptide and 0.72 for the native peptide. On the other hand, the regression lines can be extended back to the point where they equaled 100 per cent bound label. By solving these regression lines for B = 100 per cent, we obtain 19 minutes for the D-trp^{32} peptide ligand and 12 minutes for the start of the native ligand. These numbers are in good agreement with the value of 18 ± 1 minutes, the time that the spin label and membranes were mixed, kept at 4°C, and warmed from their incubation temperature of 4°C. Both the reproducibility and the fact that these data were able to successfully extrapolate to the beginning of the experiment give us confi-

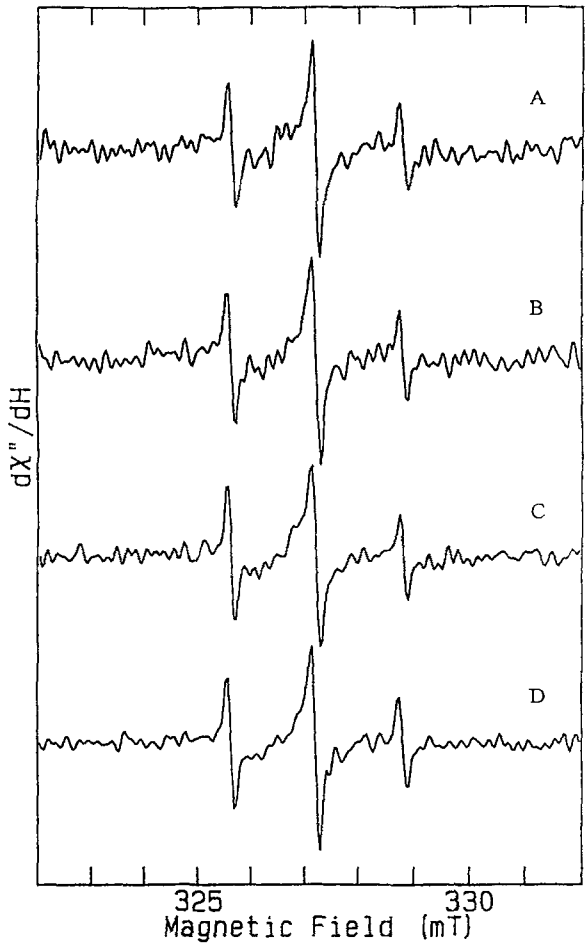


Fig. 2. Epr spectrum of proxyl-NPY labeled SK-N-MC membranes at 37°C. These data are scaled so that each is proportional to the recorded amplitudes. The linewidths become narrower as a function of time. The times from the initial mixing of the receptors and the spin label to the halfway point of the sample acquisitions are: A) 23 min., B) 27 min., C) 34 min., and D) 45 min.

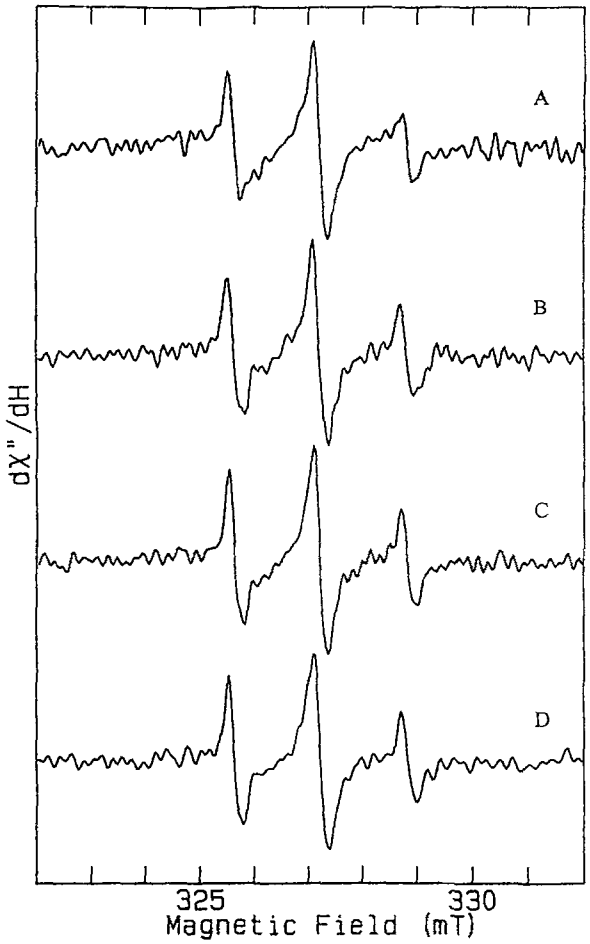


Fig. 3. Epr spectrum of d-Trp^{32}-proxyl-NPY labeled SK-N-MC membranes at 37°C. These data are scaled so that each is proportional to the recorded amplitudes. The linewidths become narrower as a function of time. The times from the initial mixing of the receptors and the spin label to the halfway point of the sample acquisitions are: A) 23 min., B) 27 min., C) 34 min., and D) 45 min.

dence in the accuracy of the slopes of these regression lines, which are their only parameters relevant to the binding of the neuropeptide. These results are interpreted to mean that the neuropeptides were released by the receptor into the solution at this rate. Chemical modification would produce an altered epr spectrum and this was not seen. The epr spectra of the labeled membranes, after one hour at 37°C, had changed to a spectrum identical to that of the spin labeled neuropeptide dissolved in water.

Because these epr results provide the "off rates" for the previously measured binding constants, the "on rates" for binding are calculable by dividing the "off rates" by the K_i's. The value obtained for k_{on} for [Ac-Tyr^{1}N$^{\varepsilon 4}$-proxyl]-NPY, was 3.3×10^5 sec^{-1} M^{-1} and for [Ac-Tyr^1, N$^{\varepsilon 4}$-proxyl,-D-Trp^{32}]-NPY it was 1.2×10^3 sec^{-1} M^{-1}. There is a large difference in the binding rates of the two neuropeptides. Because these molecules resemble one another in terms of size, solubility and structure, we would have expected the "on rates" to be very similar. The fact that they are not similar is interpreted by us to signify that the binding transition state (in the sense of a transition state theory model) is lower in energy for the [Ac-Tyr^{1}N$^{\varepsilon 4}$-proxyl]-NPY ligand than for the [Ac-Tyr^{1}N$^{\varepsilon 4}$-proxyl,-D-Trp^{32}]-NPY molecule. Therefore the effect of substituting D-Trp^{32} for L-Thr^{32} is to make it more difficult to pass through this transition state in the binding of the neuropeptide. This interpretation contrasts with the possible experimental alternative result where the on-rates are equal, signifying that

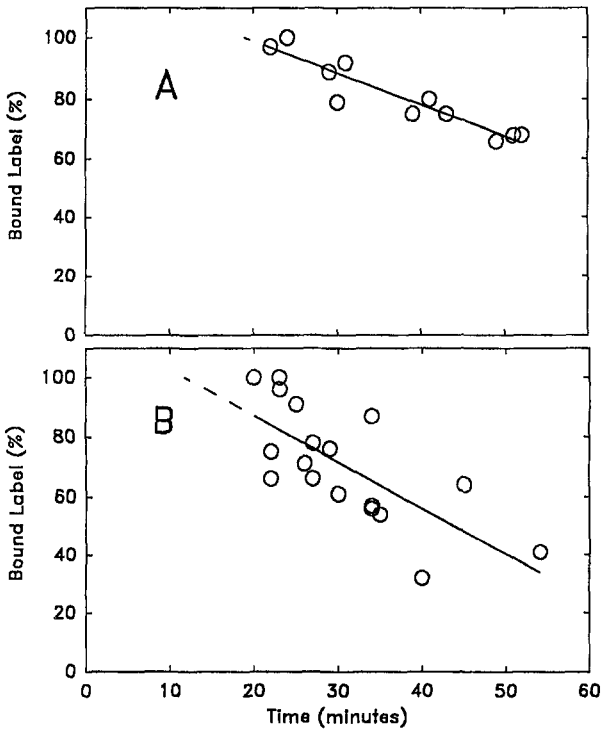


Fig. 4. Percent of the bound spin label as a function of time after mixing the labeled neuropeptides with the SK-N-MC membranes, superimposed by their regression lines. The regression equations were: B = 120-1.04T (R = 0.94) for the D-Trp^{32} containing peptide (A) and B = 118-1.56T (R = 0.72) for the unmodified NPY. The regression lines are extended back in time (dashed portion) to where they equal 100 on the ordinate.

the transition state has the same energy for both NPY and D-Trp^{32}-NPY molecules. In this case the differences in binding would have to manifest themselves in vastly different neuropeptide off rates.

Spin label experiments were also performed on the SK-N-MC cell suspensions. Unfortunately, these experiments were not successful in determining the bound to free ratios of the spin-labeled neuropeptides because the spin-label was chemically reduced, annihilating the nitroxide signal by the cellular metabolic chemistry before a significant change in the epr linewidth could be measured. In our experience, most living cells at 37°C will use the dissolved oxygen in the cell slurry within five to ten minutes after they are added to the flat cell. The time depends on the thickness of the cell slurry. At this point, the reducing excess in the cells resulting from the anaerobic conditions begins to chemically reduce the nitroxide spin label. The epr signal can be recovered by aeration of the sample, proving that the loss of the signal is due to chemical reduction. The result of this chemistry is that the signal will disappear before the labeled peptide comes off of the cell receptor. It was not possible to poison the enzymatic pathways and inhibit this reduction, although cyanide and iodoacetamide were added to the cell suspension in an attempt to block the reductive pathways involving heme proteins. Rather than attempting to inhibit the complex of reactions that could be contributing to the reduction of the spin label, we simply abandoned the intact cell experiments in favor of experiments using membrane preparations.

This study indicates that the L-threonine to D-tryptophan substitution of residue 32 of NPY greatly destabilizes the transition state for binding of this ligand to the Y_1 receptor. Thus, for these ligands binding to the receptor, there may be a "guidance system" that allows the ligand to bind to intermediate locations before it reaches the ultimate binding site. For small flexible molecules binding to proteins it has been suggested that such molecules change their conformation on binding. Furthermore, the bound conformation may be considerably above the global energy minimum (Nicklaus 1995). If this hypothesis is valid for small flexible molecules binding to proteins, it should certainly be valid in the case of the even more flexible small peptides binding to a protein receptor and provides additional support for the validity of our analysis of NPY and D-Trp NPY binding to the Y_1 receptor.

If these ligands are "strained" in the binding process, then the lessening of this strain becomes an attractive mechanism for the promotion of the binding. This interpretation is consistent with our results and suggests that the substitution of a D-trp at residue 32 is in accord with such a mechanism in its binding behavior.

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