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Pharmacological characterization of the inhibitory effects of neurotensin on the rabbit ileum myenteric plexus preparation

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1 Neurotensin in picomolar concentrations caused a concentration-related inhibition of the spontaneous contractile activity of the longitudinal muscle from the rabbit isolated ileum. Neurotensin was approximately 100 times more potent than adrenaline and about 10000 times as active as adenosine triphosphate (ATP) in producing similar relaxations.

2 The neurotensin-induced inhibitory effect did not follow activation of adrenoceptors or P_1-purinoceptors since the effect of the neuropeptide was not antagonized by a combination of phentolamine plus (−)-propranolol, nor by pretreatment with theophylline.

3 Tetrodotoxin did not reduce the potency of neurotensin in relaxing the rabbit ileum, suggesting that the neurotensin-induced inhibition is not neuronally mediated. The inhibitory responses of neurotensin were blocked non-competitively by apamin.

4 The inhibitory effect of neurotensin was short-lived with the subsequent development of tachyphylaxis, which was not crossed to the inhibitory action of adrenaline or ATP. Similarly, when tachyphylaxis to adrenaline or to ATP was established, the inhibitory action of neurotensin was unaffected. Desensitization was characterized by a gradual shift of the neuropeptide concentration-response curve to the right and downwards.

5 Preincubation of rabbit ileum strips with 10 nM dynorphin (1–13) significantly increased the inhibitory action of neurotensin.

Introduction

Neurotensin is a basic tridecapeptide first isolated and chemically characterized from the hypothalamus; subsequently, the peptide has been localized in a variety of brain nuclei where it may act as a neurotransmitter (for a recent review see Nemeroff, Luttinger & Prange, 1983). In the periphery, substantial amounts of neurotensin-like immunoreactivity have been detected in the gastrointestinal tract, particularly in the small intestine of man. The highest levels of the neuropeptide are found in the N cells of the ileal mucosa with a minor proportion of the neurotensin distributed along nerve fibres of the intestinal plexus. (Polak, Sullivan, Bloom, Buchan, Facer, Brown & Pearse, 1977; Leeman & Carraway, 1982). Although little is known at present concerning the role of neurotensin in the functioning of the digestive system, several lines suggest that this peptide is of physiological importance. Neurotensin may act as local hormone on the human intestine since plasma neurotensin-like immunoreactive levels rise sharply following a fatty meal (Blackburn, Bloom & Polak, 1978; Mashford, Nilsson, Rokaeus & Rosell, 1978; Rossell & Rokaeus, 1979). In addition, neurotensin influences electrolyte transport, in the mucosa of the ileum increasing transepithelial potential differences and short circuit current (Kachur, Miller, Field & Rivier, 1982). Neurotensin may also modulate intestinal peristaltis; electrophysiological studies on single myenteric neurones from the guinea-pig (Williams,

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Katayama & North, 1979) showed that picomoles of neurotensin produce concentration-dependent increases in neuronal cell firing in a large proportion of the myenteric neurones. Furthermore, the potent contractile activity of neurotensin in the guinea-pig ileum is neuronally mediated and under opiate control (Huidobro-Toro & Way, 1982). Dynorphin, the novel opioid peptide endogenous to the intestines (Goldstein, Fischli, Lowney, Hunkapiller & Hood, 1981; Tachibana, Araki, Ohya & Yoshida, 1982), is a potent non-competitive antagonist of the neurotensin excitatory response in the ileum of the guinea-pig (Zhu, Huidobro-Toro, Lee, Loh & Way, 1982; Huidobro-Toro & Zhu, 1983; Huidobro-Toro, 1983).

The object of the present investigation was to characterize the nature and mechanism of the neurotensin response of the ileal musculature of the rabbit as compared to that of the guinea-pig, and to examine further the interaction of dynorphin with neurotensin on the rabbit small intestine.

Methods

Adult male New Zealand rabbits (2–2.5 kg), purchased from Simonsen (Gilroy, CA) were used throughout. The standard Purina Chow diet was supplemented twice a week with fresh vegetables and fruit.

Preparation of the longitudinal-muscle-myenteric plexus preparation from the rabbit ileum for measurement of contractile activity

Rabbits were killed by a blow on the neck; the abdomen was opened by a midline incision and the terminal portion of the ileum was removed and placed in a modified Ringer solution of the following composition (mM): NaCl 154, KCl 5.66, $CaCl_2$ 2.54, glucose 2.77, $NaHCO_3$ 5.95 and choline chloride 0.002. The last 10 cm of the ileum was always discarded. Longitudinal muscle-myenteric plexus preparations were dissected according to the technique described by Yoshimura, Huidobro-Toro, Lee, Loh & Way (1982). The intestinal strips were set up in 10 ml tissue baths containing Ringer solution at 37°C gassed with 95% O_2 and 5% CO_2 to maintain a pH of 7.4 The tension of the longitudinal muscles was measured with a Grass FT 03C force-displacement transducer connected to a multichannel Grass model 7D polygraph recorder. Preparations were subjected to an initial tension of about 1 g. Tissues were allowed to equilibrate for 1 h before drug application; during this equilibration interval, the tissues were washed with 30–40 ml of Ringer solution every 15 min.

Quantification of the inhibitory responses; concentration-response curves

In the determination of concentration-effect curves, the activity or each concentration of agonist was obtained after incubation of the tissues with the drugs for 2 to 5 min. After recording the inhibitory response caused by application of neurotensin for 5 min, the tissues were washed (40–50 ml of drug-free Ringer solution) and the preparations were retested usually 20 min later with a higher concentration of neurotensin or the specific compound under study. The response to neurotensin, adrenaline or ATP was quantified by integration of the area of inhibition produced by the different concentrations of agonists during a fixed 5 min period of time (Chow & Marshall, 1981). Integration of the area of inhibition was done manually by cutting the inhibitory response from the tension tracing recording (g-min cm^{-2}) and weighing each piece of paper. The inhibitory effect was expressed as g of paper.

The concentration of each compound required to cause a 50% inhibition (IC_{50}) was calculated by interpolation from the respective concentration-response curves.

Effect of phentolamine, propranolol, theophylline and tetrodotoxin

Concentration-response curves for neurotensin were performed in the absence and in the presence of 12 μM phentolamine, 12 μM (−)-propranolol, 12 μM phentolamine plus 12 μM propranolol or 50 μM theophylline added directly to the tissue chambers 2 min before the agonist. The IC_{50} of each agonist was determined in the same preparation before and after the application of each antagonist. Concentration-response curves to neurotensin were also determined before and after tetrodotoxin (TTX: 100 and 300 nM for 2 min). All these experiments were repeated on at least four separate ileum strips obtained from different rabbits; each tissue was used for only one drug.

Desensitization protocols

Two concentrations of neurotensin were used: a priming concentration, which was followed by a second application of the same or higher concentration of the neuropeptide 6 min later, without washout. To test for crossed desensitization between neurotensin and adrenaline, ileum preparations were desensitized to the inhibitory action of 13 nM neurotensin followed 3 min later by the application of 66.3 nM adrenaline. Conversely, tissues desensitized to the inhibition caused by 66.3 nM adrenaline were tested 3 min after the second application of the

catecholamine with 13 nM neurotensin. In an additional set of experiments, tachyphylaxis of the ileum to 136 μM ATP was established, then neurotensin was applied.

In an independent set of experiments, the effect of a priming concentration of neurotensin on the effect of subsequent applications of the neuropeptide was tested. Tissues were pretreated for 6 min with 1.0, 5.24 or 52.4 nM neurotensin, and then concentration-response curves to the neuropeptide were derived. The results compare the potency of neurotensin in control preparation with that in desensitized tissues.

Antagonism of neurotensin inhibition by apamin

Separate ileum preparations were exposed to 5.24 nM neurotensin alone, or to the neuropeptide in the presence of 3.7, 11.2 or 37 nM apamin, 2 min before neurotensin. In a parallel set of experiments, the action of 15 nM apamin was evaluated by performing a neurotensin concentration-response curve before and after a 2 min preincubation with the bee venom. Four separate ileum preparations were used in each protocol; results compare the action of neurotensin with and without apamin.

Influence of dynorphin

Neurotensin concentration-response curves were obtained before and after a 2 min incubation with 10 nM dynorphin-(1-13). The biological activity of dynorphin-(1-13) was checked before the experiment by bioassay on the guinea-pig ileum (Yoshimura *et al.*, 1982).

Statistical analysis

The paired two tail Student's t test was used to compare the neurotensin concentrations required to cause 50% inhibition (IC_{50}) before and after drug treatments. P values less than 0.05 were considered to be significant.

Drugs and chemicals

Neurotensin, adrenaline bitartrate, adenosine triphosphate disodium salt (ATP), tetrodotoxin (TTX), apamin and thophylline were purchased from Sigma Chemical Co. (St. Louis, MO). Dynorphin-(1-13) was obtained commercially from Peninsula Laboratories (San Carlos, CA); this neuropeptide was dissolved in acidified methanol as detailed by Goldstein, Tachibana, Lowney, Hunkapiller & Hood (1979). The amount of solvent used did not itself affect the muscular activity of the ileum. All drug solutions were prepared freshly before the experiment as 1 mg ml^{-1} stock solutions. Adrenaline was dissolved in 0.01 N HCl and ATP was buffered to pH 7.4. Aliquots of neurotensin, tetrodotoxin and apamin (all 1 μg μl^{-1}) were stored at −40°C for at least 6 months without significant loss in biological activity. Drug concentrations refer to the final molarity of the bases or peptides.

Results

Inhibitory action of neurotensin, adrenaline and ATP on rabbit ileum

The application of picomolar amounts of neurotensin caused a concentration-dependent inhibition of the pendular movements of the longitudinal muscle of the rabbit ileum ($n = 28$ rabbits). The inhibitory action of neurotensin was not sustained, but faded within 2–3 min, and the level of muscular activity prior to its addition returned. The inhibition was in all cases proportional to the concentration of neurotensin applied; the larger the concentration of the peptide the more profound was the inhibition of the spontaneous activity and this was sometimes accompanied by a relaxation of the muscle. Neurotensin

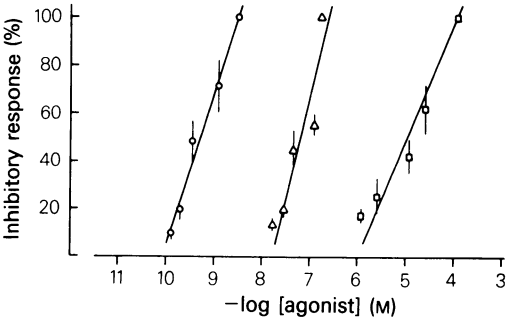

Figure 1 Concentration-response curves to neurotensin, adrenaline and adenosine triphosphate (ATP): Longitudinal muscles from the rabbit ileum containing the attached myenteric plexus were set up for isometric recordings. Increasing concentrations of neurotensin (○), adrenaline (△) or ATP (□) were added to the tissue chambers at 20 min intervals. The inhibition caused by each concentration was quantified and expressed as a percentage of the area of inhibition caused by 6 nM neurotensin, 150 nM adrenaline or 130 μM ATP. The area of inhibition generated by 6 nM neurotensin was 34.8 ± 2.8 g; that of 150 nM adrenaline was 37.7 ± 19.7 and that of ATP was 31.9 ± 12.2 g. The neurotensin IC_{50} was 0.7 nM ($n = 12$) whereas the IC_{50}s for adrenaline and ATP were 80 nM ($n = 8$) and 10,000 nM ($n = 4$) respectively. The concentration-response curve for neurotensin was derived from 12 ileal preparations, that of adrenaline from 8 and that of ATP from 4 separate ileum tissues.

Table 1 Lack of effect of several pharmacological treatments on the neurotensin-induced inhibitory response of the longitudinal muscle of the rabbit ileum myenteric plexus

	n	*Apparent neurotensin* IC_{50} (nM) ($\bar{x} \pm$ s.e.)
I. Control	8	0.75 ± 0.04
+ 12 μM phentolamine + 12 μM (−) propranolol	8	0.95 ± 0.13
II. Control	4	1.27 ± 0.18
+ 50 μM theophylline	4	2.06 ± 0.50
III. Control	8	1.06 ± 0.11
+ 100 nM tetrodotoxin	4	1.81 ± 0.77
+ 300 nM tetrodotoxin	4	0.72 ± 0.06

No significant differences were detected in the potency of neurotensin following the adrenoceptor, purinoceptor or Na^+-channel blockers.

produced a brief and short-lived rebound excitation in only 5 out of 28 preparations; this effect was not concentration-related. The inhibitory response to neurotensin was highly reproducible and was always reversed on washing.

Figure 1 shows the neurotensin concentration-response curve from 12 separate ileum preparations; a true maximal effect was not obtained due to the method of measurement of the response. The concentration of the neuropeptide for half maximal inhibition was approximately 0.7 nM. In as much as there were variations in the magnitude of the inhibition caused by the larger concentrations of the peptide and considering the variability from different segments of the ileum and from one animal to another, the IC_{50} values derived from concentration-response curves varied by 10–15%. Figure 1 also shows the concentration-response curves to ad-

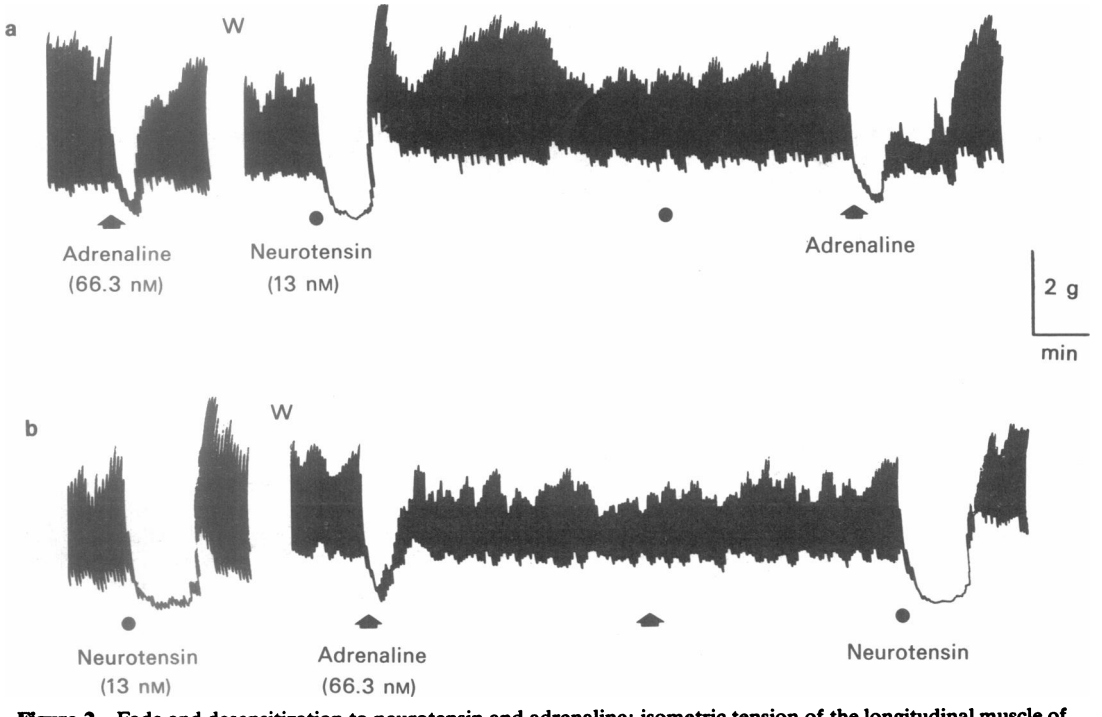

Figure 2 Fade and desensitization to neurotensin and adrenaline: isometric tension of the longitudinal muscle of the rabbit ileum. At the arrows applications of 66.3 nM adrenaline, W = wash and dots signify the addition of 13 nM neurotensin. Traces (a) and (b) were derived from a same ileal strip; between (a) and (b) 20 min and 4 washes with 10 ml Tyrode solution.

renaline and ATP. The catecholamine was approximately 100 times less potent than neurotensin in inhibiting the ileum, wheras ATP was about 10,000 times less potent. The inhibitory response to all three agonists was transient and faded within 2–3 min to base line values (Figure 2 and 3). Independent of the potency of each agonist, the concentrations causing the largest relaxations produced about the same area of inhibition (see legend to Figure 1).

Effect of adrenoceptor and purinoceptor antagonists

Antagonists of either adrenoceptors or purinoceptors did not significantly modify the potency of neurotensin in inhibiting the muscular activity of the rabbit ileum (Table 1). Preincubation of the tissues with 12 μM phentolamine, 12 μM (−)-propranolol, or both antagonists together (both at 12 μM) did not significantly alter the effectiveness of neurotensin. When tissues were pretreated with 50 μM theophylline there was no reduction in the inhibitory potency of the neuropeptide. At the concentration of phentolamine, propranolol or theophylline tested, none of these compounds influenced the frequency or the amplitude of the spontaneous contractions of the rabbit ileum, whereas the inhibitory responses to 44–110 nM adrenaline or 4–13 μM ATP were blocked in control experiments.

Pretreatment with tetrodotoxin (TTX)

A 2 min preincibation of the rabbit ileum with 100 or 300 nM TTX, did not significantly reduce the potency of neurotensin in inhibiting the pendular movements of the ileum (Table 1). TTX did not alter the amplitude of the pendular spontaneous activity of the longitudinal smooth muscles of ileum. The effectiveness of the sample of TTX utilized was simultaneously determined on the guinea-pig ileum; these concentrations abolished the contractile effects of transmural electrical stimulation of the tissue (0.15 Hz, 5 ms duration, 60 V).

Fade and desensitization to neurotensin: specificity of action

Since the inhibitory effect of neurotensin was transient, the possibility that the response was tachyphylactic was next investigated. Inhibition of the ileum by 13 nM neurotensin was followed 6 min later by its complete ineffectiveness, whereas 66.3 nM adrenaline caused an inhibition equivalent

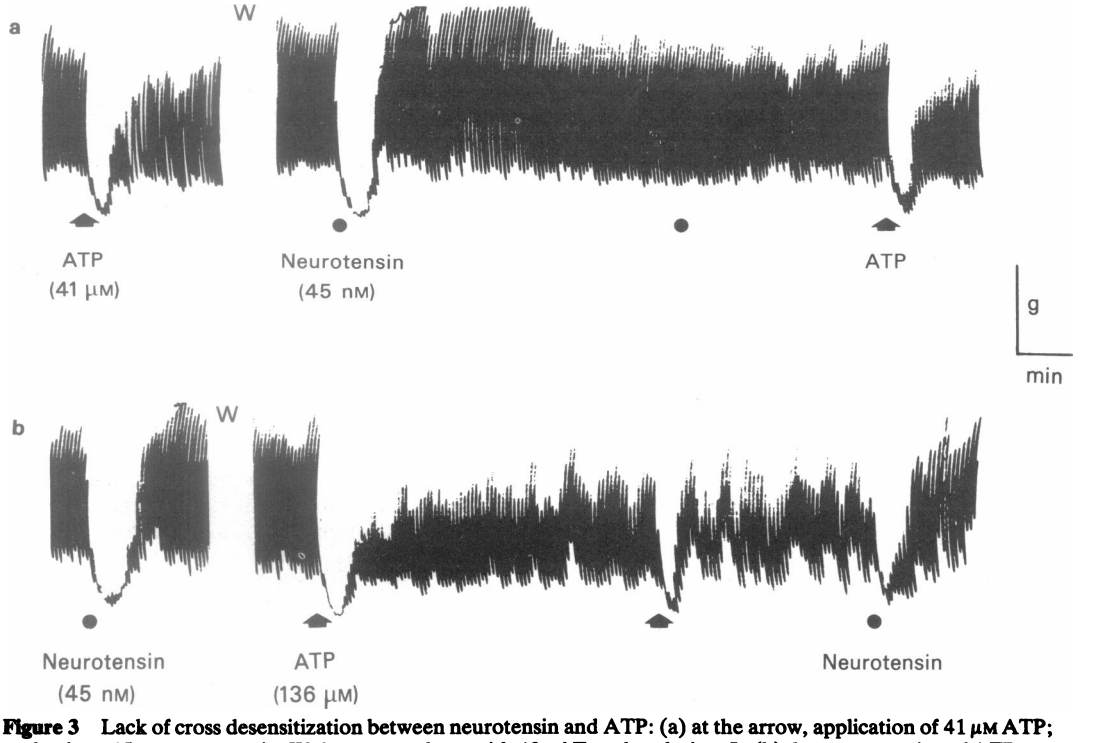

Figure 3 Lack of cross desensitization between neurotensin and ATP: (a) at the arrow, application of 41 μM ATP; at the dots, 45 nM neurotensin. W denotes washout with 40 ml Tyrode solution. In (b) the concentration of ATP was increased to 136 μM. The time between (a) and (b) was 20 min. Both traces were derived from the same preparation.

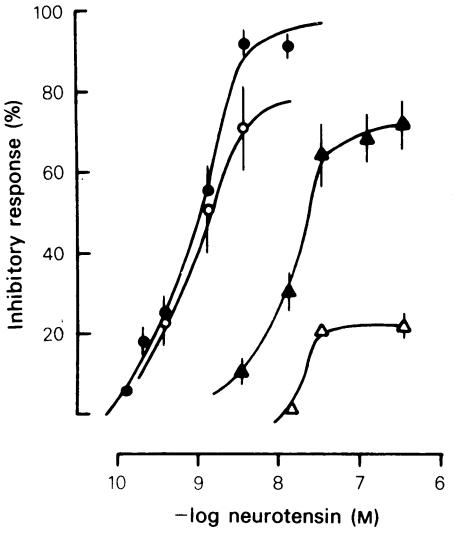

Figure 4 Desensitization of the ileum to neurotensin: neurotensin concentration-response curves were obtained from ileum preparations prior to (●) and following a 6 min pretreatment with either 1, (○) 5.24 (▲) or 52.4 nM (△) neurotensin. The points refer to the mean inhibitory response and the vertical bars the s.e.mean. The control curve was derived from 12 different preparations derived from 3 animals: $n = 4$ for the other lines.

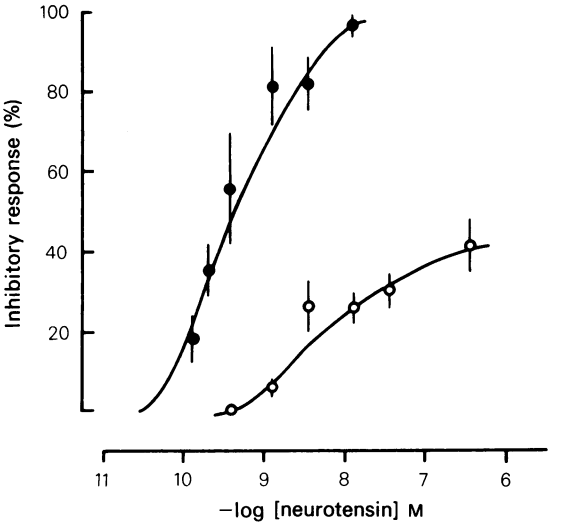

Figure 6 Effect of apamin on the neurotensin concentration-curve: neurotensin concentration-response curves on rabbit ileum preparations prior to (●) and 2 min after the application of 15 nM apamin (○). Points represent the mean inhibitory response and bars the s.e.mean ($n = 4$).

to that in the control (Figure 2a). Similarly, the ileum could be rendered tachyphylactic to adrenaline within a 5 min incubation period, but there was no loss of response to neurotensin (Figure 2b).

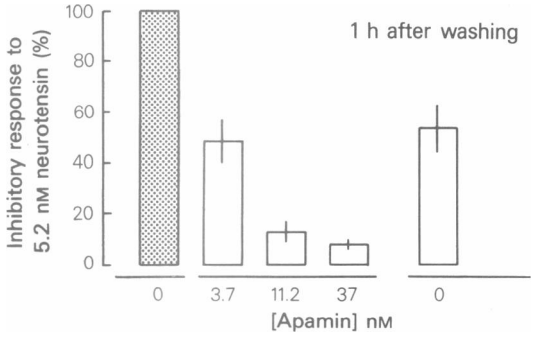

Figure 5 Antagonism of neurotensin by apamin: the area of inhibition caused by 5.2 nM neurotensin in four separate rabbit ileum strips was measured and expressed as 100% response (stippled colum). After washing, the tissues were incubated with apamin for a 2 min period, followed by the addition of 5.2 nM neurotensin. Columns indicate the mean percentage response and the bars the s.e.mean. The response of the ileum to neurotensin following an hour washing is depicted on the right.

Figure 3a shows that preparations desensitized to neurotensin responded normally to the inhibitory effect of ATP. In addition, Figure 3b illustrates that qualitatively, some degree of desensitization developed to the relaxant action of ATP. When ATP was used to desensitize the ileum, the inhibitory effect of neurotensin was found to be normal. Thus, desensitization was specific for each drug, probably signifying the occupancy of different receptors by each agonist.

Preincubation of intestinal segments with a low concentration of neurotensin (1 nM) did not significantly modify the concentration-response curve to a second application of the peptide. When the priming concentration of neurotensin was increased to 5.24 nM, there was a greater than 20 fold displacement of the neurotensin concentration-response curve to the right with a significant reduction ($P < 0.01$) of the maximal inhibitory effect of the peptide (Figure 4). With 52.4 nM neurotensin there was an almost complete loss in tissue response to a second application of the neuropeptide. As shown in Figure 4, the neurotensin concentration-response curve was markedly flattened, reaching a plateau at about 20% of inhibition ($P < 0.001$). After washing (40 ml min^{-1}), the sensitivity of the tissues to neurotensin recovered in a time-dependent fashion.

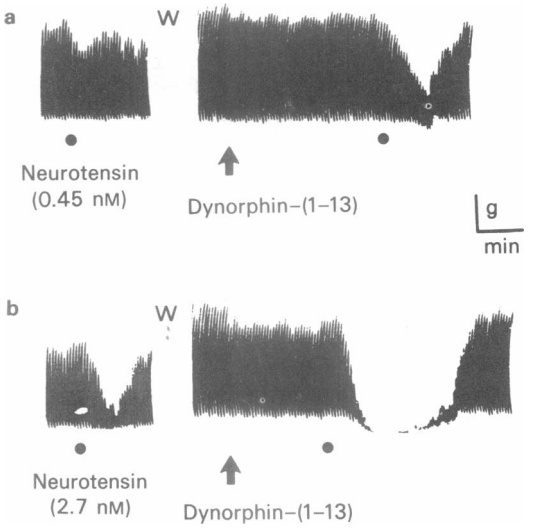

Figure 7 Dynorphin-neurotensin interaction: at the dots, application of neurotensin: upper panel (a), 045 nM; lower panel (b), 2.7 nM. At the arrows, application of 10 nM dynorphin. W indicates that tissues were washed with 40 ml drug-free Tyrode solution. Time between (a) and (b) was 30 min.

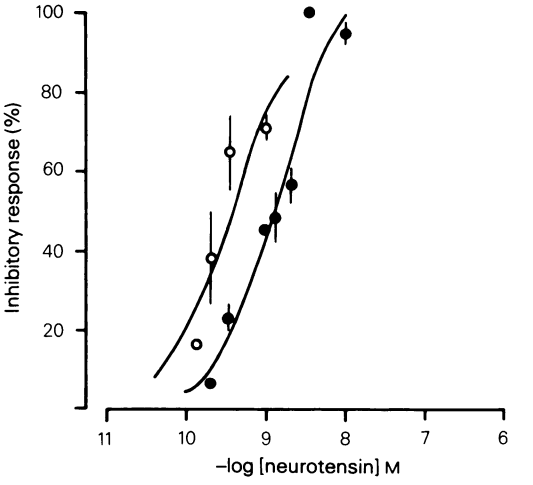

Figure 8 Effect of dynorphin-(1-13) on the concentration-response curve to neurotensin: neurotensin concentration-response curves were obtained in 8 ileum preparations before (●) and after 2 min incubation of the ileum with 10 nM dynorphin-(1-13) (○). Points are the mean inhibitory responses, and the bars are the s.e.mean ($n = 8$).

Apamin blockade of the neurotensin-induced inhibition

Apamin, the 19-amino acid bee venom toxin, was a powerful antagonist of the neurotensin-induced inhibitory response. As shown in Figure 5, a 2 min preincubation of the ileum with 3.7 nM apamin caused more than a 50% reduction in the inhibition induced by 5.2 nM neurotensin; increasing the concentration of apamin to 11.2 or 37 nM almost abolished the neurotensin-induced inhibition. In a parallel series of experiments, pretreatment of the tissues with 15 nM apamin caused a non-parallel displacement of the neurotensin concentration-response curve to the right (Figure 6). The antagonism produced by apamin was slowly reversible in that the inhibitory activity of 5.2 nM neurotensin recovered by little more than 50% 1 h after the apamin treatment (Figure 5). Apamin did not influence the amplitude or rate of the pendular movements of the ileum except at the highest concentration (37 nM), where there was a slow rise in the muscle tone. The action of apamin was not selective for neurotensin; the bee venom also reduced the inhibitory action of 44–110 nM adrenaline or 4–13 μM ATP.

Modification of the neurotensin sensitivity by dynorphin

Pretreatment of the tissues for 2 min with 10 nM dynorphin caused a small reduction in the pendular movements of the ileum and produced a significant ($P < 0.05$) potentiation of the inhibitory response to neurotensin (Figure 7). Figure 8 shows that the pretreatment of the ileum with dynorphin (10 nM) displaced the neurotensin concentration-response curve to the left in a parallel fashion and significantly increased the neurotensin potency from 0.93 ± 0.04 nM ($n = 8$) to 0.48 ± 0.07 nM ($n = 4$, $P < 0.01$). This effect of dynorphin was reversible on washing.

Discussion

It is likely that the inhibitory action of neurotensin on the rabbit ileum longitudinal muscle follows a direct effect of the neuropeptide on neurotensin inhibitory receptors, located on the smooth muscle membrane. Several arguments substantiate this conclusion. The neurotensin-induced inhibition was not antagonized either by adrenoceptor or purinoceptor blocking agents, suggesting that neither the release of endogenous catecholamines or purines nor the occupation of their receptor sites is involved in this response. The neurotensin-induced muscle relaxation was not antagonized by tetrodotoxin, which indicated that the inhibition was not of neuronal origin. The neurotensin inhibitory response was potently antagonized by apamin, a toxin with the recognized affinity and

selectivity for blocking Ca^{2+}-dependent K^+ channel induced hyperpolarizations in several biological preparations (Vladimirova & Shuba, 1978; 1980; Banks, Brown, Burgess, Burnstock, Claret, Cocks & Jenkinson, 1979; Brown & Burnstock, 1981).

The specificity of the desensitization experiments is entirely consistent with the view that neurotensin acts on selective inhibitory receptors distributed along the rabbit ileum. Based on the present results, using adrenoceptor or purinoceptor receptor antagonists and the desensitization experiments, it is possible to conclude that the neurotensin-induced inhibition is due to occupation of receptors that are specific for the neuropeptide and different from the sites activated by catecholamines or purine derivates. In the presence of desensitizing concentrations of neurotensin (5.2 and 52 nM), the concentration-response curve to neurotensin was shifted to the right and downwards, indicating a concentration-dependent loss of agonist potency and of the maximal effect. These results could be interpreted to indicate that there is a relatively small population of neurotensin inhibitory receptors, and therefore a relatively low receptor reserve capacity on the rabbit ileum. Desensitization, which is a reversible process, is apparently related to a temporal inactivation of a fraction of the active population of receptors rather than to fast metabolism of the neuropeptide by the tissues or to a metabolic muscular effect (Huidobro-Toro & Foree, 1980; Huidobro-Toro, Chelala, Bahouth, Nodar & Musacchio, 1982; Kachur, *et al.*, 1982). Desensitization could be demonstrated with relatively low concentrations of neurotensin; for example, the incubation of the tissues with 5 times a median effective concentration resulted in a 20 fold loss in potency and in a considerable reduction of the maximal inhibitory response. In this respect, the short-lived response of the ileum to neurotensin may be due to this desensitization phenomenon (Huidobro-Toro & Foree, 1980; Huidobro-Toro *et al.*, 1982).

It has been previously demonstrated that dynorphin and related opioid peptides potently antagonize the neurotensin-induced contractile response on the guinea-pig ileum (Zhu *et al.*, 1982; Huidobro-Toro, Zhu, Lee, Log & Way, 1983; Huidobro-Toro & Zhu, 1983; Huidobro-Toro, 1983). In the present experiments, dynorphin increased the inhibitory action of neurotensin on the rabbit ileum by an as yet unknown mechanism.

An interesting aspect of the pharmacology of neurotensin concerns the differences in the muscular responses to neurotensin in different animal species. Whereas neurotensin contracts the ileum of the guinea-pig via a neuronally-mediated effect (Kitabgi & Freychet, 1978; Huidobro-Toro & Way, 1982) in the rabbit and rat ileum the action of neurotensin is essentially inhibitory. In the fundus of the rat stomach and the taenia coli of the guinea-pig, neurotensin causes a contraction that is not sensitive to tetrodotoxin or to opioid peptides (Kitabgi & Freychet, 1978; Quirion, Regoli, Rious & St. Pierre, 1980; Kitabgi, 1982; Huidobro-Toro, 1983; Kullak & Huidobro-Toro, unpublished results). This implies that the neuropeptide probably activates excitatory, non-neuronal mechanisms related to excitatory receptors on the smooth muscle membrane. Interestingly, both excitatory and inhibitory actions of neurotensin can be obtained on different portions of the gastrointestinal tract of the guinea-pig. For instance, while neurotensin is a potent stimulant of the ileum of the guinea-pig, it relaxes the colon (Kitabgi & Vincent, 1981). This duality of action may indicate that there are two independent populations of excitatory and inhibitory neurotensin receptors. The present findings support the notion that neurotensin activates inhibitory neurotensin receptors located on the smooth muscle membrane surface, stimulation of which leads to muscle hyperpolarization that is related to the activation of a Ca^{2+}-dependent K^+ channels, as indicated by the results with apamin (Jenkinson, 1981; 1982).

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