Mast Cell Binding of Neurotensin

I. IODINATION OF NEUROTENSIN AND CHARACTERIZATION OF THE INTERACTION OF NEUROTENSIN WITH MAST CELL RECEPTOR SITES

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Neurotensin was iodinated at equimolar concentrations of peptide, iodide, and chloramine-T, producing a labeled peptide with a specific activity of 1000 to 2000 Ci/mmol. Rat mast cells specifically and reversibly bound 1.27 pmol of neurotensin/10⁶ cells with a reversible affinity, Kd, of 154 nM. Optimum specific binding occurred between pH 6.8 and 7.2 under hypotonic conditions and dropped sharply as buffer concentration increased beyond 10 μM. The divalent cations Ca²⁺ and Mg²⁺ prevented binding with 50% inhibition at 1.5 and 4 mM, respectively. Binding was strongly and equally inhibited by the sodium and potassium salts of chloride, bromide, and iodide, and to a lesser degree by LiCl. Maximum binding of 125I-neurotensin occurred within 10 min at 0°, and within 1.5 to 2 min binding was reduced to half-maximum in the presence of excess unlabeled neurotensin or upon 20-fold dilution in buffer. Both CaCl₂ and NaCl were able to dissociate 60% of the total bound neurotensin; half the label bound was removed in 4 to 6 min. EDTA inhibited the binding only at high concentrations and no requirement was found for sulfhydryl groups, ATP, or a glycoprotein in the binding of neurotensin.

Neurotensin, a tridecapeptide (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH), has been isolated from the hypothalamus (1, 2) and immunoreactive neurotensin has been detected in other distinct areas of rat brain (3-5) as well as in the gastrointestinal tract (6-8). A direct central nervous system effect of neurotensin is seen in its ability to produce hypothermia and increased vasopermeability, and gut contraction, and these actions are mimicked by histamine, such as histamine, increased vasopermeability, and gut contraction, and these actions are reversed by the H₁-receptor blocker, diphenhydramine (6, 16, 18). Rat mast cells, which line the peritoneal and thoracic cavities (19), release histamine in response to the vasoactive peptides bradykinin and substance P (20). In light of these observations, it has been suggested that the extra-CNS receptors that are involved in histamine regulation with a simple cellular model we utilized mast cells in these binding studies. A preliminary report of these data has been published (10).

EXPERIMENTAL PROCEDURES

Materials — Neurotensin was synthesized by solid state methods and purified to homogeneity by procedures developed in this laboratory (21-23). Carrier-free Na¹²⁵I was purchased from Amersham/Searle, Sephadex LH-20 (swelled in methanol) from Pharmacia, Whatman glass-fiber filters (GF/C) from Reeves Angel, and disposable chromatography columns from Bio-Rad. Chloramine-T (Eastman) and sodium metabisulfitte (Fisher) were analytical reagent grade. Crystalline bovine serum albumin bacteriological grade (Fraction V) was from Nutritional Biochemical Corp. Silica gel chromatogram sheets were a product of Eastman. Mature male rats were obtained from the Charles River Co. ¹³¹I-Neurotensin was kindly prepared for us (in December, 1975) by J. L. Mordret, Centre D'Etudes Nucléaires, Gif-Sur-Yvette, France, by means of an iodide exchange reaction and had a specific activity of approximately 77 Ci/mmol.

Thin Layer Chromatography and Paper Electrophoresis — Electrophoresis was performed in Veronal buffer, pH 8.5, using a Beckman-Horváth cell at 300 V and 40 mA for 45 min at 20°. Thin layer...
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Neurotensin was iodinated using equimolar ratios of peptide, Na"I, and CT (24). To 10 μl of 0.4 mM sodium phosphate, pH 6.75, 4.9 μl of neurotensin and 1 μCi of Na"I were added and the reaction was initiated by the rapid mixing of 10 μl of CT (0.13 μg) in 0.4 M sodium phosphate buffer, pH 6.75. After 30 s at room temperature, 10 μl of sodium metabisulfite (0.35 μg; 4 molar excess) was added to terminate the reaction. Iodinated neurotensin was isolated from Sephadex LH-20 columns (10 × 0.7 cm with sintered polyethylene discs), pre-equilibrated with 5 to 10 column volumes BAW (10:2:1) and eluted by the same at a flow rate of 9 to 10 ml/h.

Isolation of Mast Cells—Mast cells were isolated from both the peritoneal and thoracic cavities of 20 to 40 guillotined and exsanguinated rats according to method of Johnson and Moran (19). The cells were washed thrice, in isotonic saline and once in 0.32 M sucrose. They were stored at 4°C in 0.32 M sucrose containing 0.02% NaN₃ at concentrations of 0.2 to 1.2 × 10⁹ cells/ml (and used without further purification). The proportion of mast cells ranged from 30 to 50% as determined by direct counting using methylene blue and eosin-hematoxylin stains for mast cells and non-mast cells, respectively.

Radioceptor Assay—The binding assay, carried out in triplicate, contained approximately 20 to 120 fmol of ¹²⁵I-neurotensin (neutralized to pH 6.5 to 7.0) of Peak I (Fig. 1), 10 μM Tris/acetate buffer, pH 7.0, 0.1 g of bovine serum albumin, 80 mM sucrose, and 1 to 6 × 10⁶ mast cells in a total volume of 100 μl. After 15 min at 0°C, the peptide/cell mixture was rapidly filtered using presoaked (2% albumin) glass fibers and washed once with 0.5 ml of cold 2% albumin within 3 to 4 s. The specific binding is the difference in counts per min between tubes containing varying amounts of unlabeled neurotensin and those containing 6 nmol of unlabeled neurotensin obtained was 1000 to 2000 Ci/mmol.

The labeled peptide was analyzed for homogeneity by thin layer chromatography and paper electrophoresis. Thin layer chromatography of ¹²⁵I-neurotensin revealed the following: (a) a single major radioactive spot from Peak I was evident which migrated ahead of neurotensin in BAW and BPyA (Fig. 2). More than one spot was usually, but not always, found in BBA; the significance of this is not known. (b) Peak II was qualitatively similar to the first in terms of the radioiodinated peptide. (c) The largest radioactive peak (III) migrated near the solvent front, ahead of Na"I.

Paper electrophoresis confirmed that most of the label in Peak I and II in bound to a molecule that remains at the origin while the negatively charged iodide migrates anodally (Fig. 3). The electrophoresis pattern for Peak III was superimposable on that of Na"I, and thus represents an unknown iodine-containing molecule that also appears to be present in the commercial Na"I solution. The amount of free non-peptide-bound radiiodide in the ¹²⁵I-neurotensin peaks averaged 5.4%.

The specific binding of ¹²⁵I-neurotensin was constant through the peak tubes of Peak I and decreased thereafter. The fraction with the highest radioactivity in Peak I was used in all the binding experiments.

pH Optimum and Buffer Concentration—Using several buffers over a pH range from 4 to 8.8, a sharp optimum in the specific binding of ¹²⁵I-neurotensin to mast cells was observed around neutrality, with good agreement at the overlapping pH values (Fig. 4A). A similar optimum pH was found for the binding of neurotensin to rat brain synaptosomes (25) and release of histamine by vasoactive peptides from the mast cells (20). Varying the concentration of Tris/acetate buffer, pH 7.0, from 10 to 150 mM, gave no true maximum; specific binding decreased precipitously with increasing buffer concentration beyond 10 mM with approximately 10 to 15% binding left at isotonicity (Fig. 4B).

Effect of Ions on Binding—In order to determine the effect of
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FIG. 2. Thin layer chromatography of \( ^{125}\text{I}\)-neurotensin. Peak fractions from the Sephadex LH-20 column in Fig. 1 were chromatographed with 50 μg of unlabeled neurotensin (dashed circles) in the three solvent systems listed under "Experimental Procedures" and exposed to x-ray film for 3 to 5 days.

FIG. 3. Paper electrophoresis of approximately 1-μl aliquots of the peak fractions from Fig. 1. Migration is toward the anode on the right. The paper strips were air-dried and cut into 0.5-cm wide sections. The vertical dashed line represents the origin. A, Fraction 4; B, Fraction 12; and C, Fraction 36. Na \( ^{125}\text{I} \) is given by the dotted line in C.

FIG. 4. A, optimal pH for the binding of \( ^{125}\text{I}\)-neurotensin to mast cells. The buffers were 25 mM, except phosphate (8.3 mM): sodium acetate (Δ), sodium phosphate (□), Tris/acetate (▲), and Tris/HCl (●). They were incubated with \( 5.8 \times 10^6 \) mast cells and 10.4 fmol of \( ^{125}\text{I}\)-neurotensin. B, the effect of buffer concentration on the binding of \( ^{125}\text{I}\)-neurotensin to mast cells and 38.8 fmol of \( ^{125}\text{I}\)-neurotensin. The points are the average of the triplicate assays from two experiments. For these data, 1 fmol of \( ^{125}\text{I}\)-neurotensin bound was equal to 7,150 cpm.

FIG. 5. A, effect of MgCl\(_2\) (●), CaCl\(_2\) (○), and EDTA (△) on the binding of \( ^{125}\text{I}\)-neurotensin to mast cells. Disodium EDTA was neutralized before use. B, the dissociation of \( ^{125}\text{I}\)-neurotensin from mast cells in the presence of CaCl\(_2\). The femtomoles bound are given as the difference between the controls without labeled neurotensin and those containing 20 mM CaCl\(_2\) in the presence of 6 nmol of neurotensin after steady state conditions had been reached (cf. A) using \( 1.3 \times 10^6 \) cells and 128 fmol of \( ^{125}\text{I}\)-neurotensin in A and 93 fmol of \( ^{125}\text{I}\)-neurotensin in B.

FIG. 6. A, influence of NaCl (○), KCl (●), and LiCl (□) on the interaction of \( ^{125}\text{I}\)-neurotensin with mast cells. Specific binding is the differential between controls without the salts and those assays with the salts both in the absence and presence of 6 nmol of neurotensin using \( 1.3 \times 10^6 \) cells and 128 fmol of \( ^{125}\text{I}\)-neurotensin. B, dissociation of \( ^{125}\text{I}\)-neurotensin from mast cells in the presence of 30 mM NaCl as a function of time. The experiment utilized \( 1.3 \times 10^6 \) cells and 93 fmol of \( ^{125}\text{I}\)-neurotensin and was conducted as given in the legend to Fig. 5.
of the ionic milieu on the binding of neurotensin, the effects of the major extracellular, as well as intracellular cations were investigated separately. Both CaCl₂ and MgCl₂ inhibit the specific binding of neurotensin (Fig. 5A): CaCl₂ decreased the binding by 50% at 1.5 mM, comparable to the effect of CaCl₂ on the binding of neurotensin to synaptosomes (25). At 20 mM CaCl₂, approximately 30% of the 125I-neurotensin remained bound. MgCl₂ also prevented the interaction of 125I-neurotensin with its receptor site, although it was slightly less effective than CaCl₂.

The monovalent cations, Na⁺ and K⁺, strongly interfered with the binding of neurotensin (Fig. 6A) and exhibited the same degree of inhibition as chloride, bromide, or iodide salts. Although LiCl also served as an antagonist for binding of neurotensin to mast cells, the extent of its interference was less than that of NaCl or KCl (Fig. 6A).

Association and Dissociation of Neurotensin from Mast Cells—The time to reach maximum specific binding of 125I-neurotensin to mast cells at 0°C was 10 min (Fig. 7A) and the binding remained constant for at least 60 min. Although the specific binding found at 22°C (room temperature) was comparable to that at 0°C, ice bath temperatures were used to ensure minimum degradation by proteolytic enzymes that might be released from damaged cells. Compared to 0°C, incubations at 37°C reduced by one-third the amount of specifically bound 125I-neurotensin. Bacitracin (2.8 µg), which inhibits the degradation of TRF and LRF in vitro (26), had no effect upon the reduction of 125I-neurotensin at 37°C. In fact, at both 22 and 37°C, the addition of bacitracin suppressed binding by as much as 40%.

After reaching the maximum level of binding (i.e., steady state conditions), 50% of the labeled neurotensin could be dissociated from its receptor binding site in approximately 1.5 min by the addition of excess neurotensin (6 nmol) and 100% dissociated by 10 min (Fig. 7B). Upon dilution in a 20-fold excess of buffer (containing bovine albumin), which reduced the ratio of labeled neurotensin bound to mast cells, dissociation of bound 125I-neurotensin was evident after 2 min; the maximum amount displaced in 10 min, however, was only 70% of the total bound. These dissociation effects are comparable to those seen with synaptosomes, but are smaller for mast cells by factors of 4 to 6 (25).

The addition of CaCl₂ to the 125I-neurotensin-mast cell complex at steady state conditions brought about its dissociation: 56% of the bound neurotensin was displaced at the end of 10 min, although 50% dissociated in approximately 5 min (Fig. 5B). NaCl also caused the dissociation of bound 125I-neurotensin from mast cells (Fig. 6B), an effect similar to that observed with CaCl₂; 66% of the bound neurotensin was displaced in 3 to 4 min.

Influence of Other Factors on Binding—Appreciable inhibition of binding was seen at a concentration of EDTA greater than 10 mM and none at 2 mM or less (Fig. 5A).

Iodoacetamide, N-ethylmaleimide, and dithiothreitol at concentrations up to 5 mM had no effect on the binding of neurotensin to mast cells. From these results, it appears that there is no obvious interaction of the peptide with membrane sulfhydryl groups. The requirement for a glycoprotein in the binding of a variety of peptide hormones, including neurotensin,* to synaptosomes has not been detected (27), and none was found to be involved here in the interaction with mast cells; the binding data were identical in the presence or absence of 20 µg of concanavalin A. ATP (3 mM) did not affect the binding of 125I-neurotensin to mast cells, whereas the binding to synaptosomes was reduced. Treatment with 0.1% Triton X-100 and centrifugation through a 5 to 25% (w/w) sucrose gradient did not yield a soluble binding component from mast cells as determined by our assay method.

Affinity Constant of Neurotensin for Mast Cells—the affinity of 125I-neurotensin for the mast cell receptor site was determined in the presence of increasing concentrations of unlabeled neurotensin under the optimal conditions observed for binding (Fig. 8).

In the calculation of the data, it was assumed that there were two types of binding sites; one representing specific binding and the other unsaturatable, nonspecific binding.

If we let: $(HR)_{total}$ = concentration of peptide bound to both types of sites; $(R_i) =$ total concentration of specific receptor sites; $(HR) =$ concentration of specifically bound unlabeled peptide; $(H*R_i) =$ concentration of specifically bound labeled peptide; $(HR) =$ concentration of nonspecifically bound peptide; $(H) =$ concentration of free (i.e., unbound) unlabeled peptide; $K_p =$ dissociation constant for the peptide-receptor complex, then, $(HR)_{total} = (1/K_p)(H)(R_i) - (HR) - (H*R_i)$

* L. H. Lazarus and M. H. Perrin, unpublished observations.
concentration or inclusion of cations is surprising considering the ionic environment in which the mast cells exist in situ and the isotonic medium used for histamine release (20). However, this phenomenon of maximum binding under hypotonic conditions in vitro has been observed in other systems, such as the binding of 125I-LRF and 3H-TRF to rat anterior pituitary cells, neurotensin binding to synaptosomes (25) and adrenocorticotropic hormone binding to adrenal cells. It is interesting that both CaCl₂ and NaCl prevent the binding of 125I-neurotensin and dissociate bound neurotensin from the receptor site. This suggests that these cations might counteract the ionic conditions necessary for binding, either by coupling with a negatively charged area in the binding site or associating with the peptide. The steady state conditions appear to be an equilibrium system since dilution of 125I-neurotensin by either neurotensin, buffer, or cations reduces binding.

Just as the binding of neurotensin to rat brain synaptosomes was seen to be stereospecific (25), so is the binding to mast cells (10). This is detailed in the following paper (29). It is shown that the binding of neurotensin to mast cells specifically involves the COOH-terminal portion of the peptide (10) and that numerous neurotensin analogs and other biologically active peptides are ineffective in displacing bound 125I-neurotensin. Mast cells therefore appear to be an excellent source for the study of extraneural receptor sites in the rat.

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