MOLECULAR PHARMACOLOGY, 18, 11-19

Neurotensin Binding to Extraneural and Neural Receptors: Comparison with Biological Activity and Structure–Activity Relationships

PATRICK KITABGI, CLAUDINE POUSTIS, CLAUDE GRANIER,* JURPHAAS VAN RIETSCHOTEN,* JEAN RIVIER,† JEAN-LOUIS MORGAT‡ AND PIERRE FREYCHET

Institut National de la Santé et de la Recherche Médicale (INSERM) *Groupe de Recherches sur les Hormones Polypeptidiques et la Physiopathologie Endocrinienne, U 145, et Laboratoire de Médecine Expérimentale, Faculté de Médecine, Université de Nice, Chemin de Vallombrose, 06034 Nice Cédex, France; *Laboratoire de Biochimie, Faculté de Médecine Nord, Boulevard Pierre Dramard, 13326 Marseille Cédex 3, France; †Laboratories for Neuroendocrinology, The Salk Institute for Biological Studies, La Jolla, California 92037; and ‡Service de Biochimie, Centre d'Etudes Nucléaires de Saclay, B.P. No. 2, 91190 Gif-sur-Yvette, France*

Received December 3, 1979; Accepted January 31, 1980

SUMMARY

KITABGI, P., C. POUSTIS, C. GRANIER, J. VAN RIETSCHOTEN, J. RIVIER, J.-L. MORGAT AND P. FREYCHET. Neurotensin binding to extraneural and neural receptors: comparison with biological activity and structure–activity relationships. *Mol. Pharmacol.* 18: 11-19 (1980).

The binding of [^{3}H]neurotensin to a cell line (HT 29) derived from a human colon carcinoma was characterized and compared with [^{3}H]neurotensin binding to rat brain synaptic membranes. Both systems were used as radioreceptor assays for neurotensin and 18 neurotensin synthetic analogs, and the binding affinities thus derived were compared to the biological potencies obtained from the peptide abilities to contract isolated longitudinal smooth muscle strips of the guinea pig ileum. Tritiated neurotensin bound specifically and reversibly to HT 29 cells. The characteristics of [^{3}H]neurotensin binding to cells at 24°C were those of a simple, bimolecular reaction involving one class of noncooperative binding sites. A K_d value of 1.5 nM was independently obtained from association kinetic and equilibrium experiments; total binding capacity was 37 fmol/10^6 cells (22,000 neurotensin-binding sites/cell). Peptides structurally not related to neurotensin did not affect [^{3}H]neurotensin binding. These binding characteristics were very similar to those observed for the binding of [^{3}H]neurotensin to rat brain synaptic membranes. When the binding affinities of neurotensin and neurotensin analogs were compared in the extraneural (HT 29 cells) and neural (brain membranes) systems, a highly significant correlation between the two binding systems was observed. A highly significant correlation was also found when the biological potencies of neurotensin and neurotensin analogs were compared with their binding affinities in either the neural or the extraneural radioreceptor assay. The positive charge on both arginyl residues 8 and 9 and the L-configuration of Arg^9 were important for binding and biological activity. An aromatic residue in the L-configuration was required in position 11 of the neurotensin molecule. The side-chain methyl groups of Ile^{12} and carboxy-terminal residue Leu^{13}, as well as the presence of Leu^{13} in the L-configuration, were required for activity.

INTRODUCTION

Neurotensin is a hypotensive, gut-contracting peptide originally isolated from calf hypothalamus and subsequently from calf small intestine (1, 2); peptides isolated from both sources have been shown to have the same amino acid sequence (3, 4). Neurotensin, a tridecapeptide, exhibits a broad spectrum of pharmacological effects both in the central nervous system and in peripheral tissues (see (5) for review). Brain tissues have been found to possess specific binding sites with a high affinity for ^{125}I-neurotensin (6, 7) and [^{3}H]neurotensin (8); the properties of these sites have suggested that they are involved in the pharmacological responses to neurotensin in brain (6–8). Therefore, the neurotensin binding sites in brain provide a useful tool for investigating the structural

This investigation was supported by Grant ATP 71.78.103 from the Institut National de la Santé et de la Recherche Médicale (INSERM, Paris, France) and by a grant from the University of Nice. Claudine Poustis is a recipient of a Research Fellowship from the Délégation Générale à la Recherche Scientifique et Technique (DGRST, Paris).

0026-895X/80/040011-09$02.00/0

requirements for the interaction of the peptide with its neural receptors. The question has been raised as to whether neurotensin receptors are different in brain and peripheral tissues. ^{125}I-Neurotensin has been shown to bind to mast cells (9); however, the high K_d value (154 nM) observed may suggest that these binding sites are not pharmacologically relevant. Recently, we have reported that [^{3}H]neurotensin binds in a specific manner to a crude membrane preparation from intestinal smooth muscle (10), but the full characterization of these binding sites was hindered by the small number of high-affinity sites in this membrane preparation.

In the present paper, we report on the specific, high-affinity binding of [^{3}H]neurotensin to a cell line (HT 29) derived from a human colon carcinoma (11). This cell line has been shown to specifically bind the vasoactive intestinal peptide (12), insulin (13), and epidermal growth factor (14). It will be shown here that HT 29 cells provide a suitable system to study the interaction of neurotensin with a nonneural binding site, and to compare extraneural and neural neurotensin binding with regard to the ability of a variety of synthetic neurotensin analogs to inhibit the binding of [^{3}H]neurotensin.

We have also attempted to substantiate the pharmacological relevance of these binding sites by comparing the binding potencies of neurotensin and its analogs in the neural and extraneural radioreceptor assays with their biological potencies in a bioassay. For this purpose, an *in vitro* system should be preferred to the *in vivo* bioassays that have been described so far (15–17) since differential degradation of neurotensin and its analogs is likely to occur *in vivo*, which could interfere with the determination of the peptide potency (16, 17). At present, the only *in vitro* effects of neurotensin that can be measured in terms of concentration–response relationships are those on the contractility of gastrointestinal smooth muscles (10, 15, 18–20) and cardiovascular preparations (21, 22). Recently we have shown that the neurotensin-induced contraction in the longitudinal smooth muscle of the guinea pig ileum is mediated through the release of acetylcholine and that longitudinal smooth muscle strips that are incubated with the anticholinesterase agent neostigmine provide a suitable *in vitro* bioassay for neurotensin (23). We have used this bioassay to determine the EC_{50} of neurotensin and its analogs and we have compared these values with the K_d values derived from the peptide ability to inhibit the binding of [^{3}H]neurotensin to the HT 29 cell line and to brain membranes.

It has been recognized from *in vivo* studies that the COOH-terminal region of neurotensin is essential for biological activity (15). Therefore, the neurotensin analogs tested in the present study present modifications of the parent molecule that bear essentially on the COOH-terminal region extending from residues 8 to 13 of the peptide. The role of the arginyl residues 8 and 9, tyrosyl residue 11, and leucyl-carboxy-terminal residue 13 was particularly examined.

TABLE 1

List of neurotensin analogs

No.	Compound	Abbreviation
1	Neurotensin[a]	NT
2	(3-D-Tyrosine)-neurotensin	D-Tyr^{3}NT
3	(8-D-Arginine)-neurotensin	D-Arg^{8}NT
4	(9-D-Arginine)-neurotensin	D-Arg^{9}NT
5	(8-D-Arginine, 9-D-arginine)-neurotensin	D-Arg^{8,9}NT
6	(11-D-Tyrosine)-neurotensin	D-Tyr^{11}NT
7	(11-Phenylalanine)-neurotensin	Phe^{11}NT
8	(11-D-Phenylalanine)-neurotensin	D-Phe^{11}NT
9	(11-D-Leucine)-neurotensin	D-Leu^{11}NT
10	(13-D-Leucine)-neurotensin	D-Leu^{13}NT
11	Neurotensin (8–13)hexapeptide	NT 8–13
12	Neurotensin (9–13)pentapeptide	NT 9–13
13	Neurotensin (10–13)tetrapeptide	NT 10–13
14	Neurotensin (1–12)dodecapeptide	NT 1–12
15	(12-Alanine)-neurotensin (8–13)hexapeptide	Ala^{12}NT 8–13
16	(13-Alanine)-neurotensin (8–13)hexapeptide	Ala^{13}NT 8–13
17	Acetyl-neurotensin (8–13)hexapeptide	Ac-NT 8–13
18	Acetyl-(8-citrulline)-neurotensin (8–13)hexapeptide	Ac-Cit^{8}NT 8–13
19	Acetyl-(9-citrulline)-neurotensin (8–13)hexapeptide	Ac-Cit^{9}NT 8–13

[a] Amino acid sequence of NT: pGlu–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–Tyr–Ile–Leu-OH.

MATERIALS AND METHODS

Peptides. The neurotensin analogs used in this study are listed in Table 1. Neurotensin (NT)[1] and analogs 1 to 12 were synthesized by one of us (J. R.); their synthesis has been reported in detail elsewhere (16). Neurotensin was also purchased from Beckman, Bioproducts Department (Geneva, Switzerland). Peptides 11 to 19 were synthesized by two of us (C. G. and J. V. R.). The synthesis of analog 14 has been reported elsewhere (8). Analogs 11 to 13 and 15 to 19 were synthesized by the solid-phase method previously described for the synthesis of apamine (24). Analogs 17 to 19 were prepared by acetylation of NT 8–13, Cit^8-NT 8–13, and Cit^9-NT 8–13 with acetic anhydride in 0.1 M Tris–HCl, pH 9.0, for 90 min. The acetylated peptides were purified on carboxymethyl-cellulose equilibrated in ammonium acetate and lyophilized. Tyrosine was then deacetylated by treatment with 0.5 M hydroxylamine, pH 7.4, for 60 min, and the peptides were desalted on Bio-Gel P2. Amino acid analysis of the peptides is shown in Table 2. For all peptides, only one spot was detected after high-voltage paper electrophoresis.

Tritiated neurotensin was prepared by one of us (J. L. M.) as previously described (8) and was shown to have the same biological activity as synthetic unlabeled neurotensin (8). Two batches of [^{3}H]neurotensin (specific activity, 77 and 65 Ci/mmol) were used in the studies reported here.

Cell culture and suspension. Dulbecco's modified Eagle's medium, fetal calf serum, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS), trypsin–EDTA solution, and penicillin–streptomycin solution were purchased

[1] Abbreviations used: NT, neurotensin; Cit, citrulline; PBS, phosphate-buffered saline; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KRH, Krebs–Ringer–Hepes solution; BSA, bovine serum albumin; Ac, acetyl.

TABLE 2

Amino acid analysis of synthetic neurotensin analogs

The results are the mean molar ratios of three and two different analyses after acid hydrolysis (AH) and enzymatic hydrolysis (EH), respectively. Names of peptides are abbreviated as explained in Table 1, where the amino acid sequence of neurotensin is also indicated.

Amino acid	NT 8–13		NT 9–13		NT 10–13		Ala[12]NT 8–13		Ala[13]NT 8–13		Cit[8]NT 8–13		Cit[9]NT 8–13	
	AH	EH	AH	EH	AH	EH	AH	EH	AH	EH	AH	EH	AH	EH
Proline	0.99	1.02	1.00	1.03	0.99	1.00	1.02	1.07	1.00	1.04	1.00	1.05	1.02	0.97
Citrulline											ND[a]	1.01[b]	ND[a]	1.03[b]
Alanine							1.07	1.09	1.10	1.07				
Isoleucine	1.00	1.02	0.97	1.00	1.00	1.02			0.97	1.00	0.97	0.99	0.97	0.97
Leucine	1.02	1.04	1.01	1.03	1.01	1.03	0.98	1.00			1.01	1.01	1.02	0.99
Tyrosine	0.81	0.94	0.80	0.94	0.71	0.93	0.66	0.94	0.78	0.94	0.61	0.94	0.80	0.93
Arginine	1.99	1.96	1.01	0.99			1.92	1.89	1.92	1.94	1.01	0.99	1.04	1.04

[a] ND = not determined as it eluted in two peaks.

[b] Calculated using the extinction coefficient of arginine.

from Grand Island Biological Company (Scotland). HT 29 cells (11) (obtained from Dr. A. Zweibaum, INSERM, Paris, France) were routinely grown in 75-cm^2 plastic culture flasks in 20 ml Dulbecco's modified Eagle's medium with 10% fetal calf serum in the presence of 100 μU/ml penicillin and streptomycin in air with 5% CO_2 at 37°C. Cells in confluent monolayer cultures were passaged once a week as follows: cells were harvested by incubation for 3 min at 37°C with 0.05% trypsin, 0.5 mM EDTA in 0.13 M PBS, pH 7.2, and washed three times with culture medium before seeding. For neurotensin binding experiments, cells were used between the 5th and 15th weekly passages; within these limits, the passage number of the culture did not appear to affect neurotensin binding. Cells were seeded 2 days in advance at a density of 15 to 20 × 10^6 cells/75-cm^2 flask in 20 ml of medium; this yielded 30 to 40 × 10^6 cells/flask on the day of the binding assay. Preliminary experiments indicated that the specific binding of [^{3}H]neurotensin to cells that were detached from the monolayer by treatment with the trypsin solution for periods up to 45 min was not affected when compared to that of cells resuspended by incubation with a trypsin-free solution (0.5 mM EDTA in Ca^{2+}- and Mg^{2+}-free PBS, pH 7.2) (not shown). Therefore, for all binding experiments reported herein, HT 29 cells were harvested by a 3-min incubation at 37°C with the trypsin solution, collected by centrifugation, washed three times, and resuspended in a Krebs–Ringer Hepes solution (KRH) (mM: 114 NaCl; 4 KCl; 2.5 $CaCl_2$; 1.2 KH_2PO_4; 1.2 $MgSO_4$; 20 Hepes), pH 7.4, containing 1% bovine serum albumin (BSA) (Fraction V, Sigma). Cell number was determined by counting the cell suspension in a Nageotte chamber. Cell viability was estimated by trypan blue exclusion and was greater than 95%.

Binding of [^{3}H]neurotensin to HT 29 cells. For kinetic studies of association, incubations were carried out at 24°C in a final volume of 250 μl of KRH (1% BSA) with cells and [^{3}H]neurotensin as indicated. At each time point, bound neurotensin was separated from free by filtration on Millipore filter (EAWP 1 μm) as follows: the 250-μl incubation mixture was diluted in 1 ml of chilled KRH (1% BSA) and immediately filtered. The tube and filter were washed with a total of 4 ml of chilled KRH (1% BSA). The whole filtration procedure did not exceed 15 s. Filters where then placed in counting vials that contained 5 ml of scintillation liquid (Unisolve 1, Koch–Light Laboratories) and counted for radioactivity in a liquid scintillation counter.

For kinetic studies of dissociation, cells were incubated with [^{3}H]neurotensin at 24°C. After 20 min of incubation, cells were rapidly collected by centrifugation, and the cell pellet was resuspended with chilled KRH (1% BSA) in a volume equal to the initial incubation volume. The specific binding of [^{3}H]neurotensin at time 0 of dissociation was measured on 250-μl-aliquot portions of the cell suspension using the filtration procedure described above. The cell suspension was then diluted with KRH (1% BSA) at 24°C in a final volume equivalent to 20 times the initial incubation volume, and dissociation was allowed to proceed at 24°C. At the times indicated, 5-ml-aliquot portions of the diluted cell suspension were filtered as described above and filters were counted for radioactivity.

For equilibrium studies, cells were incubated for 30 min at 24°C in a final volume of 250 μl of KRH (1% BSA) with [^{3}H]neurotensin at the concentrations indicated. At the end of the incubation period, incubation mixtures were centrifuged for 90 s at 1000*g* in 1.5-ml conical tubes containing 1 ml of chilled KRH (1% BSA). The supernatants were discarded and the pellets were washed once with 1 ml of chilled KRH (1% BSA); the pellets were then resuspended in 100 μl of water, dissolved in 1.2 ml of scintillation liquid (Unisolve 1), and counted for radioactivity.

TABLE 3

Integrity of [^{3}H]neurotensin after exposure to HT 29 cells

Labeled neurotensin that had been exposed or not to HT 29 cells was tested for its ability to bind to fresh HT 29 cells or to rat brain synaptic membranes as described under Materials and Methods. In both binding assays the final concentration of [^{3}H]neurotensin (either exposed or not exposed) was 0.7 nM. Each value is the mean ± SE of triplicate determinations.

[^{3}H]Neurotensin	Specific binding	
	To HT 29 cells (fmol/10^6 cells)	To synaptic membranes (fmol/mg protein)
Not exposed to cells	16.4 ± 0.2	7.8 ± 0.4
Exposed to cells	15.0 ± 0.6	7.2 ± 0.2

Preliminary experiments indicated that the binding of [^{3}H]neurotensin was a linear function of cell concentration up to 8×10^6 cells/ml, and insensitive to pH variations in the range 6.8–8 (not shown). All binding experiments were done with 3×10^6 cells/ml at pH 7.4. The degradation of [^{3}H]neurotensin following exposure to HT 29 cells was measured in the incubation medium as follows: 1.5 nM [^{3}H]neurotensin was incubated in KRH (1% BSA) for 30 min at 24°C in the absence (control) or presence of 3×10^6 cells/ml (experimental). Cells were then collected by centrifugation. The integrity of [^{3}H]-neurotensin in control or experimental medium was tested by measuring the ability of the labeled peptide to rebind to fresh HT 29 cells (3×10^6 cells/ml) over 30 min at 24°C, and to bind to rat brain synaptic membranes as described below. Table 3 shows that [^{3}H]neurotensin exposed to cells had a binding activity similar to that of the control [^{3}H]neurotensin. This suggests that by these criteria the binding activity of the tritiated peptide remained virtually unaltered following exposure to HT 29 cells under the conditions of the standard binding assay (i.e., 30-min exposure to 3×10^6 cells/ml at 24°C). All data have been corrected for nonspecific binding, i.e., the amount of radioactivity bound in the presence of 1 μM unlabeled neurotensin. With [^{3}H]neurotensin at 1.4 nM, the nonspecific binding represented less than 10% of total binding. Unless indicated otherwise, the specific binding has been expressed as femtomoles of [^{3}H]neurotensin bound per 10^6 cells.

Binding assay of unlabeled neurotensin and neurotensin analogs with HT 29 cells. The constants K_i for the inhibition of [^{3}H]neurotensin binding by unlabeled neurotensin and neurotensin analogs were obtained from competition experiments in which cells (3×10^6/ml) were incubated at equilibrium (30 min, 24°C) with a fixed concentration of [^{3}H]neurotensin (1–1.5 nM) and increasing concentrations of unlabeled neurotensin or analog. Bound neurotensin was separated from free by the centrifugation method described above for equilibrium binding studies. Values of K_i were calculated from the concentration of unlabeled peptide that produces a 50% inhibition (IC_{50}) of the specific [^{3}H]neurotensin binding using the relation

$$K_i = IC_{50} \frac{K_d^*}{K_d^* + [L^*]}$$

where K_d^* is the dissociation constant and [L*] the concentration of [^{3}H]neurotensin. The binding potency relative to that of neurotensin was then calculated for each analog as (K_i NT/K_i A) × 100, where K_i NT and K_i A are the inhibition constants of unlabeled neurotensin and analog binding, respectively.

Binding assay of unlabeled neurotensin and neurotensin analogs with rat brain synaptic membranes. The properties of the binding of [^{3}H]neurotensin to rat brain synaptic membranes have been reported elsewhere (8). In this binding assay, [^{3}H]neurotensin at 1.5–2 nM was incubated for 30 min at 24°C in 250 μl of 50 mM Tris–HCl, pH 7.5, containing 1% BSA, 0.4 mg/ml membrane protein, and varying concentrations of unlabeled neurotensin or analog. Bound peptide was separated from free by filtration on Millipore filters (EGWP 0.2 μm) (8). The inhibition constants K_i for the binding of unlabeled neurotensin and analogs and their binding potencies relative to that of neurotensin were calculated as described above for the binding assay with HT 29 cells.

Biological assay of neurotensin and neurotensin analogs. Longitudinal smooth muscle strips (4–5 cm in length) were dissected from the guinea pig ileum and set up in a 10-ml organ bath for isometric tension recording as previously described (10). After equilibration of the preparations in normal Tyrode solution, (mM: 136.8 NaCl; 2.7 KCl; 1 $MgSO_4$; 0.4 NaH_2PO_4; 11.9 $NaHCO_3$; 3.6 $CaCl_2$; 5.5 glucose) at pH 7.4, 0.1 μM neostigmine methylsulfate (Sigma) was added to the reservoir of Tyrode solution, and concentration–response curves for neurotensin and neurotensin analogs were obtained as described elsewhere (23). This bioassay takes advantage of the fact that neostigmine enhances the effectiveness of the contracting effect of neurotensin fourfold over that observed in the absence of the anticholinesterase agent, without altering the potency of the peptide (23). Each experiment consisted of full concentration–response curves for neurotensin and a maximum of three neurotensin analogs. From these curves, half-maximally effective concentrations of peptides (EC_{50}) were determined and potencies relative to that of neurotensin were calculated as (EC_{50} NT/EC_{50} A) × 100, where EC_{50} NT and EC_{50} A represent half-maximally effective concentrations of neurotensin and analog, respectively.

RESULTS

Characteristics of [^{3}H]neurotensin binding to HT 29 cells. In the following section, it is assumed that [^{3}H]-neurotensin interacts with specific binding sites on HT 29 cells according to a simple, reversible bimolecular reaction and that at equilibrium, the concentration of the reactants obeys the law of mass action.

Figure 1 shows the specific binding of [^{3}H]neurotensin to HT 29 cells at 24°C as a function of time for two [^{3}H]neurotensin concentrations, 1.5 and 3.8 nM. The specific binding increased with time and reached a plateau

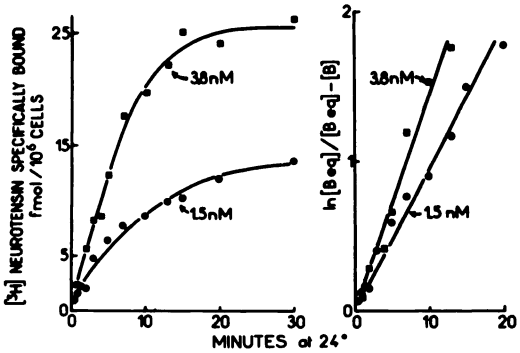

FIG. 1. *Binding of [^{3}H]neurotensin as a function of time*
Left panel: [^{3}H]Neurotensin at 1.5 or 3.8 nM was incubated with HT 29 cells (3×10^6 cells/ml) at 24°C and at the times indicated, bound was separated from free by filtration as described under Materials and Methods. Right panel: the data have been plotted according to the equation of a pseudo-first-order reaction as explained in the text. Each point is the mean of three determinations in a typical experiment.

TABLE 4

Kinetic constants of [^{3}H]neurotensin binding to HT 29 cells

Data were obtained from time courses of association of [^{3}H]neurotensin to HT 29 cells as explained in the text.

Slope (k_1[L] + k_{-1})		Constant		
[L] = 1.5 nM	[L] = 3.8 nM	k_1	k_{-1}	$K_d = k_{-1}/k_1$
0.08 min^{-1}[a]	0.14 min^{-1}[b]	2.6 × 10^7 l · mol^{-1} min^{-1}	0.04 min^{-1}	1.5 nM

[a] Mean value from three experiments.
[b] Mean value from two experiments.

by 20–30 min and 15–20 min with 1.5 and 3.8 nM [^{3}H] neurotensin, respectively (Fig. 1, left). The binding remained stable until 60 min at both ligand concentrations, and the nonspecific component did not exceed 10% of total binding throughout the experiment (not shown). Because bound ligand represented less than 5% of total ligand with both [^{3}H]neurotensin concentrations and since degradation of free ligand in the presence of HT 29 cells did not exceed 7–8% of total after a 30-min incubation (Table 3), it can be approximated that the concentration of free labeled neurotensin remained constant over the time course of association. Accordingly, the data can be analyzed as a pseudo-first-order reaction by the equation

$$\ln \frac{[B_{eq}]}{[B_{eq}] - [B]} = (k_1[L] + k_{-1})t,$$

where $[B_{eq}]$ is the concentration of bound [^{3}H]neurotensin at equilibrium, [B] is the concentration of bound peptide at a given time t, [L] is the concentration of free labeled ligand, k_1 is the rate constant of association, and k_{-1} is the rate constant of dissociation. When $\ln([B_{eq}]/[B_{eq}] - [B])$ was plotted as function of time (Fig. 1, right), straight lines were obtained. The slope values derived from several time courses of association are shown in Table 4 together with values of k_1, k_{-1}, and $K_d = k_{-1}/k_1$.

Figure 2 represents a time course of dissociation of [^{3}H]neurotensin from HT 29 cells. Results have been plotted according to the equation $\ln([B]/[B_0]) = -k_{-1}t$, in which $[B_0]$ is the concentration of bound peptide at time 0 of dissociation. A straight line was obtained, indicating that the dissociation of [^{3}H]neurotensin is a first-order process during at least the first 45 min of the study (Fig. 2). However, after 60 min, only 60–65% of the ligand had dissociated, and during the following 60 min only 10–20% further dissociated (not shown), suggesting that some irreversible process was occurring after a prolonged incubation of the peptide with HT 29 cells. Therefore, only the initial 45 min of the dissociation experiments were taken into account for the calculation of k_{-1}. A value of 0.023 min^{-1} (mean from three experiments) was found for k_{-1}, which compares reasonably well with the value of 0.04 min^{-1} derived from association experiments (Table 4).

At equilibrium, the specific binding of [^{3}H]neurotensin to HT 29 cells as a function of increasing concentrations of labeled ligand is illustrated by Fig. 3. This binding was saturable. A Scatchard analysis of the data yielded a straight line, indicating that neurotensin binds to a single class of binding sites without cooperative interaction (Fig. 3, right). A total binding capacity of 37 fmol/10^6 cells, corresponding to 22,000 neurotensin binding sites per cell, and a K_d of 1.4 nM (mean from four experiments) were obtained from equilibrium binding data. It should be noted that this K_d value (1.4 nM) agrees remarkably well with that (1.5 nM) derived from association experiments (Table 4).

The binding of [^{3}H]neurotensin to HT 29 cells was not affected by a large excess (micromolar concentration) of unlabeled biologically active peptides unrelated to neurotensin (Table 5) some of which (insulin and epidermal growth factor) have been shown to specifically bind to this cell line (13, 14). In contrast, unlabeled neurotensin at 1 μM inhibited the binding of labeled neurotensin by 95% (Table 5).

Thus, the data indicate that [^{3}H]neurotensin binds to HT 29 cells with a high affinity in a specific, reversible, and saturable manner. The characteristics of the interaction of labeled neurotensin with HT 29 cells appear to be those of a simple, bimolecular reaction as suggested by the linearity of the Scatchard plot and the good agreement between the kinetic and equilibrium determinations of the K_d value. In addition, the parameters of [^{3}H]neurotensin binding in HT 29 cells are very similar to those of [^{3}H]neurotensin binding in rat brain synaptic membranes (8).

Comparison of the effects of neurotensin and neurotensin analogs in binding and biological assays. The competitive inhibition of the binding of [^{3}H]neurotensin to HT 29 cells by increasing concentrations of unlabeled neurotensin is shown in Fig. 4A. From this inhibition

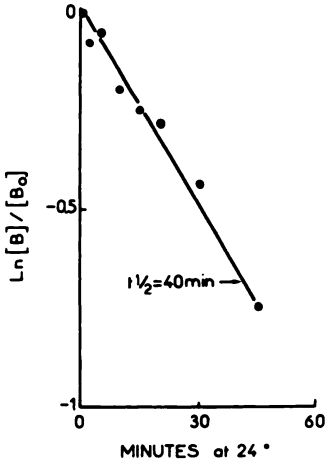

FIG. 2. *Time course of dissociation of [^{3}H]neurotensin from HT 29 cells*

Dissociation was induced by a 20-fold dilution of HT 29 cells (3 × 10^6 cells/ml) that had previously been exposed to 3 nM [^{3}H]neurotensin for 20 min. The nonspecific binding, which has been subtracted from each experimental point, was determined throughout in a simultaneous experiment where unlabeled neurotensin (1 μM) was added to the incubation medium at the beginning of the association period. At the times indicated, [^{3}H]neurotensin that remained bound to cells was measured as described under Materials and Methods. Results have been plotted according to the equation of a first-order reaction as explained in the text. Each point is the mean of three determinations in a typical experiment.

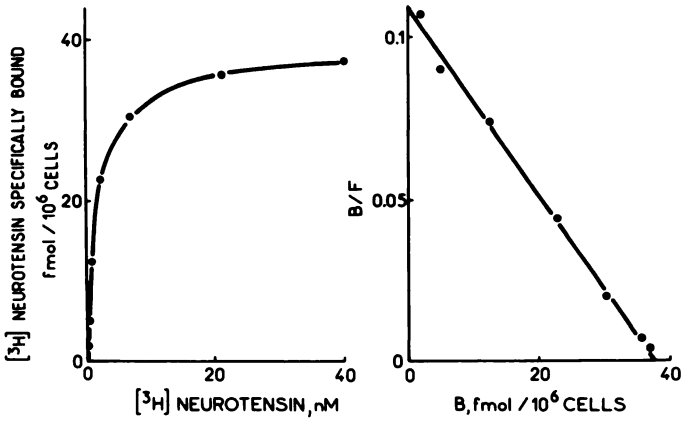

FIG. 3. *Binding of [^{3}H]neurotensin to HT 29 cells as a function of increasing concentrations of labeled ligand*

Left panel: the specific binding was measured after a 30-min incubation of HT 29 cells (3×10^6 cells/ml) at 24°C as described under Materials and Methods. Right panel: the data have been plotted according to Scatchard analysis. Each point is the mean of triplicate determinations in a typical experiment.

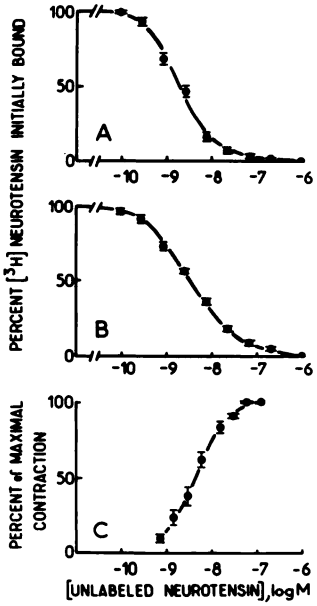

FIG. 4. *Competitive binding assays and bioassay of unlabeled neurotensin*

(A, B) Competitive inhibition of [^{3}H]neurotensin binding to HT 29 cells (A), or rat brain synaptic membranes (B), by increasing concentrations of unlabeled neurotensin. Binding assay conditions were as described under Materials and Methods. Binding is expressed as the percentage of initial binding of [^{3}H]neurotensin, i.e., the binding of [^{3}H]neurotensin in the absence of unlabeled neurotensin. (C) Concentration–response curve for the contraction of the longitudinal smooth muscle strip of the guinea pig ileum by neurotensin. Bioassay conditions were as described under Materials and Methods. Results are expressed as the percentage of maximal contraction induced by neurotensin. For all three assays, each point is the mean ± SE of seven separated experiments.

curve and the corresponding IC_{50} value, the K_i for the binding of unlabeled neurotensin to HT 29 cells was calculated (as indicated under Materials and Methods) as 1.2 ± 0.2 nM (Table 6). This value is very close to the K_d value (1.4 nM) obtained for [^{3}H]neurotensin as expected if unlabeled neurotensin has the same biological activity as the tritiated peptide (8). The competitive inhibition curve of [^{3}H]neurotensin binding to rat brain synaptic membranes by unlabeled neurotensin and the concentration–response curve for the contracting effect of neurotensin in longitudinal smooth muscle strips of the guinea pig ileum are shown in Figs. 4B and C, respectively. The concentration dependencies for both neurotensin binding to brain synaptic membranes and neurotensin effect on intestinal smooth muscle and the corresponding K_i and EC_{50} values were very similar to the concentration dependency and the K_i for neurotensin binding to HT 29 cells (Fig. 4 and Table 6).

Competitive inhibition curves of [^{3}H]neurotensin binding to HT 29 cells and brain synaptic membranes and concentration–response curves for the concentration of intestinal smooth muscle were established for a variety of neurotensin analogs; the K_i, EC_{50}, and relative potencies derived are indicated in Table 6. All of those analogs with a measurable level of activity were found to produce inhibition curves parallel to that of neurotensin in the two binding assays and were as effective (i.e., produced the same maximal response) as neurotensin in the bioassay. The results in Table 6 indicate that there is a good general agreement for the K_i, EC_{50}, and potency (relative to that of neurotensin) of neurotensin analogs in the three assay systems.

The extent of this correlation was further analyzed in Fig. 5. There was a highly significant correlation between the K_i values of the peptides in the two competitive binding assays (Fig. 5A). The correlation was also highly significant when the biological potencies of the peptides were compared with their binding potencies in either of the competitive binding assays (Figs. 5B and C).

TABLE 5

Competitive inhibition of [^{3}H]neurotensin binding by unlabeled peptides

Binding is expressed as the percentage of initial binding of [^{3}H]-neurotensin, i.e., the binding of [^{3}H]neurotensin in the absence of unlabeled peptide.

Unlabeled peptide at 1 μM	Percentage initial binding of [^{3}H]neurotensin[a]
None	100
Neurotensin	4.6 ± 0.5
Insulin	105.0 ± 2.0
Glucagon	101.0 ± 2.0
Somatostatin	98.0 ± 1.0
Epidermal growth factor	83.0 ± 1.0
Substance P	96.0 ± 2.0
Bradykinin	96.0 ± 2.0

[a] Each value is the mean ± SE of triplicate determinations.

DISCUSSION

Because neurotensin is present in both the central nervous system and the gastrointestinal tract and exerts pharmacological effects on both neural and extraneural targets, it was of interest to compare the properties of the interaction of neurotensin with extraneural (HT 29 cells) and neural (rat brain synaptic membranes) binding sites. The characteristics of [^{3}H]neurotensin binding to HT 29 cells and rat brain membranes were very similar

TABLE 6

Comparison of the binding and biological activities of neurotensin and its analogs

K_i, EC_{50}, and relative potency were calculated as explained under Materials and Methods using K_d values of 1.5 nM[a] and 2 nM[b] for the binding of [^{3}H]neurotensin to HT 29 cells and rat brain synaptic membranes, respectively.

Compound[c]	Binding assay						Bioassay (smooth muscle)		
	HT 29 cells			Synaptic membranes					
	n[d]	K_i (nM)	Potency	n[d]	K_i (nM)	Potency	n[d]	EC_{50} (nM)	Potency
NT	7	1.2 ± 0.2[e]	100	13	1.9 ± 0.2[e]	100	12	5.0 ± 1.0[e]	100
D-Tyr^{3}NT	1	1.4	70	2	2	95	3	4.6	110
D-Arg^{8}NT	2	3	40	2	5.3	36	5	5.6	90
D-Arg^{9}NT	2	500	0.24	2	400	0.5	4	125	4
D-Arg^{8,9}NT	2	600	0.2	2	400	0.5	5	125	4
D-Tyr^{11}NT	2	100	1.2	2	200	1	3	280	1.8
Phe^{11}NT	3	30	4	2	10	19	4	15	33
D-Phe^{11}NT	1	>4000	<0.05	2	2000	0.1	3	2500	0.2
D-Leu^{11}NT	1	>4000	<0.05	2	>4000	<0.05	2	>5000	<0.1
D-Leu^{13}NT	2	400	0.3	2	500	0.4	3	455	1.1
NT 8–13	3	1	120	4	13	15	3	25	20
NT 9–13	2	90	1.3	2	200	1	3	170	3
NT 10–13	2	>4000	<0.05	2	>4000	<0.05	2	>5000	<0.1
NT 1–12	2	>4000	<0.05	2	>4000	<0.05	2	>5000	<0.1
Ala^{12}NT 8–13	2	300	0.4	2	>2000	<0.1	2	>5000	<0.1
Ala^{13}NT 8–13	2	400	0.3	2	>2000	<0.1	2	>5000	<0.1
Ac-NT 8–13	2	1	120	3	1.1	170	3	5.6	90
Ac-Cit^{8}NT 8–13	2	27	4.4	3	90	2.1	3	55	9
Ac-Cit^{9}NT 8–13	2	20	6	3	85	2.3	3	30	17

[a] Value derived from kinetic and equilibrium studies as explained in the text.

[b] Value obtained from Kitabgi *et al.* (8).

[c] Abbreviations as explained in Table 1.

[d] Number of experiments.

[e] Mean ± SE of n determinations.

with regard to both kinetic parameters and specificity, as evaluated by the ability of unlabeled neurotensin and 18 synthetic analogs to compete with [^{3}H]neurotensin binding.

The present work was also designed to evaluate the pharmacological relevance of neural and extraneural neurotensin binding sites by comparing for neurotensin and neurotensin analogs the biological activity *in vitro* with the binding activity in the two binding assays. For this purpose, we have taken advantage of the fact that neostigmine enhances the magnitude of the contracting effect of neurotensin on intestinal smooth muscle (23), thereby allowing a convenient *in vitro* bioassay. A highly significant correlation was found between the biological potency of the peptides in intestinal smooth muscle and their potency to inhibit [^{3}H]neurotensin binding[2] in either the HT 29 cell or the brain membrane-binding assay. These findings indicate that neurotensin binding sites in HT 29 cells have properties similar to those of receptors involved in the pharmacological response to the peptide. Thus, HT 29 cells represent a valuable pharmacological tool for studying neurotensin interactions with extraneural binding sites.

Our results differ in several respects from those reported by Lazarus *et al.* (9, 25) for the binding of ^{125}I-neurotensin to mast cells, taken as a source for the study of extraneural neurotensin receptors. Thus, these authors have reported a K_d value of 154 nM for the binding of neurotensin to mast cells, which is two orders of magnitude above the K_d values found in the present study and well above the range of neurotensin concentrations that are biologically effective *in vitro* ((10, 15, 18–23, 26, 27), present study). Also, the binding potencies of several neurotensin analogs (D-Arg^{8}NT, D-Arg^{9}NT, D-Tyr^{11}NT, D-Phe^{11}NT, D-Leu^{13}NT, and NT 9–13) in the mast cell-binding assay (25) markedly differed from those observed, in the present study, with both the neural (rat brain membranes) and extraneural (HT 29 cell line) binding assays as well as with the *in vitro* bioassay (intestinal smooth muscle). Finally, bradykinin (a peptide structurally not related to neurotensin) was on a molar basis equipotent to neurotensin in inhibiting ^{125}I-neurotensin binding to mast cells (25) whereas it did not affect [^{3}H]neurotensin binding to HT 29 cells. It is presently unknown whether these discrepancies are related to methodological differences, such as the use of ^{125}I-neurotensin (9) instead of [^{3}H]neurotensin (present study), or whether neurotensin binding to mast cells occurs through a receptor which is different from that involved in binding to neural and extraneural preparations and in biological activity on intestinal smooth muscle, as documented in the present study.

It should be noted that the biological potency of several neurotensin analogs markedly differed when measured *in vitro* or *in vivo*. Particularly, the potencies (relative to neurotensin taken as 100) of analogs D-Tyr^{11}NT, D-

[2] In the remaining part of the discussion, the potency of unlabeled neurotensin and analogs to inhibit [^{3}H]neurotensin binding will be referred to as their binding potency.

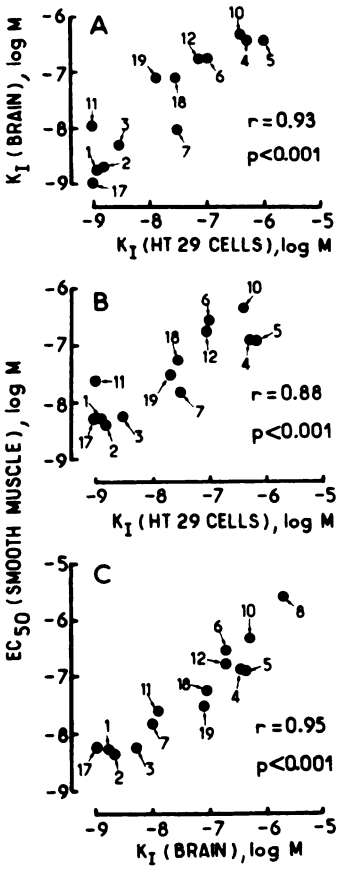

FIG. 5. *Correlation between the binding affinity and the biological potency of neurotensin and neurotensin analogs*

(A) Correlation between the K_i of binding of neurotensin and analogs to HT 29 cells and to rat brain synaptic membranes. (B) Correlation between the K_i of binding of neurotensin and analogs to HT 29 cells and the EC_{50} of the peptides in eliciting contractions of the longitudinal smooth muscle of the guinea pig ileum. (C) Correlation between the K_i of binding of neurotensin and analogs to rat brain synaptic membranes and the EC_{50} of the peptides in eliciting smooth muscle contraction. K_i and EC_{50} values have been taken from Table 6. The number affected to each point refers to compounds listed in Table 1. Only those peptides with measurable K_i and EC_{50} have been included in this figure.

Phe[11]NT, and D-Leu[11]NT were 1000, 1000, and 30, respectively,[3] when measured *in vivo*, as compared to 1.8, 0.2, and 0.1 when measured *in vitro* (see Table 6). Such large discrepancies might be explained by an enhanced resistance of these analogs to enzymatic degradation leading to a prolonged action *in vivo*, as suggested by Lazarus *et al.* (25) and by Rivier *et al.* (16), and this would suggest that an inactivation process of neurotensin involving tyrosyl residue 11 is occurring *in vivo*. Greater relative potencies were also found for analogs D-Arg[9]NT and NT 9–13 *in vivo* (50 and 20, respectively[3]) than *in vitro* (4 and 3, respectively; see Table 6). These examples point to the difficulties encountered when comparing drug activities *in vitro* and *in vivo*. In particular, such factors as distribution in extracellular fluids, diffusion and access to receptor sites, and inactivation are likely to differ *in vitro* and *in vivo*.

From the data shown in Table 6 it is clear that the smallest neurotensin partial sequence which behaves as a full agonist is the NT 9–13 pentapeptide. Indeed, the NT 10–13 tetrapeptide has no detectable activity whereas NT 9–13 has 3% of the biological potency of neurotensin. The addition of Arg[8] yields the NT 8–13 hexapeptide which is as potent as neurotensin in binding to HT 29 cells and has 15–20% of the potency of neurotensin in binding to brain membranes[4] and in eliciting smooth muscle contraction. It is of interest that the acetylation of NT 8–13 produces a peptide (Ac-NT 8–13) which is as potent as neurotensin in all three assay systems. These observations could be explained by the presence in brain membranes and intestinal smooth muscle, but not in HT 29 cells, of an aminopeptidase that would partially degrade NT 8–13. Whatever the explanation, these results indicate that the COOH-terminal sequence 8–13 of neurotensin satisfies the structural requirements for the full potency and effectiveness of the peptide *in vitro*. The role of the two arginyl residues (8 and 9) was investigated with regard to both the conformation and the charge of these residues. The results in Table 6 indicate that the positive charge on both residues is important for binding and biological activity as demonstrated by the reduced potency of the analogs Ac-Cit[8]NT 8–13 and Ac-Cit[9]NT 8–13; citrulline is sterically equivalent to arginine, without a positive charge on the side chain. In addition, the L-configuration of residue Arg[9] is required as indicated by the markedly decreased potency of analogs D-Arg[9]NT and D-Arg[8,9]NT, in contrast to D-Arg[8]NT which retains almost full binding and biological potency *in vitro* (40–90%). Thus, according to the criterium of configuration Arg[9] appears to play a greater role than Arg[8] for the recognition of neurotensin by its receptors.

The results with analogs bearing modifications on residue 11 point to the important role played by Tyr[11]. The low potency of D-Tyr[11]NT (1–2%), the virtual lack of activity of D-Phe[11]NT and D-Leu[11]NT, and, in contrast, the substantial potency of Phe[11]NT (20–30%) indicate that an aromatic residue in the L-configuration is required at position 11 of the neurotensin molecule for both receptor binding and biological activity, as previously reported by Rivier *et al.* (25). In addition, the decreased potency of Phe[11]NT suggests that the hydroxyl group of Tyr[11] may be involved in the interaction of neurotensin with its receptor.

The presence of a leucyl residue in the L-configuration at the C-terminus is necessary for the activity of neurotensin as evidenced by the very weak potency of D-Leu[13]NT (0.3–1%) and the lack of activity of NT 1–12. Finally, the side-chain methyl groups of Leu[13] and Ile[12] are strongly involved in the recognition (possibly through hydrophobic interactions) of neurotensin by its receptor, as indicated by the barely detectable activity of Ala[12]NT

[3] Values taken from Rivier *et al.* (16); the biological activity of the peptides was measured as their ability to induce hypothermia in cold-exposed rats 60 min after intracisternal administration.

[4] We have previously reported a potency of NT 8–13 greater than that of neurotensin (8). Using two other preparations of NT 8–13 from two different sources, we have not confirmed this earlier finding in the present study.

8–13 and Ala[13]NT 8–13 compared to the activity of NT 8–13.

The two binding assays and the biological assay used in this study appear to represent valuable pharmacological tools for investigating the structural requirements for neurotensin interaction with its biologically important receptor. These systems should help in designing and evaluating new synthetic neurotensin analogs with superagonist or antagonist properties. Such analogs would be of considerable interest for investigating the physiological role and mechanisms of action of neurotensin.

ACKNOWLEDGMENTS

We would like to thank Dr. A. Zweibaum for kindly supplying us with HT 29 cells. We also wish to thank G. Visciano for expert technical assistance and J. Duch for excellent secretarial assistance.

REFERENCES

1. Carraway, R., and S. E. Leeman. The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. *J. Biol. Chem.* **248:** 6854–6861 (1973).
2. Kitabgi, P., R. Carraway and S. E. Leeman. Isolation of a tridecapeptide from bovine intestinal tissue and its partial characterization as neurotensin. *J. Biol. Chem.* **251:** 7053–7058 (1976).
3. Carraway, R., and S. E. Leeman. The amino acid sequence of a hypothalamic peptide, neurotensin. *J. Biol. Chem.* **250:** 1907–1911 (1975).
4. Carraway, R., P. Kitabgi and S. E. Leeman. The amino acid sequence of radioimmunoassayable neurotensin from bovine intestine. Identity to neurotensin from hypothalamus. *J. Biol. Chem.* **253:** 7996–7998 (1978).
5. Bissette, G., P. Manberg, C. B. Nemeroff and A. J. Prange, Jr. Neurotensin, a biologically active peptide. *Life Sci.* **23:** 2173–2182 (1978).
6. Uhl, G. R., J. P. Bennett and S. H. Snyder. Neurotensin, a central nervous system peptide: apparent receptor binding in brain membranes. *Brain Res.* **130,** 299–313 (1977).
7. Lazarus, L. H., M. R. Brown and M. H. Perrin. Distribution localization and characteristics of neurotensin binding sites in the rat brain. *Neuropharmacology* **16,** 625–629 (1977).
8. Kitabgi, P., R. Carraway, J. Van Rietschoten, C. Granier, J. L. Morgat, A. Menez, S. Leeman and P. Freychet. Neurotensin: specific binding to synaptic membranes from rat brain. *Proc. Natl. Acad. Sci. U. S. A.* **74,** 1846–1850, (1977).
9. Lazarus, L. H., M. H. Perrin and M. R. Brown. Mast cell binding of neurotensin. I. Iodination of neurotensin and characterization of the interaction of neurotensin with mast cell receptor sites. *J. Biol. Chem.* **252:** 7174–7179 (1977).
10. Kitabgi, P., and P. Freychet. Neurotensin: contractile activity, specific binding, and lack of effect on cyclic nucleotides in intestinal smooth muscle. *Eur. J. Pharmacol.* **55:** 35–42 (1979).
11. Fogh, J. and G. Trempe. New human tumor cell lines, in *Human Tumor Cells in Vitro* (J. Fogh, ed.). Plenum, New York, 115–141, 1975.
12. Laburthe, M., M. Rousset, C. Boissard, G. Chevalier, A. Zweibaum and G. Rosselin. Vasoactive intestinal peptide: A potent stimulator of adenosine 3′: 5′-cyclic monophosphate accumulation in gut carcinoma cell lines in culture. *Proc. Natl. Acad. Sci. U.S.A.* **75:** 2772–2775 (1978).
13. Forgue-Lafitte, M. E., A. Horvat and G. Rosselin. Insulin binding by a cell line (HT 29) derived from human colonic cancer. *Mol. Cell. Endocrinol.* **14:** 123–130 (1979).
14. Kitabgi, P., C. Poustis, A. Zweibaum and P. Freychet. Peptide receptors in colonic tumor cells: specific binding of epidermal growth factor to the HT 29 cell line. in *Hormone Receptors in Digestion and Nutrition* (G. Rosselin, P. Fromageot and S. Bonfils, eds.). North-Holland, Amsterdam, 255–260, 1979.
15. Carraway, R., and S. E. Leeman. Structural requirements for the biological activity of neurotensin, a new vasoactive peptide, in *Peptides: Chemistry, Structure and Biology* (R. Walter and J. Meienhofer, eds.). Ann Arbor Science, Ann Arbor, Michigan 679–685, 1975.
16. Rivier, J. E., L. H. Lazarus, M. H. Perrin and M. R. Brown. Neurotensin analogues. Structure-activity relationships. *J. Med. Chem.* **20:** 1409–1412 (1977).
17. Loosen, P. T., C. B. Nemeroff, G. Bissette, G. B. Burnett, A. J. Prange, Jr. and M. A. Lipton. Neurotensin-induced hypothermia in the rat: structure-activity studies. *Neuropharmacology* **17:** 109–113 (1978).
18. Rökaeus, Å., E. Burcher, D. Chang, K. Folkers and S. Rosell. Actions of neurotensin and (Gln[4])-neurotensin on isolated tissues. *Acta Pharmacol. Toxicol.* **41:** 141–147 (1977).
19. Segawa, T., M. Hosokawa, K. Kitagawa and H. Yajima. Contractile activity of synthetic neurotensin and related polypeptides on guinea-pig ileum. *J. Pharm. Pharmacol.* **29:** 57–58 (1977).
20. Kitabgi, P., and P. Freychet. Effects of neurotensin on isolated intestinal smooth muscles. *Eur. J. Pharmacol.* **50,** 349–357 (1978).
21. Quirion, R., F. Rioux and D. Regoli. Chronotropic and inotropic effects of neurotensin on spontaneously beating auricles. *Can. J. Physiol. Pharmacol.* **56:** 671–673 (1978).
22. Quirion, R., F. Rioux, D. Regoli and S. St-Pierre. Neurotensin-induced coronary vessels constriction in perfused rat hearts. *Eur. J. Pharmacol.* **55:** 221–223 (1979).
23. Kitabgi, P., and P. Freychet. Neurotensin contracts the guinea-pig longitudinal ileal smooth muscle by inducing acetylcholine release. *Eur. J. Pharmacol.* 56: 403–406 (1979).
24. Van Rietschoten, J., C. Granier, H.. Rochat, S. Lissitzky and F. Miranda. Synthesis of Apamin, a neurotoxic peptide from bee venom. *Eur. J. Biochem.* **56:** 35–40 (1975).
25. Lazarus, L. H., M. H. Perrin, M. R. Brown and J. E. Rivier. Mast cell binding of neurotensin. II. Molecular conformation of neurotensin involved in the stereospecific binding to mast cell receptor sites. *J. Biol. Chem.* **252:** 7180–7183 (1977).
26. Kitabgi, P., G. Hamon and M. Worcel. Electrophysiological study of the action of neurotensin on the smooth muscle of the guinea-pig taenia coli. *Eur. J. Pharmacol.* **56:** 87–93 (1979).
27. Dolais-Kitabgi, J., P. Kitabgi, P. Brazeau and P. Freychet. Effect of neurotensin on insulin, glucagon, and somatostatin release from isolated pancreatic islets. *Endocrinology* **105:** 256–260 (1979).

Send reprint requests to: P. Kitabgi, INSERM U 145, Faculté de Médecine, Chemin de Vallombrose, 06034 Nice Cedex, France.