NEUROTENSIN STIMULATES EXOCYTOTIC HISTAMINE SECRETION FROM RAT MAST CELLS AND ELEVATES PLASMA HISTAMINE LEVELS

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SUMMARY

1. Neurotensin stimulated histamine release and granule extrusion when applied to isolated rat peritoneal mast cells.
2. This secretory response was prevented by the removal of calcium or energy and was not accompanied by the release of lactic dehydrogenase.
3. The secretory response produced by neurotensin was prevented by prior treatment of mast cells with cromoglycate.
4. The intravenous injection of neurotensin into anaesthetized rats produced a rapid and significant increase in the level of blood histamine that was dependent upon the dose of neurotensin.
5. Treatment of rats with compound 48/80, 24 hr before neurotensin, abolished the elevation in blood histamine caused by neurotensin. The intravenous injection of cromoglycate 1–2 min before neurotensin greatly reduced the response to neurotensin.
6. The intradermal injection of neurotensin (0.03–30 p-mole) increased capillary permeability in rats pre-treated intravenously with Evans Blue. This response was abolished by the antihistamine, diphenhydramine. Increasing the dose of neurotensin to 300 p-mole partially overcame this inhibition by diphenhydramine.
7. Our results demonstrate that neurotensin can elicit an exocytotic secretory response from isolated rat peritoneal mast cells and elevate histamine levels in blood. It is suggested that some of neurotensin’s physiological effects may be due to stimulation of mast cell secretion.

INTRODUCTION

Neurotensin is a vasoactive tridecapeptide from brain and gut first isolated and characterized by Carraway & Leeman (1973, 1976). Given intravenously, neurotensin produces a variety of physiological effects (Carraway & Leeman, 1973; Fernstrom,

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Carraway & Leeman, 1980), several of which are mimicked by histamine (Douglas, 1975a). A major cellular store of histamine is the mast cell (Selye, 1965; Beaven, 1976). This cell is found in many tissues of the body, usually closely associated with blood vessels, and is in particularly high numbers in the skin, gut and lungs (Selye, 1965). Recently neurotensin has been shown to bind to specific receptor sites on the mast cell surface (Lazarus, Ferrin & Brown, 1977a). Here we report that neurotensin stimulates the isolated rat mast cell to extrude granules and release histamine by exocytosis. When given intradermally it produces a classic passive cutaneous anaphylactic reaction, and when given intravenously neurotensin elicits a prompt and significant increase in the level of blood histamine. A preliminary account of some of our results has been published (Cochrane, Lansman, Paterson, Carraway & Leeman, 1979).

METHODS

Peritoneal mast cells were obtained from male Sprague-Dawley rats (200–400 g) as previously described (Cochrane & Douglas, 1974), washed three times and incubated in Ca-Locke solution (mm): 150 NaCl, 5 KCl, 0.5 CaCl₂, 10 HEPES (N-2-hydroxethylpiperazine-N'-2-ethanesulphonic acid). For Ca-free Locke, CaCl₂ was omitted and EGTA (ethyleneglycol bis-(β-aminoethyl ether)-N,N',N'-tetraacetic acid) added. All solutions were adjusted to pH 7.2-7.3 and contained glucose (3.6 mm) and bovine serum albumin (BSA, 1 mg/ml). For some experiments the mixed peritoneal washings, containing some 5% mast cells, were purified by density gradient centrifugation through BSA (Chabot, Riback & Cochrane, 1981) to contain approximately 90% mast cells. For light microscopy, suspensions of mast cells were pipetted in Sykes-Moore culture chambers and fields observed as previously described (Cochrane & Douglas, 1974). Histamine release from isolated mast cells was assayed by the fluorometric method of Kremzner & Wilson (1961) as previously described (Cochrane, Douglas, Mouri & Nagazato, 1975). Histamine secretion (supernatant histamine) from isolated mast cells is expressed as a percentage of total histamine (cell plus supernatant).

For intravenous injections, animals were anaesthetized with sodium pentobarbitone (Nembutal, 40 mg/kg). Compounds for intravenous injection were dissolved in 0.9% saline and injected via the tail vein. The injection volume was always less than 0.4 ml. Following the injection of the test substance, the animals were decapitated and the blood collected into ice-cold, heparinized (10 units/tube) plastic tubes, and centrifuged at 900 g for 10 min at 4°C. The plasma fraction was then carefully removed and stored frozen until assayed.

Blood levels of histamine were determined by the specific, radio-enzymatic assay for histamine initially described by Taylor & Synder (1972) and modified by Beaven, Jacobson & Horakova (1972), and by Shaff & Beaven (1979). For this assay, the enzyme, histamine-N-methyl-transferase, was isolated from rat kidney following the procedure outlined by Shaff & Beaven (1979) and stored at −20°C until use. S-adenosyl-L-[methyl-3H]methionine (ICN Pharmaceuticals, Inc., 9 Ci/m-mole) was used as the methyl donor and [3H]methylhistamine extracted into chloroform, dried and counted according to Shaff & Beaven (1979). Histamine standards (histamine-free base or histamine dihydrochloride, Sigma) were run with each assay and the amount of histamine present in the plasma samples determined from the standard curve. The assay was linear over the range of 0.1–4.0 ng histamine. Samples containing greater amounts of histamine were appropriately diluted with sodium phosphate buffer (pH 7.9) before being assayed.

Changes in vascular permeability were determined by the passive skin reaction (Ishizaka, 1978). Rats were anaesthetized, the hair removed from the abdominal area, and a 1.0% solution of Evans Blue in 0.9% saline injected intravenously (1 ml/kg). Ten minutes later, the compounds to be tested were injected intradermally (0.1 ml/site) and the reaction allowed to develop for 10 min. The rat was then killed, the skin overlying the abdominal area removed, visually inspected and photographed. The blued responses were measured and the areas calculated.

Lactic dehydrogenase (LDH) was measured using a commercially available colorimetric assay kit (Sigma Chemical Company). LDH release (supernatant LDH) is expressed as a percentage of total LDH (cellular and supernatant). Neurotensin (Sigma) was stored frozen at 0.5 mg/ml in saline and diluted to the appropriate concentration immediately prior to use. Compound 48/80 (Burroughs
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Wellcome), cromoglycate (cromolyn sodium) (Fisons), and Evans Blue (Fisher) were prepared fresh each day. The antihistamine, diphenhydramine hydrochloride, Benadryl, (50 mg/ml) was obtained in injectable form from Parke Davis and appropriately diluted with saline. Amidated neurtensin was obtained from Dr Karl Folke, University of Texas at Austin, Texas. Differences between means were tested for significance using Student's t test.

RESULTS

Responses to neurtensin

Isolated rat mast cells bathed in Ca-Locke solution and viewed by phase-contrast microscopy are typically spherical and highly refractile with regular cellular outlines (Cochrane & Douglas, 1974). With the addition of neurtensin (1 x 10^-5 M), the cells promptly (within 1 min) undergo the classical degranulating or 'bubbling' secretory response that is identical to that elicited by antigen (Anderson, Slorach & Uvnas, 1973), compound 48/80 (Bloom & Haegermark, 1965; Cochrane & Douglas, 1974) and other mast cell secretagogues (Lansman & Cochrane, 1980). This response has been shown by electron microscopy to be the result of exocytosis (Singleton & Clarke, 1965; Röhl, Anderson & Uvnas, 1971). Accompanying this extrusion of granules is the release of histamine (Fig. 1). Histamine release was detectable at 10^-4 M-neurtensin (P < 0.05, 5.2 ± 0.6 %, mean ± S.E. of mean, n = 4, versus 2.8 ± 0.1 %, n = 4 without neurtensin) and increased as the concentration of neurtensin was raised, showing a plateau from 10^-7 to 10^-5 M-neurtensin (the values of histamine release for 10^-7, 10^-6 M-neurtensin were not significantly different). With further increases in neurtensin concentration, histamine release increased sharply with no apparent saturation even at 10^-3 M-neurtensin (Fig. 1). When purified populations of mast cells were used, an identical dose–response relationship was seen. The amidated form of neurtensin (10^-3 - 10^-2 M), shown by Folkes, Chang, Humphries, Carraway, Leeman & Bowers (1976) to have less than 0.5 % of the biological effect of neurtensin, and by Lazarus, Perrin, Brown & Rivier (1977) to be ineffectual in binding to the mast cell surface, failed to elicit histamine release except at very high concentrations (10^-4 M). Lactate dehydrogenase did not accompany the release of histamine in response to neurtensin. The level of LDH in the supernatant from mast cells incubated at 37 °C for 10 min in Ca-Locke solution was 61 ± 0.8 %, n = 3. Spontaneous histamine release was 4.1 ± 0.5 %, n = 3. With the addition of a high concentration of neurtensin (10^-4 M), histamine release rose to 35.2 ± 3.1 %, n = 3, while the level of LDH remained steady (7.4 ± 0.8 %, n = 3). Sonication of mast cells released 81 ± 6 % of the LDH and 90 ± 4 % of the histamine (n = 3).

Effect of calcium

The secretory response of the mast cell (Douglas, 1975b; Foreman & Mongar, 1972b; Goth & Johnson, 1975), like other exocytotic secretory responses (Douglas, 1968; Rubin, 1974), requires a source of calcium. In the present experiments, simply removing the calcium from the bathing solution reduced but did not abolish the secretory response elicited by neurtensin. Thus, histamine release from mast cells in Ca-Locke solution in response to 10^-5 M-neurtensin was 25.0 ± 1.7 %, mean ± S.E., n = 3; the removal of calcium reduced the histamine released to 91 ± 0.8 %, n = 3. However, when the chelating agent, EGTA (1 mM), was included in the Ca-free Locke
and the mast cells washed twice and incubated for 1 hr at 37 °C in Ca-free Locke containing 0·5 mM-EGTA, histamine release in response to neurotensin was reduced to control levels (4·0 ± 0·9, n = 3). The reintroduction of calcium (but not magnesium) with neurotensin to such chelated mast cells restored the secretory response (Fig. 2). As the concentration of calcium was increased, histamine release in response to neurotensin (10⁻⁸ M) increased and reached a maximum value when 1·5 mM-calcium was introduced (Fig. 2). With further increases in calcium, the amount of histamine released in response to neurotensin declined (Fig. 2). This inhibition by calcium of the secretory response initiated by neurotensin was also evident in mast cells not exposed to EGTA. Thus mast cells incubated in 2 mM-Ca-Locke solution released only 8·4 ± 1·0% (n = 3) histamine when exposed to neurotensin (10⁻⁸ M) while cells incubated in 0·5 mM-Ca-Locke released 25·0 ± 1·7% histamine (n = 3) in response to neurotensin (10⁻⁸ M).

Fig. 1. Histamine release from rat mast cells in response to increasing concentrations of neurotensin. Mast cells were washed three times and bathed in Ca-Locke solution at 37 °C for 10 min. Neurotensin was then added and the incubation continued for 10 min. Each point represents the mean ± S.E.M. of mean of n experiments (in parentheses). Where not present, the symbol covered the error bars in this and in all subsequent Figures.
Effect of inhibitors

Like calcium, the removal of a source of energy abolishes exocytotic secretion (Sakei, 1964; Douglas, 1968; Rubin, 1974; Diamant, 1975). When mast cells were incubated for 10 min in glucose-free Locke solution containing both antimycin A ($10^{-6}$ M) and deoxyglucose ($10^{-5}$ M) and neurotensin ($10^{-6}$ M) subsequently added, histamine release was prevented (5.0 ± 0.7%, $n = 3$). If glucose was included with antimycin A ($10^{-6}$ M) and deoxyglucose removed, the secretory response to neurotensin was restored (19.8 ± 1.0%, $n = 3$).

![Graph showing histamine release in response to calcium concentration](image)

**Fig. 2.** Histamine release from mast cells incubated in chelating agent and bathed in Ca-free Locke, in response to the addition of neurotensin and varying concentrations of calcium. Mast cells were washed twice with Ca-free Locke solution containing EGTA (1 mM), incubated for 1 hr in Ca-free Locke containing EGTA (1 mM) and resuspended in Ca-free Locke containing 0.5 mM-EGTA. Neurotensin ($1 \times 10^{-6}$ M) and calcium (at the indicated concentrations) were then added and the incubation continued for 10 min. Mean ± S.E. of mean of three.

Cromoglycate has been shown to inhibit histamine release from rat mast cells stimulated by a variety of secretagogues including antigen (Garland, 1973), compound 48/80 (Orr, Hall, Gwilliam, & Cox, 1971; Spataro & Bosmann, 1976), dextran (Garland & Mongar, 1974) or ionophore A23187 (Johnson & Bach, 1975). The addition of cromoglycate (100 μM, a concentration used by other investigators: Spataro & Bosmann, 1976; Theoharides, Sieghart, Greengard & Douglas, 1980) 10 min before neurotensin ($10^{-6}$ M) inhibited histamine release from isolated mast cells bathed in Ca-Locke by 70 ± 2% $n = 3$. 
Effect of neurotensin injected intravenously

The intravenous injection of neurotensin to anaesthetized rats dramatically elevated plasma histamine levels (11·6 ± 1 ng/10 μl., n = 8) when compared to control, saline injected animals (0·71 ± 0·1 ng/10 μl., n = 10) (Fig. 3). This effect was significant at 1·0 n-mole/kg, and with further increases in the dose of neurotensin histamine levels continued to rise (Fig. 3). In two of four trials, the injection of 0·5 n-mole/kg significantly elevated the level of histamine above the control value. However, the mean of these four trials was not significantly different from control. The levels of plasma histamine in animals injected with saline was 0·71 ± 0·1 ng/10 μl. (mean ± s.e., n = 10). This value is higher than the control value previously reported by Beaven et al. (1972; 0·04 ± 0·02 ng/10 μl.). The reason for this difference is unknown. We have noted, however, that blood histamine levels vary for different rat colonies.

In the experiments described above, blood was collected 3 min after the injection of neurotensin. However, peak histamine levels (17·5 ± 2 ng/10 μl., n = 3) were evident by 1 min after the injection of neurotensin. Histamine levels then declined...
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to 11.6 ± 1 ng/10 μl., n = 8 at 3 min, 98 ± 0.9 ng/10 μl., n = 3 at 15 min after
neurotensin and 2.7 ± 0.4 ng/10 μl., n = 3 at 30 min after the injection of neurotensin.
Accompanying the injection of neurotensin were classic signs of histamine shock:
intense vasodilation of surface blood vessels, most evident in the ears and feet, and
a marked increase in the hematocrit.

Tests for assay specificity

The radio-enzymatic assay we have used to measure plasma histamine levels has
been shown to be very specific for histamine (Taylor & Snyder, 1972; Shaff & Beaven,
1979). To assure the specificity of our measurements, the following tests were made.
The response produced by neurotensin was compared to that produced by the
intravenous injection of the classic histamine releaser, compound 48/80. At a dose of
1 mg/kg, this mast cell secretagogue elevated histamine levels to 52 ± 7 ng/10 μl.,
(mean ± s.e.; n = 3) which was about 70 times higher than saline-injected control
values. This effect of compound 48/80 was dependent on the concentration injected,
was rapid in onset (by 1 min), and the animals treated with 48/80 showed the classic
signs of massive histamine release also seen with the injection of neurotensin.
Secondly, known concentrations of histamine were added to samples of plasma, the
sample quickly frozen, and then assayed for histamine. The recovery of histamine
in the samples was > 95%. Finally, we added compounds that might be expected
to interfere with the assay system. Epinephrine (1 μg), 5-hydroxytryptamine (1 μg),
histidine (1 μg), heparin (10 units) or neurotensin (10 n-mole) were assayed alone or
in the presence of added histamine. None of these substances registered in the assay
and none affected measurements of added histamine.

Effect of pre-treatment with compound 48/80 or disodium cromoglycate

The elevation of blood histamine levels caused by the injection of neurotensin could
reflect a stimulatory effect of neurotensin on tissue mast cells similar to its effect on
isolated peritoneal mast cells; however, it could also represent a stimulation of
histamine secretion from other histamine-containing cells. To help to distinguish
between these alternatives we have used compound 48/80, a selective secretagogue
for mast cells (Morrison, Roser, Henson & Cochrane, 1974) and cromoglycate, a
specific inhibitor of mast cell histamine release (Orr et al. 1971; Garland, 1973;
Spataro & Bosmann, 1976) that is widely used in the treatment of allergic asthma
and to relieve the symptoms associated with systemic mastocytosis (Sorter, Austin
& Wasserman, 1979).

In the first set of experiments, compound 48/80 was used to deplete mast cells of
their histamine stores. All animals (both treated and control) were injected with
diphenhydramine hydrochloride (1 mg/kg) to prevent complications from severe
histamine shock. Thirty minutes later, compound 48/80 (1 mg/kg) or saline (0.4 ml.)
was given intraperitoneally. The following day (24 hr later) the animals were
anaesthetized and given neurotensin (5 or 10 n-mole/kg) or saline (0.3 ml) intra-
venously. Blood was collected 3 min later and assayed as before. The results of these
experiments are shown in Fig. 4. Neurotensin markedly elevated blood histamine
levels in saline pre-treated animals (Fig. 4A); however, it was ineffective in 48/80
pre-treated animals (Fig. 4B).
In the second set of experiments, animals were injected intravenously with cromoglycate (1 mg/kg). One to two minutes later, either neurotensin (5 n-mole/kg) or saline (0.3 ml) was injected intravenously. After 3 min the blood was collected and assayed as before. In animals pre-treated with saline, neurotensin increased plasma histamine levels about 30-fold, from a resting level of $0.63 \pm 0.05$ ng/10 $\mu$l., $n = 3$ to $18.1 \pm 1.2$ ng/10 $\mu$l., $n = 4$. Pre-treatment with cromoglycate reduced this elevation in histamine to $4.7 \pm 2$ ng/10 $\mu$l., $n = 6$.

![Graph showing plasma histamine levels](graph.png)

**Fig. 4.** Plasma histamine levels in response to neurotensin in animals pretreated 24 hr previously with saline (A) or compound 48/80, 1 mg/kg (B). Neurotensin (5 or 10 n-mole/kg) or saline (0.3 ml) were given intravenously as before. Mean ± S.E. of mean of $n$ experiments, quoted within the column.

**Effect of neurotensin injected intradermally**

The intradermal injection of neurotensin markedly increased the vascular permeability in rats treated with Evans Blue as indicated by its ability to stimulate the extravasation of the dye-albumin complex (Pl. 1). Anaesthetized rats received an intravenous injection of Evans Blue, and 10 min later 0.1 ml saline, neurotensin (three different concentrations), compound 48/80 or histamine, with or without the antihistamine diphenhydramine hydrochloride, were injected intradermally. After
10 min the animals were killed, the skin rapidly removed and its underside examined and photographed. The result of such an experiment is shown in Pl. 1. While the intradermal injection of saline (site 1) caused minimal blueing, the injection of neurotensin produced a marked response which increased in size and intensity as the concentration of neurotensin was raised (site 2 = 0.03 p-mole; site 3 = 30 p-mole; site 4 = 300 p-mole). In four separate experiments, the size of the blueing responses were (mean ± s.e.): 56.4 ± 8.6 mm², 132.4 ± 13.2 mm² and 307.3 ± 53.9 mm² for the low, medium, and high doses of neurotensin, respectively. Compound 48/80 (site 5, 0.5 µg) and histamine (site 6, 0.5 µg) also produced blueing responses. When diphenhydramine hydrochloride (2 µg) was included with each of the test substances, the response was completely abolished in all but the highest neurotensin concentration (site 4a).

DISCUSSION

Our results show that neurotensin can stimulate isolated rat peritoneal mast cells to extrude granules and release histamine. Judged by phase-contrast microscopy, this secretory response is identical in appearance to that elicited by antigen (Anderson et al. 1973), compound 48/80 (Bloom & Haegermark, 1965; Cochrane & Douglas, 1974), and other mast cell secretagogues (Lansman & Cochrane, 1980), responses that are known to result from exocytosis (Singleton & Clark, 1965; Röhllich et al. 1971). Like these other secretory responses, the histamine secretion stimulated by neurotensin is concentration-dependent, requires a source of calcium and energy, and is not accompanied by the release of LDH.

The initial plateau of histamine release in response to neurotensin at about 18% has its half-saturation point near 5 × 10^{-5} M-neurotensin Lazarus et al. (1977a) have shown that iodinated neurotensin binds to receptor sites on the mast cell with a $K_D = 154$ nm. The reason for this apparent discrepancy is unknown. High doses of neurotensin (above $10^{-5}$ M) are not cytotoxic; there is no LDH release and the histamine secretion observed is calcium-dependent. The secretory response to high doses of neurotensin may reflect the general stimulatory effect of basic peptides observed by Johnson & Erós (1973).

The inhibition of the secretory response to neurotensin by relatively low levels of calcium in the extracellular fluid is consistent with the finding of Lazarus et al. (1977a), that calcium inhibits the binding of $^{[11]}$T-neurotensin to mast cells. Moreover, similar inhibitory effects of calcium on secretion have been previously observed in mast cells (Foreman & Monger, 1972b; Grossman & Diamant, 1974), nerves (Cooke & Quastel, 1973), and neurosecretory terminals (Douglas & Poisner, 1964).

Our results suggest that stimulation of mast cell secretion is responsible for elevating blood levels of histamine following the injection of neurotensin. Thus pre-treatment with either compound 48/90 or cromoglycate prevented or greatly reduced the ability of neurotensin to elevate plasma histamine levels. Compound 48/80 has been shown to selectively bind to mast cells in mixed peritoneal cell washings (Morrison et al. 1974) and is without effect on a variety of other secretory cells including chromaffin cells, cells of the exocrine pancreas and nerve terminals (D. E. Cochrane, unpublished results). Cromoglycate has been shown to be an effective inhibitor of histamine release from mast cells stimulated by a variety of
agents including, as we have shown here, neurotensin (Orr et al. 1971; Garland, 1973; Garland & Mongar, 1974; Spataro & Bosmann, 1976).

Our results also show that the increase in cutaneous vascular permeability caused by an intradermal injection of neurotensin is blocked by antihistamine (Pl. 1). Given neurotensin’s ability to release histamine from isolated mast cells (Fig. 1), it seems reasonable to suggest that neurotensin causes an increase in cutaneous vascular permeability by releasing histamine from skin mast cells. At this time, it is not known whether neurotensin is normally present within skin or if it might be delivered to skin in quantities sufficient to affect mast cells. One possibility is that neurotensin or a neurotensin-like material is contained within cells of the skin and stimuli causing vasodilation release of this material.

The findings we have reported here suggest that some of the other biological actions of neurotensin might be mediated via release of active substances such as histamine from mast cells. The effect of neurotensin on gastric acid secretion (Fernstrom et al. 1980), for example, could possibly be due to actions on gastric mast cells and not due to a direct effect of neurotensin on the parietal cells. It is possible that actions on mast cells could be a major pathway by which neurotensin exerts its effects within the body.

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EXPLANATION OF PLATE

Passive cutaneous responses showing the stimulatory effect of neurotensin and its prevention by antihistamine. Rats were anaesthetized and pre-treated intravenously with Evans Blue (1%, 0.1 ml./100 g body weight). Ten minutes later, substances to be tested were injected (0.1 ml.) intradermally and the reaction allowed to develop for 10 min. Site 1 = saline, sites 2, 3, and 4 = neurotensin (0.03, 0.30 and 300 pmole, respectively), site 5 = compound 48/80 (0.5 μg), and site 6 = histamine (0.5 μg). Note the marked reaction surrounding injection sites 2, 3, and 4, and its increasing intensity from 2 to 4. Contrast this reaction to that surrounding site 1 (saline) and compare with sites 5 and 6. Inclusion of the antihistamine diphenhydramine (2 μg/0.1 ml.) with the saline, neurotensin, compound 48/80 or histamine completely abolished the responses to the low (2a) and medium (3a) doses of neurotensin and those produced by compound 48/80 (5a) and histamine (6a), and largely prevented that produced by the high dose of neurotensin (4a). Identical results were obtained in three additional experiments (see text for area measurements).