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Neurotensin: Specific binding to synaptic membranes from rat brain

([^{3}H]neurotensin/receptor binding/structure-activity relationships)

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ABSTRACT **The binding of neurotensin to synaptic membranes from rat brain was studied at 24° with the use of [^{3}H]-neurotensin. The binding was found to be highly specific, saturable, and reversible. Values for K_D of 2 nM and 0.9 nM were derived from equilibrium and kinetic experiments, respectively. Virtually no degradation of neurotensin was observed in the incubation medium after exposure to synaptic membranes under the conditions of the binding studies. Competitive inhibition of [^{3}H]neurotensin binding by partial sequences of neurotensin revealed that the addition of the residue arginine-8 to the neurotensin-(9-13)-pentapeptide increases about 500-fold the relative binding potency, whereas the remaining portion of the NH_2-terminal region is mainly responsible for full pharmacological potency; the COOH-terminal leucyl residue is essential for binding.**

Neurotensin is a tridecapeptide: <Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu (1) that has recently been isolated from hypothalamus (2) and small intestine (3), and exhibits several pharmacological properties (1, 4). This peptide is widely distributed throughout rat brain (5). Its physiological function is as yet unknown, as is the case for other peptides that are present both in the central nervous system and the gastrointestinal tract, such as vasoactive intestinal polypeptide (6), somatostatin (7), and substance P (8, 9). There is some evidence, however, that substance P may serve as a neurotransmitter or modulator.

The data presented in this report indicate that neurotensin binds to synaptic membranes from rat brain. The properties of this binding strongly suggest the presence of biologically important synaptic receptors for neurotensin and thus a possible function, for this peptide, at the synaptic level.

MATERIALS AND METHODS

Neurotensin and Partial Sequences. Synthetic neurotensin was purchased from Beckman, Bioproducts Department (Geneva, Switzerland). The COOH-terminal partial sequences of neurotensin, i.e., the (4-13)-decapeptide, the (6-13)-octapeptide, the (8-13)-hexapeptide, the (9-13)-pentapeptide, and the (10-13)-tetrapeptide were synthesized according to the Merrifield solid phase procedure (10), and the products were purified by chromatography on Sephadex and preparative high-voltage paper electrophoresis (2). These peptides were the same as those used in the pharmacological studies reported elsewhere (4). The neurotensin-(1-12)-dodecapeptide was synthesized by the solid phase procedure of Merrifield (10) using the methodology described for the synthesis of apamin (11). After hydrofluoric acid cleavage, the crude peptide was purified successively by gel filtration on Sephadex G-15, ion exchange chromatography on carboxymethyl-cellulose (CM 52, Whatman), and by desalting on Bio-Gel P-4. The homogeneity of the peptide was assessed by amino acid analysis after acid hydrolysis: $Asp_{1.0}$, $Glu_{2.0}$, $Pro_{2.1}$, $Ile_{1.0}$, $Leu_{1.0}$, $Tyr_{1.6}$, $Lys_{1.0}$, $Arg_{2.0}$, and by high voltage paper electrophoresis at pH 6.5. The somewhat low value obtained for tyrosine is probably the result of degradation during acid hydrolysis and could have been avoided by the addition of phenol. The neurotensin-(1-10)-decapeptide was obtained as follows: 500 μg of synthetic neurotensin was treated with carboxypeptidase A (10%, wt/wt) in 0.2 M ammonium bicarbonate at 37° for 200 min. Amino acid analysis of an aliquot portion of the mixture showed that Leu, Ile, and Tyr were released in equimolar amounts. The neurotensin-(1-10)-decapeptide was purified on Bio-Gel P-2. Amino acid analysis of the peptide agreed with the loss of the three COOH-terminal amino acids of neurotensin.

Preparation of [^{3}H]Neurotensin. [^{3}H]Neurotensin was obtained by substituting tritium for iodine in molecules of triiodoneurotensin. Purification of iodoneurotensin and UV tritiation were carried out as previously described (12). Substitution of iodine by tritium led to [^{3}H]neurotensin with a specific radioactivity of 77 Ci/mmol, a value close to that expected (87 Ci/mmol) for the introduction of three tritium atoms per peptide. The titration pK of the tyrosyl residues in [^{3}H]neurotensin was identical to that observed with synthetic unlabeled neurotensin. Neurotensin and [^{3}H]neurotensin exhibited the same chromatographic and electrophoretic properties. [^{3}H]-Neurotensin was found to be as potent as native neurotensin in eliciting hyperglycemia when injected intravenously in rats. A single batch of [^{3}H]neurotensin was used in the studies reported here.

Preparation of Synaptic Membranes. Male Wistar rats (200–220 g) were decapitated. Hypothalami, thalami, and brain stems were rapidly dissected out, yielding a total of *ca* 600 mg of nervous tissue per rat. Synaptic membranes from these combined parts of the brain were prepared following the procedure of Jones and Matus (13). By this method, a fraction highly enriched in synaptic plasma membranes is recovered from the interface of a two-step sucrose density gradient on which a hypotonically lysed crude mitochondrial fraction has been separated by simultaneous sedimentation and flotation centrifugation. Tissue from twenty rats (about 12 g) yielded *ca* 30 mg of membrane protein, as assayed by the method of Lowry *et al.* (14). Membrane pellets were kept frozen at −80° with no loss of binding activity over periods of time exceeding 1 month.

Binding Assay. Unless otherwise indicated, incubations were carried out for 30 min at 24° in 50 mM Tris·HCl, pH 7.5, containing 1% (wt/vol) bovine serum albumin, membrane protein at 0.4 mg/ml, and 2 nM (120,000 cpm/ml) [^{3}H]neurotensin. Unlabeled peptides were added at the concentrations indicated.

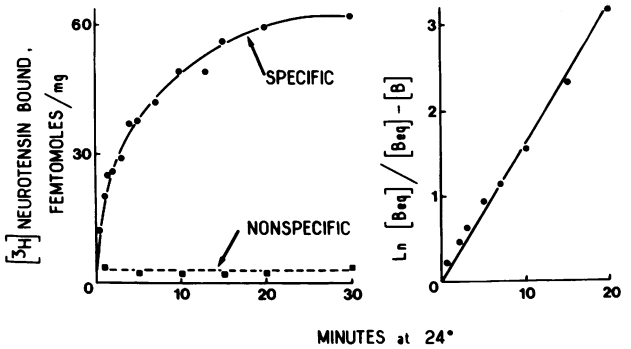

FIG. 1. Time course of binding of [^{3}H]neurotensin to synaptic membranes. (*Left*) 1.8 nM [^{3}H]neurotensin was incubated at 24° with membrane protein at 0.4 mg/ml. Specific (●) and nonspecific (■) binding were measured at the times indicated, as described in *Materials and Methods*. (*Right*) The kinetics of neurotensin binding was linearized according to the equation of a pseudo-first-order reaction as explained in the *text*. Each point is the mean of three separate experiments.

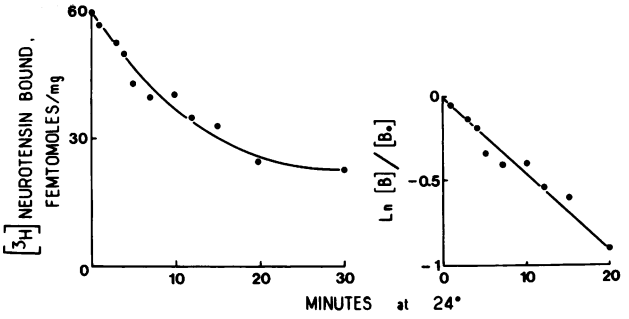

FIG. 2. Time course of dissociation of [^{3}H]neurotensin. (*Left*) 2 nM [^{3}H]neurotensin was first incubated at 24° with membrane protein at 0.4 mg/ml. After 30 min, 1 μM unlabeled peptide was added in a negligible volume (1% of incubation total volume). The [^{3}H]-neurotensin that remained specifically bound was measured by filtration at the times indicated after the addition of unlabeled neurotensin (time 0 on figure), as described in *Materials and Methods*. The nonspecific binding, which has been subtracted from each experimental point, was determined throughout in a simultaneous experiment where unlabeled peptide at 1 μM was added to the incubation medium at the beginning of the first incubation period. Each point is the mean of three separate experiments. Eighty to ninety percent of the [^{3}H]neurotensin was dissociated from membranes 120 min after addition of unlabeled neurotensin (not shown). (*Right*) dissociation was linearized according to the equation of a first-order kinetic and assuming that reassociation of [^{3}H]neurotensin with synaptic membranes was inhibited by the excess of unlabeled neurotensin.

Bound neurotensin was separated from free by filtration on a Millipore filter (EGWP 02500, 0.2 μm) as follows: 250 μl of incubation mixture was diluted in 1 ml of chilled incubation buffer and immediately filtered. The tube and filter were washed with a total of 4 ml of chilled buffer. Before use, the filters were soaked in buffer for 2 hr at 4°. The filtration procedure, including the washing step, did not exceed 30 s. The filters were then placed in counting vials that contained 6 ml of scintillation liquid (Unisolve 1, Koch-Light Laboratories) and counted in a Packard scintillation counter at a ^{3}H counting efficiency of 37%. In these conditions, the radioactivity recovered on the washed filter in the absence of membranes was less than 0.05% of the total and was subtracted from the data. The specific [^{3}H]neurotensin binding was obtained by subtracting, from the total binding, the nonspecific binding defined as the amount of radioactivity bound in the presence of a large excess (1 μM) of unlabeled neurotensin. Duplicate or triplicate determinations of [^{3}H]neurotensin binding varied by less than 10% of the mean value. Unless indicated otherwise, binding is expressed as the amount of neurotensin specifically bound per mg of membrane protein.

Degradation of Neurotensin Exposed to Synaptic Membranes. The integrity of neurotensin in the incubation medium after exposure to membranes was tested by three different methods: gel filtration, ability to rebind to synaptic membranes, and radioreceptor assay. For the first two methods, 20 nM [^{3}H]neurotensin was incubated at 24° with 0.4 mg/ml of membrane protein. After 30 min, the membranes were sedimented by centrifugation at 40,000 × *g* for 15 min at 4°; an aliquot portion of the supernatant was applied to a Sephadex G-25 (fine) column (0.9 × 30 cm) that had been equilibrated and was eluted at 4° with 50 mM Tris·HCl, pH 7.5, containing 0.5% (wt/vol) bovine serum albumin. The column had previously been calibrated with [^{3}H]neurotensin (control). In both control and experimental conditions, the radioactivity eluted from the column represented 89% of the total applied. Aliquot portions of the supernatant that contained [^{3}H]neurotensin exposed to membranes were incubated with fresh membranes so as to obtain labeled peptide at 2 nM, and this binding was compared to that of control [^{3}H]neurotensin in the usual binding assay conditions. For radioreceptor assay, unlabeled neurotensin at 100 nM was incubated at 24° with membrane protein at 0.4 mg/ml. After 30 min, membranes were sedimented as described above. Various dilutions of the supernatant were then incubated with fresh synaptic membranes (0.4 mg/ml) and 2 nM [^{3}H]neurotensin. The amount of unlabeled neurotensin was measured by comparing the inhibition of [^{3}H]neurotensin binding by these dilutions to that obtained with known concentrations of intact unlabeled peptide.

Other Materials. Synthetic substance P and somatostatin were purchased from Beckman. Insulin was a gift from the Novo Research Institute (Copenhagen, Denmark). Sephadex G-25 (fine) was purchased from Pharmacia Chemical Co. Other chemicals were of reagent grade.

RESULTS

Time Courses of Association and Dissociation. [^{3}H]Neurotensin bound specifically and reversibly to synaptic membranes (Figs. 1 and 2). At 24° and with 1.8 nM [^{3}H]neurotensin, the specific binding increased with time and reached a plateau at about 20 min (Fig. 1 *left*). The nonspecific binding remained constant throughout and did not exceed 5% of the total binding. Because bound neurotensin did not exceed 1.5% of the total [^{3}H]neurotensin at this concentration of ligand, and because virtually no degradation of the peptide was observed in the incubation medium after exposure to membranes (see below), it can be assumed that the concentration of free neurotensin in the medium remains constant with time. Therefore, the interaction of neurotensin with synaptic membranes can be analyzed as a pseudo-first-order reaction by the equation

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

in which [Beq] is the concentration of bound neurotensin at equilibrium, [B] is the concentration of bound peptide at a given time t, [L] is the concentration of ligand, k_1 is the rate constant of association, and k_{-1} is the rate constant of dissociation. When ln ([Beq]/[Beq] − [B]) was plotted as a function of time (Fig. 1 *right*), a straight line was obtained with a slope having a value of

$$1.8 \times 10^{-9} k_1 + k_{-1} = 2.5 \times 10^{-6}\ s^{-1}$$

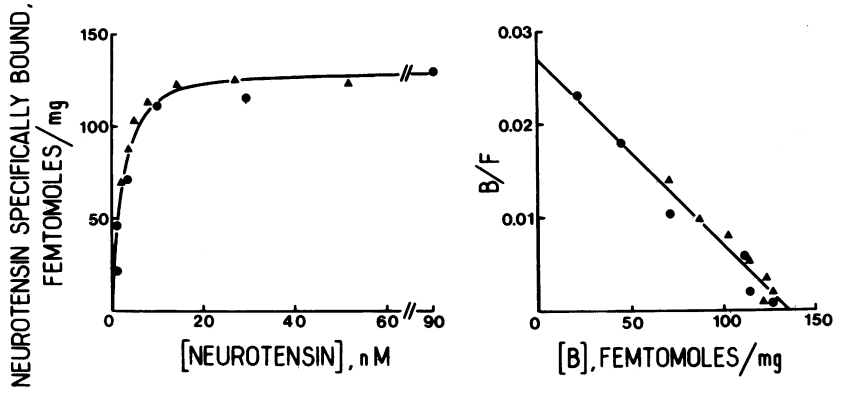

FIG. 3. Binding of neurotensin to synaptic membranes as a function of neurotensin concentration. (*Left*) The binding was measured after 30 min incubation of membranes (0.4 mg protein per ml) either with increasing concentrations of unlabeled neurotensin added to a fixed concentration (2 nM) of labeled peptide (▲), or with increasing concentrations of [^{3}H]neurotensin (●), at 24°. The concentration of neurotensin on the abscissa represents the total concentration of neurotensin in each type of experiment (labeled *plus* unlabeled neurotensin, or labeled peptide alone). The nonspecific binding has been subtracted from each point. (*Right*) The data are plotted according to Scatchard analysis: ▲, data obtained with unlabeled *plus* labeled neurotensin; ●, data obtained with labeled peptide alone. Each point is the mean of triplicate determinations in a single experiment. Similar results were obtained in five separate experiments.

The value k_{-1} was determined directly by measuring the dissociation of [^{3}H]neurotensin (Fig. 2 *left*). Dissociation was linear when plotted according to the equation ln ([B]/[Bo]) = $-k_{-1}t$, in which [Bo] is the concentration of bound peptide at time 0 (Fig. 2 *right*). The slope obtained from the dissociation data gives the rate constant of dissociation $k_{-1} = 8 \times 10^{-4}\ s^{-1}$. From the association kinetic data, the rate constant of association was calculated as $k_1 = 0.9 \times 10^6\ M^{-1}\ s^{-1}$. From the values of the rate constants, the dissociation constant was calculated as $K_D = k_{-1}/k_1 = 0.9$ nM.

Studies of Binding at Equilibrium. The concentration dependence of neurotensin binding at equilibrium was determined either by adding increasing concentrations of unlabeled neurotensin to a fixed concentration of [^{3}H]neurotensin, or in the presence of increasing concentrations of [^{3}H]neurotensin (Fig. 3 *left*). The specific binding was found to be saturable and the binding curves of [^{3}H]neurotensin alone and [^{3}H]neurotensin *plus* unlabeled peptide superimposed. Scatchard analysis of the data gave a straight line (Fig. 3 *right*), indicating that neurotensin binds to a single population of sites. The dissociation constant K_D calculated from these data has a value of 2 nM. This value of K_D agrees reasonably well with that determined from the association and dissociation rate constants (see above). A concentration of 135 fmol of binding sites per mg of membrane protein was obtained from the data shown in Fig. 3.

The specific binding of [^{3}H]neurotensin as a function of membrane protein concentration was linear up to 0.4 mg/ml (Fig. 4). The nonspecific binding did not increase significantly until protein concentration reached 0.8 mg/ml. A concentration of 0.4 mg/ml was used in the experiments reported here because it yields the highest ratio of specific binding to nonspecific binding.

Properties of Neurotensin Recovered in the Medium after Exposure to Membranes. Virtually no degradation of neurotensin was observed when the integrity of the peptide was tested in the incubation medium, after exposure to synaptic membranes under the conditions used in the binding studies. After gel filtration, 93% of the recovered radioactivity eluted as intact neurotensin after exposure to membranes, as compared to 95% for the control (unexposed) [^{3}H]neurotensin (Fig. 5). The ability of [^{3}H]neurotensin to bind to fresh membranes was only slightly lower with the peptide that had been exposed to membranes (1.10 ± 0.02% of total radioactivity) than with the control [^{3}H]neurotensin (1.25 ± 0.05% of total radioactivity). Finally, exposure of unlabeled neurotensin to synaptic membranes did not result in a significant loss of peptide as measured by radioreceptor assay (Table 1).

Competitive Inhibition of [^{3}H]Neurotensin Binding by Partial Sequences of Neurotensin. A variety of partial sequences of neurotensin were tested for their ability to inhibit the binding of [^{3}H]neurotensin to synaptic membranes (Fig. 6). With the COOH-terminal partial sequences, it was found that the (10-13)-tetrapeptide is inactive, the (9-13)-pentapeptide has about 1.5% of the potency of neurotensin, the (8-13)-hexapeptide and (4-13)-decapeptide are approximately seven times as potent as neurotensin, and the (6-13)-octapeptide is about 16 times as potent as neurotensin. Thus, the addition of the residue Arg^8 to the NH_2 terminus of the (9-13)-pentapeptide results in a considerable (500-fold) increase in binding affinity. Furthermore, the removal of up to seven residues from the NH_2 terminus of neurotensin does not result in any loss of binding activity, but rather appears to enhance the binding potency of the (4-13), (6-13), and (8-13) partial sequences. In sharp contrast, the (1-12)-dodecapeptide, which lacks the COOH-terminal leucyl residue, was found to be virtually devoid of binding ac-

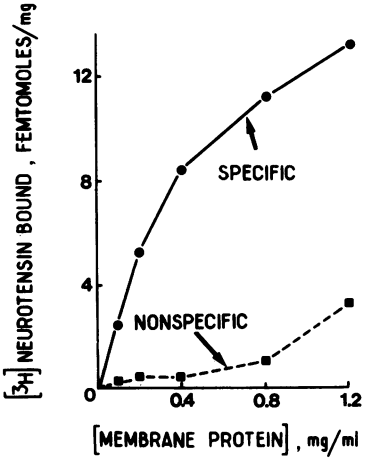

FIG. 4. Binding of [^{3}H]neurotensin (2 nM) as a function of membrane protein concentration. Specific (●) and nonspecific (■) binding were measured after 20 min incubation at 24° as described in *Materials and Methods*. The binding is expressed as the amount of [^{3}H]neurotensin bound per ml of incubation medium.

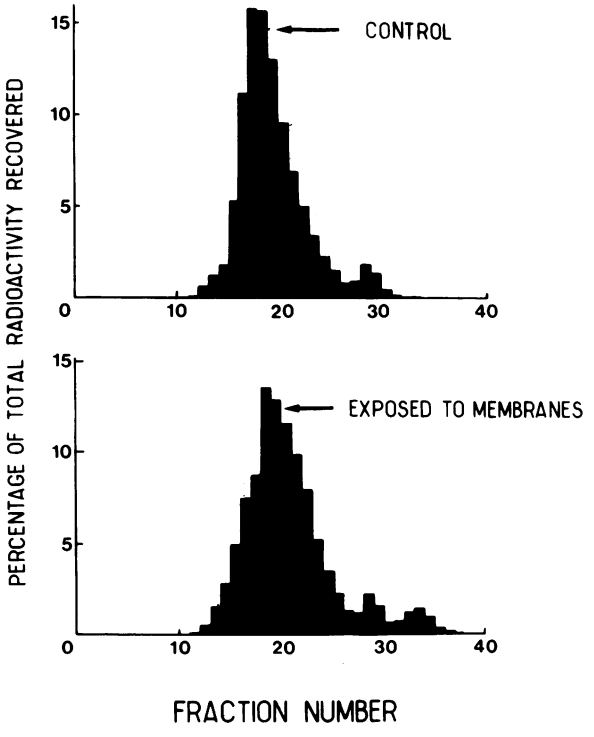

FIG. 5. Gel filtration on Sephadex G-25 (fine) of [^{3}H]neurotensin recovered in the incubation medium after exposure to synaptic membranes. (*Upper*) Elution profile of control (unexposed) [^{3}H]-neurotensin. (*Lower*) Elution profile of exposed [^{3}H]neurotensin. The percentage of total radioactivity recovered from the column is indicated for each eluted fraction (0.55 ml).

tivity, indicating the absolute requirement of the COOH-terminal residue for binding. That the COOH-terminal region of the peptide is necessary for binding is further substantiated by the complete loss of binding activity observed with the (1-10)-decapeptide (Fig. 6). High concentrations (1 μM) of substance P, somatostatin, and insulin were without effect on [^{3}H]neurotensin binding.

Table 1. Radioreceptor assay of neurotensin after exposure to synaptic membranes

Expected concentration, nM	Measured concentration, nM
0.6	0.5
1.3	1.3
2.5	2.4
5.0	4.9

Unlabeled neurotensin at 100 nM was exposed to synaptic membranes for 30 min at 24°. After centrifugation, various dilutions of the unlabeled neurotensin present in the supernatant were measured for their neurotensin content by radioreceptor assay as described in *Materials and Methods*.

DISCUSSION

The present study has shown that neurotensin binds to synaptic membranes from rat brain. Although the major part of this work was done with membranes prepared from hypothalamus, thalamus, and brain stem, a specific binding of neurotensin was also observed in synaptic membranes from cortex and cerebellum (data not shown). The binding is specific, saturable, and reversible. The nonspecific binding is a trivial portion (about 5%) of the total observed binding. The affinity of neurotensin for its binding site is high ($K_D \cong 2$ nM from equilibrium data), and the binding appears to involve only one type of site without cooperative interaction. The binding of neurotensin occurs over a range of concentrations (0.4–20 nM) that is similar to that presumably achieved in plasma when pharmacological effects are measured *in vivo* (4).

Two types of analogs can be distinguished upon examination of the relative binding potencies of partial sequences of neurotensin: (*i*) Analogs with the COOH-terminal region possess various binding potencies, depending on the length of their sequence. Thus, the data suggest that the neurotensin-(8-13)-hexapeptide Arg-Arg-Pro-Tyr-Ile-Leu represents the minimal requirement for full binding activity. In fact, it appears that the neurotensin-(4-13)-decapeptide, (6-13)-octapeptide, and

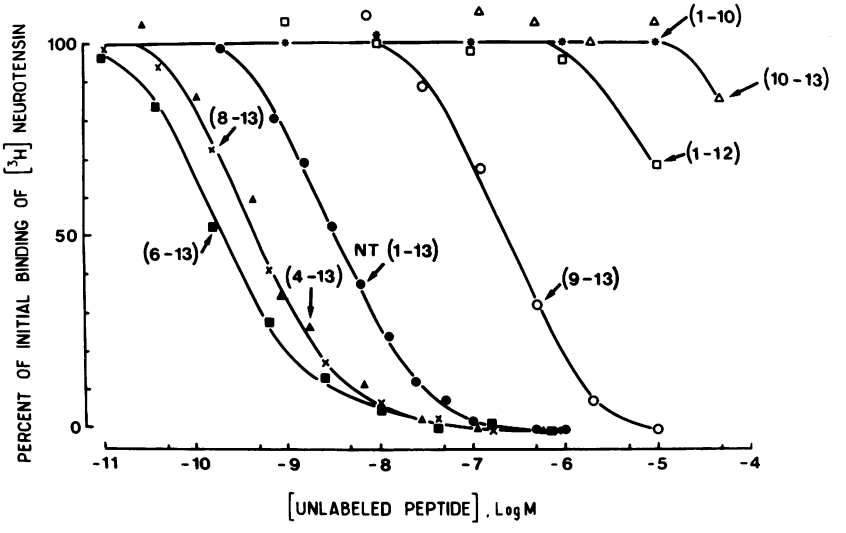

FIG. 6. Competitive inhibition of [^{3}H]neurotensin binding by unlabeled neurotensin (NT, ●) and neurotensin partial sequences: (4-13)-decapeptide (▲); (6-13)-octapeptide (■); (8-13)-hexapeptide (×); (9-13)-pentapeptide (○); (10-13)-tetrapeptide (△); (1-10)-decapeptide (✱); and (1-12)-dodecapeptide (□). Binding assay conditions were as described in *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 peptide. The nonspecific binding has been subtracted. Each point is the mean of duplicate or triplicate determinations in five separate experiments for native neurotensin, and of duplicate determinations in two separate experiments for neurotensin partial sequences.

(8-13)-hexapeptide are even more potent than native neurotensin in competing with [^{3}H]neurotensin binding to synaptic membranes. It is of interest that these relative binding potencies are greater than the corresponding pharmacological potencies *in vivo* (4). When the COOH-terminal region is reduced to five residues as in the (9-13)-pentapeptide, a dramatic decrease in binding potency is observed, indicating that Arg^8 plays an important role in binding, as anticipated from pharmacological studies *in vivo* (4). Whereas the (8-13)-hexapeptide is a prerequisite for full binding potency, the remaining portion of the native sequence, i.e., residues 1 to 7, appears to be necessary for the full expression of pharmacological activity. (*ii*) The COOH-terminal region of neurotensin is a prerequisite for binding, as evidenced by the complete loss of binding potency observed with the (1-10)-decapeptide; accordingly, this analog is also devoid of pharmacological activity. Even more striking is the almost complete loss of binding activity of the (1-12)-dodecapeptide, which points to the importance of the COOH-terminal leucyl residue in binding, and further substantiates the finding¶ that the (1-12)-dodecapeptide is pharmacologically inactive (3).

The results obtained with the partial sequences of neurotensin strongly suggest that the binding occurs at sites that are analogous to those involved in the pharmacological and/or biological effects of the peptide. The presence of such "receptor" sites for neurotensin in synaptic membranes suggests a possible role for this peptide as a transmitter or modulator of neural function. This hypothesis is supported by the finding that neurotensin is associated with the synaptosomal fraction prepared from hypothalamus (5). In this context, one might expect a process whereby the action of the peptide could be terminated, such as uptake by presynaptic nerve terminals, or degradation at, or near, the synaptic receptors. Preliminary experiments in this laboratory do not suggest an uptake of neurotensin by synaptosomes (J. P. Abita, unpublished observations). The fact that the neurotensin present in the medium was virtually not degraded after its exposure to synaptic membranes under the conditions of the binding assay does not exclude that the peptide may be degraded and inactivated after binding. Such receptor-linked degradation would certainly not have been detected in the medium, because it can be estimated that the bound neurotensin that exchanges with free neurotensin when dynamic equilibrium is reached represents less than 0.1% min^{-1} of the total peptide. Studies on the fate of membrane-bound neurotensin are required to examine this possibility.

¶ The (1-12)-dodecapeptide was isolated during the purification of intestinal neurotensin and was found to be devoid of hypotensive activity (3). Furthermore, the synthetic (1-12)-dodecapeptide that was used in the present study was devoid of hyperglycemic effect.

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