VERIFICATION OF BOTH THE SEQUENCE AND CONFORMATIONAL SPECIFICITY OF NEUROTENSIN IN BINDING TO MAST CELLS

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SUMMARY

Neurotensin (NT) analogs, modified at Arg^8 and Arg^9, were used to assess the role of Arg in NT binding to mast cells. [D-Arg^8]- and [D-Arg^9]-NT bound 4-5 times better than NT, whereas [D-$Arg^{8,9}$]-NT had the same binding affinity as NT. Binding of [Ala^8]-NT was not parallel to NT and exhibited a dissociation constant 38-fold lower than NT while [Ala^9]-NT had 32% binding. C-terminal peptides, NT_{8-13} and NT_{9-13}, had about 65% binding. These data suggest that Arg^8 plays a greater role than Arg^9 in the binding to mast cell NT receptors. Reduction of the disulfide bond in [$Cys^{2,13}$]-NT produced an analog 4-times more potent than NT, while the cyclized form had only 3% binding. Thus, a linear peptide with a free C-terminus appears to be required for binding.

NEUROTENSIN, a tridecapeptide (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) (1), produces (a) a direct CNS effect as seen by the induction of hypothermia in cold exposed rats following intracisternal administration (2,3) and (b) a variety of extraneural effects, such as hypotension, gut contraction, increased vascular permeability (1,4), hyperglycemia (4-5) and hyperglucagonemia (5). Many of these latter effects are mimicked by histamine and can be reversed by the H_1-histamine blocker, diphenhydramine (5). Mast cells, which release histamine in response to vasoactive peptides (6), represent a simple cellular model for the binding of neurotensin to extraneural receptors (7-9). That both mast cells and rat brain synaptosomal membranes (10,11) contain stereospecific receptor sites for neurotensin can be seen by the effect of analogs containing systematic replacements of the natural L-amino acids by their D-isomers: modifications in the C-terminal portion of neurotensin significantly reduce its binding affinity.

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Preliminary results obtained on the binding of peptides with D-Arg^8 and D-Arg^9 substitutions indicated that they were 5-6-fold more active (7). It appeared that the arginine residues, as well as the C-terminus carboxyl group are involved in the binding to receptors (4,9). Carraway and Leeman (4) proposed a model for NT-receptor interaction in which both C- and N-termini of the tridecapeptide would come in close proximity. To test this hypothesis, we made [Cys^{2,13}]-neurotensin. This communication investigates the role of arginine residues in the binding affinity of neurotensin to mast cells and suggests that (a) although both arginine residues are required for maximum binding, Arg^8 appears to be the more important of the two, and (b) the reduction of the disulfide bond in [Cys^{2,13}]-NT yields an analog more potent than neurotensin in the binding to mast cells.

MATERIALS AND METHODS

Neurotensin and neurotensin analogs were synthesized by solid phase methods and purified to homogeneity according to published procedures (12,13). Neurotensin was iodinated using chloramine T as oxidant at equal molar ratio of peptide, iodide (Amersham/Searle, carrier free Na^{125}I), and oxidant for 30 sec at room temperature and the iodination was terminated by a 4 molar excess of sodium metabisulfite (8). Iodinated neurotensin was isolated from a Sephadex LH-20 (pharmacia) column (10 x 0.7 cm) preequilibrated in and eluted with butanol-acetic acid-water (10:2:1) (BAW) at a slow rate of 9-10 ml/hr (8). The peak tubes of ^{125}I-neurotensin were stored at either 0° or -90°C. Our preparation of iodinated neurotensin was found to be biologically active: it produces hypotension in rats and exhibits tachyphalaxis (M.R. Brown and L.H. Lazarus, unpublished observations).

Mast cells were collected from both the peritoneal and thoracic cavities of decapitated, exsanguinated mature male rats according to Johnson and Moran (14). The cells were washed 3 times each in saline and 0.32 M sucrose and stored at 4°C in sucrose containing 0.02% NaN_3. The proportion of mast cells was found to be between 20-70% as determined by staining with methylene blue followed by eosin and hematoxylin (8). Neither erythrocytes, the main cellular contaminant in the mast cell preparation, nor lymphocytes (1M-9 cell line, donated by Jesse Roth) specifically bind neurotensin.

Specific binding was determined in triplicate 100 µl reaction assays containing 0.25-2 x 10^5 mast cells, 1 mg bovine serum albumin, 80 mM sucrose, 10 mM Tris-acetate buffer, pH 7.0, and 40-180 fmoles ^{125}I-neurotensin in the presence or absence of 6 nmoles (10 µg) unlabelled neurotensin. After incubation for 15 min at 0°C, the cell-^{125}I-neurotensin complex was rapidly filtered and washed each with 0.5 ml cold 2% bovine serum albumin through glass fiber filters (Whatman GF/C) presoaked in 2% bovine serum albumin (8). All peptides to be analyzed were diluted in 10 mg/ml bovine serum albumin used at concentrations from 10 µg to 1 ng. Specific binding to mast cells is defined as the difference in the amount of labelled neurotensin bound

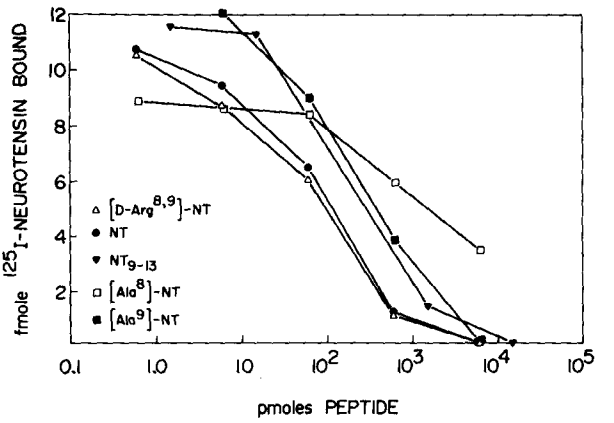

FIG. 1. Binding of neurotensin and neurotensin analogs to mast cells. This binding assay contained 173 fmoles ^{125}I-NT and 1.1 x 10^5 cells. Stock solutions of NT (•), [D-Arg^{8,9}]-NT (△), [Ala^8]-NT (□), [Ala^9]-NT (■) and NT$_{9-13}$ (▲) at 1 mg/ml in water were diluted in 10 mg/ml BSA in the range of concentration from 10 µg to 1 ng.

to mast cells in the absence and presence of a given peptide. The filters were counted in a Gamma Counter (Searle) with about 75% efficiency. The data is then plotted as the fmoles of labelled neurotensin bound versus the pmoles of unlabelled peptide used to compete with the labelled peptide (cf. Figs. 1 and 2). The concentration of a peptide that displaces 50% of the label and exhibits a curve parallel to that obtained for neurotensin is defined as its relative affinity (cf. Table 1). The biological test used to evaluate the relative potency of each of the NT analogs yields a measure of the peptide's ability to lower the body temperature of cold-exposed (4°C) rats. Potency values were calculated by 4 and 6 point bioassays. The high specificity of this test (3) as well as a discussion of the structure-activity relationships based on these data have been reported separately (7,9).

RESULTS AND DISCUSSION

The presence of D-Arg at positions 8 and 9 in neurotensin increased its affinity to mast cells 5-6-fold while the doubly substituted [D-Arg^{8,9}]-NT analog was as active as neurotensin (Fig. 1 and Table 1). It appears that although a single isomeric change in either arginine residue results in an increased affinity, the affinity of the doubly substituted analog is the same as that of neurotensin.

The decreased affinities of both [Ala^8]-NT and [Ala^9]-NT (Fig. 1 and Table 1) suggest that perhaps both arginine side chains could be involved in binding to mast cells. The fact that both the C-terminal

TABLE I. Relative Binding Affinity to Mast Cells and Relative Potency in Lowering Body Temperature of Cold-Exposed (4°) Rats of Neurotensin and its Analogs.

Peptides	Relative Binding Affinity to Mast Cells	Relative Potency for Hypothermia of Cold-Exposed Rats[d]
Neurotensin (NT)	1.00	1.00
[D-Arg^{8}]-NT[b]	5.00	1.00
[D-Arg^{9}]-NT[b]	6.40	1.00
[D-Arg^{8,9}]-NT	0.98	0.10
[Ala^{8}]-NT	0.14[a]	0.20
[Ala^{9}]-NT	0.32	1.00
NT_{8-13}	0.67	0.25
NT_{9-13}	0.60	0.20
NT + DTT[c]	1.15	-
[Cys^{2,13}]-NT	0.03	1.00
[Cys^{2,13}]-NT + DTT	4.16	-

[a]Data are averages of 50% displacement of labelled neurotensin from mast cells and includes [Ala^{8}]-NT whose binding curve does not parallel NT (Fig. 1). Values are from 2-3 separate experiments.

[b]Listed in reference 7.

[c]Final concentration of DTT (dithiothreitol) was 5 mM.

[d]Relative potency values of neurotensin and its analogs in lowering body temperature of cold-exposed rats 60 minutes following intracisternal administration of peptide. Potency values were calculated using the 4 or 6 point parallel line bioassay method. 95% confidence limits are of the order of ± 10%.

hexa- and pentapeptides, NT_{8-13} and NT_{9-13} respectively, and xenopsin, a natural analog of the C-terminal portion of neurotensin (7,9), exhibit similar binding affinities, also points to the possible equivalence of

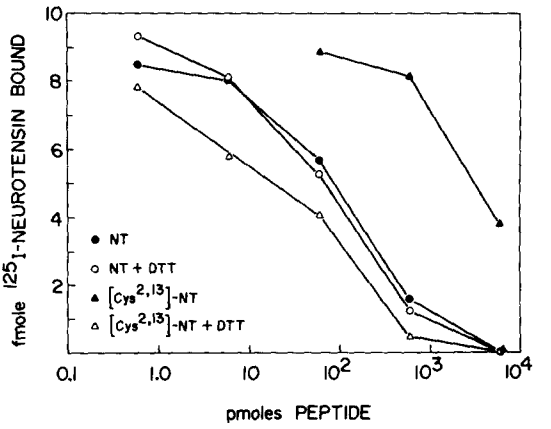

FIG. 2. Effect of dithiothreitol (DTT) on the binding [Cys^{2,13}]-NT to mast cells. The peptides were diluted as given in the legend to Fig. 1. The solid symbols are the controls without DTT while the open symbols contain 5 mM DTT: NT (• , ○) and [Cys^{2,13}]-NT (▲ , △). These assays contained 2.7 x 10^4 mast cells and 86.3 fmoles ^{125}I-NT.

these two arginine positions. However, the displacement of ^{125}I-NT by [Ala^8]-NT is not parallel to that of neurotensin (Fig. 1); a Scatchard analysis (15) of the data yields a K_D for [Ala^8]-NT of 5.88 μM which represents a decrease in the affinity by 38-fold relative to neurotensin (cf. ref. 8). This suggests that Arg^8 could be more crucially involved than Arg^9 in the binding of neurotensin. This proposal is further substantiated by (i) the full biological activity of [Ala^9]-NT and lower potency of [Ala^8]-NT in producing hypothermia (see Table 1) and (ii) the high affinity of bradykinin for mast cell receptors (7,9).

Formation of a closed ring through the introduction of cysteine residues at positions 2 and 13 leads to a marked reduction in total binding and apparent affinity for the receptor (Fig. 2), although full biological potency is observed in producing hypothermia (Table 1). These results could simply indicate either that there exist different classes of neurotensin receptors, or that the disulfide bond is reduced in vivo with the concomitant production of an active peptide. The in-

clusion of 5 mM dithiothreitol in the mast cell binding assay confirms the latter hypothesis: in fact, reduced [$Cys^{2,13}$]-NT is 4-times more active than neurotensin and represents greater than a 100-fold increase in binding over the oxidized monomeric peptide (Fig. 2 and Table 1). The possibility exists that the free sulfhydryl group interacts with the neurotensin receptor, conferring upon the analog an apparent greater relative affinity. However, two inactive peptides, both containing disulfide bonds, somatostatin and [Arg^{8}]-vasopressin (7,9), remain ineffective competitors of neurotensin in the presence of 5 mM dithiotheitol. To understand fully the effect of sulfur containing analogs of neurotensin, several C-terminal derivatives are being prepared. It should be pointed out, however, that the binding of neurotensin to mast cells and its biological effects (1-5) may involve similar, but not necessarily identical receptor sites. Furthermore, the phenomena measured in bioassays may represent secondary responses to neurotensin.

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