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Characterization of neurotensin receptors in intestinal smooth muscle using a nonpeptide antagonist

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Abstract

Neurotensin reduced substance P-induced tension in ileal muscle strips and the relaxant effect was inhibited by a nonpeptide antagonist, SR 48692, 2-[(1-(7-chloro-4-quinolinyl)-5-(2,6-dimethoxyphenyl)pyrazol-3-yl)carbonylamino]tricyclo(3.3.1.l.^{3,7})decan-2-carboxylic acid with a half-maximal concentration (IC_{50}) of 7.4 ± 2.1 nM ($n = 9$) and a dissociation constant (K_b) of 0.9 ± 0.2 nM. Neurotensin produced a contractile response in ileal muscle strips pretreated with apamin (50 nM) and in isolated chick rectums and both contractile effects were inhibited by SR 48692 with IC_{50} of 31.6 ± 9.5 nM and K_b of 3.2 ± 0.9 nM ($n = 6$) and with IC_{50} of 28.9 ± 6.9 nM and K_b of 5.4 ± 1.0 nM ($n = 7$), respectively. The K_b values for the contractile effects were not significantly different from each other, but significantly different from that for the relaxant effect, suggesting that both types of effect are mediated via distinct subtypes of neurotensin receptor in the intestinal smooth muscles. Contractile responses and excitatory junction potentials evoked by electrical stimulation of nonadrenergic, noncholinergic (NANC) nerves in isolated chick rectums were not inhibited by SR 48692 (up to 3.3 μM). This does not provide functional evidence for the idea that neurotensin acts as an unidentified excitatory neurotransmitter of NANC nerves in the avian rectum.

Keywords: Neurotensin; SR 48692; Neurotensin receptor; NANC (nonadrenergic, noncholinergic) excitatory nerve; Smooth muscle, intestinal

1. Introduction

Neurotensin, which was first isolated from bovine hypothalamus (Carraway and Leeman, 1973), is located in neural (Schultzberg et al., 1980) and non-neural (Orci et al., 1976; Polak et al., 1977; Helmstaedter et al., 1977; Sundler et al., 1977) elements of the intestine, particularly in the small intestine (Carraway and Leeman, 1976). Neurotensin exerts various actions in the intestine: it has inhibitory and excitatory effects on the peristaltic activity (Ohashi et al., 1996), and produces contractile and relaxant responses in intestinal smooth muscle (Kitabgi and Freychet, 1979; Huidobro-Toro and Zhu, 1984; Goedert et al., 1984; Allescher et al., 1992; Mule et al., 1992; Ohashi et al., 1994). Under these circumstances, many reports suggest a possible role for the peptide as a neurotransmitter (Kitabgi and Vincent, 1981; Goedert et al., 1984; Komori et al., 1986), a circulating hormone (Carraway and Leeman, 1980; Theodorsson-Norheim and Rosell, 1983) or a modulator of intestinal motor activity (Kitabgi and Vincent, 1981; Bueno et al., 1985).

Recently, Gully et al. (1993) reported the pharmacological profile of a nonpeptide neurotensin antagonist, SR 48692. Using this antagonist, the existence of neurotensin receptor subtypes has been suggested (Dubuc et al., 1994; Labbe-Jullie et al., 1994). However, in the gastrointestinal tract, neurotensin receptors and their antagonism by SR 48692 are not fully characterized yet. In a previous paper (Ohashi et al., 1994), we found that neurotensin exerts a direct action to contract the longitudinal and circular smooth muscles of guinea-pig small intestine, in addition to its well-documented indirect action brought about by the release of acetylcholine from cholinergic nerves (Kitabgi and Freychet, 1978, 1979; Kitabgi, 1982; Huidobro-Toro and Way, 1982), and enhances the voltage-dependent inward Ca^{2+} current (Ohashi et al., 1994) in ileal smooth muscle cells of the guinea-pig. Neurotensin also exerts an apamin-sensitive inhibitory action to relax longitudinal and circular smooth muscles or inhibit muscarinic receptor-mediated contraction in circular smooth muscle of the guinea-pig (Ohashi et al., 1994).

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The present study was designed to characterize neurotensin receptors mediating relaxant and contractile responses to neurotensin in muscle strips of the longitudinal layer of guinea-pig ileum and in isolated chick rectums, and to investigate a possible role of neurotensin as an unidentified excitatory neurotransmitter in the avian rectum (Komori et al., 1986, 1992).

2. Materials and methods

2.1. Animals

Guinea-pigs of either sex, weighing 350–450 g, and young male chicks (Rhodhorn, GOTO 360), aged 10–60 days, were stunned and killed by exanguination.

2.2. Muscle strips of guinea-pig ileum

The ileum was removed from a killed guinea-pig, from which intestinal segments (about 5 cm long) were obtained by cutting. The longitudinal muscle layer of the intestinal segment was peeled from the underlying circular muscle, washed in Tyrode solution (composition, mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 2.1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 5.6. A muscle strip (3 mm wide and 50 mm long) was folded in two at a right angle to the longitudinal axis to prepare a double-layered muscle strip. The ends of the muscle strip were each tied with cotton thread.

2.3. Isolated chick rectums

The rectal region of the intestine was removed with Remak's nerve from a killed chick and the lumen was flushed clean with Tyrode solution. A 10 mm of the anal cut end of Remak's nerve was detached carefully from adhering tissues and prepared for electrical stimulation. Otherwise, the nerve was carefully removed. Both ends of the isolated rectum were tied with cotton thread.

2.4. Measurement of mechanical responses

The ileal muscle strip or the isolated chick rectum was set up in a 5-ml organ bath filled with Tyrode solution, which was bubbled with air and maintained at 37 ± 1°C: the ligated oral end of the preparation was attached by thread to a force-displacement transducer (Orientic, T7-30-240) and the anal end of the preparation was fixed by thread to the bottom of the organ bath. The preparation was allowed to equilibrate under a resting load of 0.3 g for 40 min or more before starting the experiment. The isometric tension developed longitudinally was picked up by the transducer, amplified by a preamplifier (Nihon Kohden, AS1202), and recorded on a potentiometric recorder (Nippon densi kagaku, U-228). In some experiments, the isolated rectum with Remak's nerve was set up horizontally in a 25-ml organ bath filled with Tyrode solution kept at 30°C in order to limit the spontaneous activity of the preparation and bubbled with air. The anal end of Remak's nerve was placed in a bipolar suction electrode for stimulation of the nerve with a stimulator (Nihon Khoden, MSE-3). Measurement and recording of longitudinal changes in tension were made substantially in the same way as described above. Guanethidine (2 μM) and atropine (1 μM) were used to block the adrenergic and cholinergic nerve pathways.

2.5. Measurement of excitatory junction potentials

The isolated chick rectum was sectioned longitudinally along the side opposite the edge which contained the axon terminals and pinned out in an organ bath (10 ml). Excitatory junction potentials were evoked by electrical field simulation of the intramural nerves with single rectangular pulses of 0.2 ms duration at a 2 s interval at an appropriate intensity (10–30 V). A pair of silver wire electrodes (1 mm in diameter), one insulated with Araldite except for the tip and placed on the tissue, the other uninsulated and placed in the organ bath, were used for electrical field stimulation. Records of excitatory junction potentials were made intracellularly using glass microelectrodes filled with 3 M KCl, with resistances of 40–80 MΩ after blockade of the adrenergic and cholinergic nerve pathways in the same way as described above. Furthermore, to bathing solution were added isoprenaline (1.3 μM) and an inhibitor of voltage-gated Ca^{2+} channels, methoxyverapamil (D 600, 10 μM) throughout of the course of the experiments. Isoprenaline served to suppress spontaneous electrical and mechanical activities of the rectal muscle and to maintain stable electrode impalement (Komori and Ohashi, 1982). D 600 served to prevent excitatory junction potentials from triggering discharge of action potentials.

2.6. Drugs

Drugs used were bovine neurotensin and substance P (from Peptide Institute, Osaka, Japan), a neurotensin receptor antagonist, SR 48692, 2-[(1-(7-chloro-4-quinolinyl)-5-(2,6 - dimethoxyphenyl)pyrazol -3- yl)carbonylamino]tricyclo(3.3.1.l.^{3,7})decan-2-carboxylic acid, (kindly supplied by Sanofi Researche, Montpellier, France), apamin and methoxyverapamil (D 600)(from Sigma, St. Louis, USA), atropine sulphate, isoprenaline sulphate, ATP and tetrodotoxin (from Wako, Tokyo, Japan), and carbachol chloride and guanethidine sulphate (from Tokyo Kasei, Tokyo, Japan). The stock solutions of all drugs were dissolved in distilled water, made up at 1000 or more times higher concentrations than those used for the experiments, and stored at −20°C. A certain amount of the concentrated drug solution was added to the organ bath and/or the

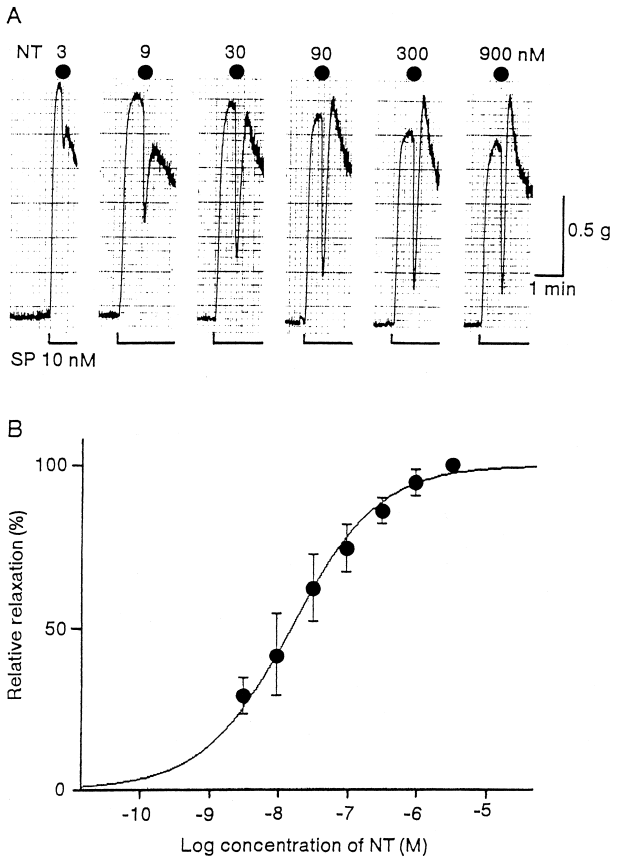

Fig. 1. Concentration–response relationship for the relaxant effect of neurotensin in longitudinal muscle strips from guinea-pig ileum. A, Relaxant responses to neurotensin (NT, 3–900 nM) in a muscle strip. The muscle tension was increased by substance P (SP, 10 nM). B, A mean concentration–response curve. Ordinate, percent change in the size of the relaxant responses. The maximum size of the relaxant response induced by 3 μM neurotensin was taken as 100%. Abscissa, −log (concentration of neurotensin). Each point represents the mean ± S.E.M. (vertical line) of 5 measurements.

reservoir to give the final desired concentration. The drug was washed away by replacing the bathing solution with fresh solution.

2.7. Calculations and statistical analysis

All data were expressed as means ± S.E.M., where *n* refers to the number of preparations or cells. The statistical significance was evaluated by using Student's paired or unpaired *t* tests. Values of $P < 0.05$ were considered significant. The concentrations of neurotensin and SR 48692 eliciting 50% of their maximal effect (EC_{50} and IC_{50}) were determined for each muscle preparation by Hill plot analysis of data points using a computer software (NEC9801RX). The antagonist dissociation constant (K_b) was calculated by the equation described by Cheng and Prusoff (1973) (the relation of K_b to IC_{50} derived from responses to an agonist at a single concentration can be expressed as follows:

$$K_b = IC_{50}/\{1 + ([A]/EC_{50})\} \quad (1)$$

where [A] is the fixed agonist concentration, EC_{50} is the neurotensin concentration causing 50% of its maximal effect, IC_{50} is the antagonist concentration reducing by 50% the effect of [A].

3. Results

3.1. The effect of SR 48692 on relaxant response to neurotensin in longitudinal muscle strips from guinea-pig ileum

Neurotensin produced a concentration-dependent reduction of substance P (10 nM)-induced tension in ileal muscle strips, as shown in Fig. 1A. The maximal effect of neurotensin was obtained, when it was applied at 3 μM. EC_{50} value, a concentration required to elicit 50% of the maximal response, was determined in five muscle strips and the mean EC_{50} was estimated to be 8.9 ± 3.3 nM

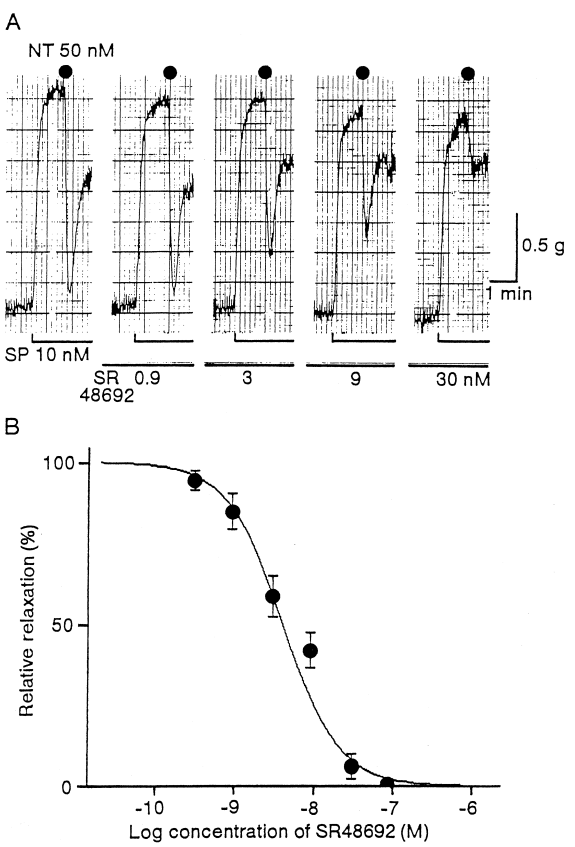

Fig. 2. The effect of SR 48692 on the relaxant response induced by 50 nM neurotensin in longitudinal muscle strips from guinea-pig ileum. A, Selective and concentration-dependent inhibition of the neurotensin-induced relaxation by SR 48692. The muscle tension was increased by substance P in the same way as in Fig. 1. B, A mean concentration-inhibition curve. Ordinate, percent change in the size of the relaxant responses. The size of the relaxant response to 50 nM neurotensin (NT) in the absence of SR 48692 was taken as 100%. Abscissa, −log (concentration of SR 48692). Each point represents the mean ± S.E.M. (vertical line) of 9 measurements.

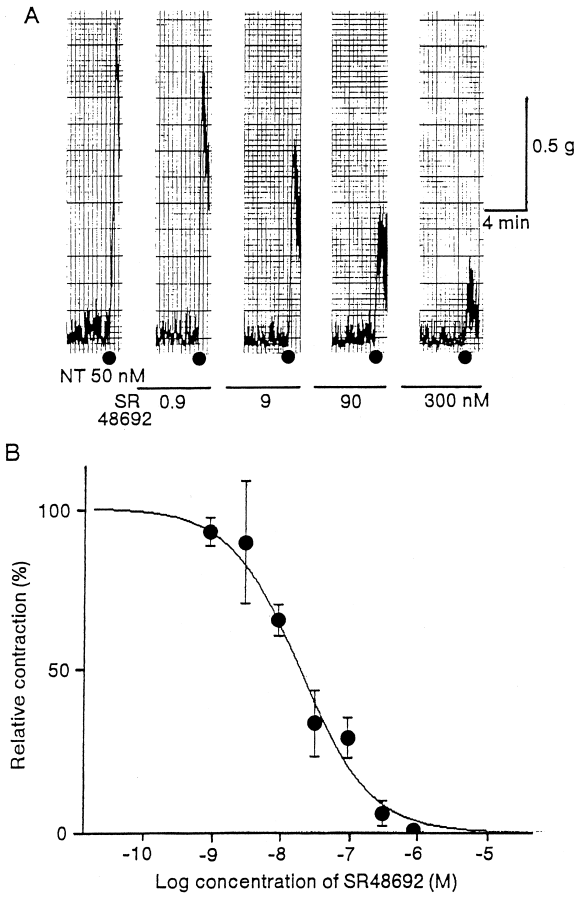

Fig. 3. The effect of SR 48692 on the contractile response induced by 50 nM neurotensin in longitudinal muscle strips from guinea-pig ileum. Contractile responses to 50 nM neurotensin (NT) were obtained in the presence of apamin (50 nM). A, Selective and concentration-dependent inhibition of the neurotensin-induced contraction by SR 48692. B, A mean concentration-inhibition curve. Ordinate, percent change in the size of the contractile responses. The size of the contractile response to 50 nM neurotensin in the absence of SR 48692 was taken as 100%. Abscissa, −log (concentration of SR 48692). Each point represents the mean ± S.E.M. (vertical line) of 6 measurements.

($n = 5$). Fig. 1B shows a mean concentration–response curve for the relaxant effect of neurotensin, in which each point represents the mean of five experiments. From the curve, neurotensin at a concentration of 50 nM was found to elicit about 70% of its maximum effect. This neurotensin concentration was chosen for studies with SR 48692. SR 48692, when applied at concentrations ranging from 0.3 to 90 nM, itself produced no detectable change in the basal tension and the tension induced by substance P, but inhibited neurotensin (50 nM)-induced relaxation. The inhibitory effect increased concentration-dependently with a concentration of 90 nM required to block completely, as shown in Fig. 2. The mean IC_{50} was determined to be 7.4 ± 2.1 nM ($n = 9$) and Hill coefficient was 1.10 ± 0.04 ($n = 9$). From Eq. (1) (see Section 2), K_b of SR 48962 was calculated by taking 50 nM of neurotensin as [A] and the mean EC_{50} of neurotensin (8.9 nM) as EC_{50}. Nine experiments gave a mean value for K_b as 0.9 ± 0.2 nM.

3.2. The effect of SR 48692 on contractile responses to neurotensin in longitudinal muscle strips from guinea-pig ileum

Neurotensin elicited a contractile response in the presence of apamin (50 nM) to serve to block its relaxant response. Neurotensin at 50 nM elicited a contractile response corresponding to about 90% of the maximal response in the concentration–response curve (EC_{50}, 5.8 nM) reported by Ohashi et al. (1994). The contractile response reached a peak tension within 1 min and then disappeared gradually. During the decay phase small tension changes were usually repeated. SR 48692 inhibited the contractile response to neurotensin in a concentration-dependent manner. The threshold concentration of SR

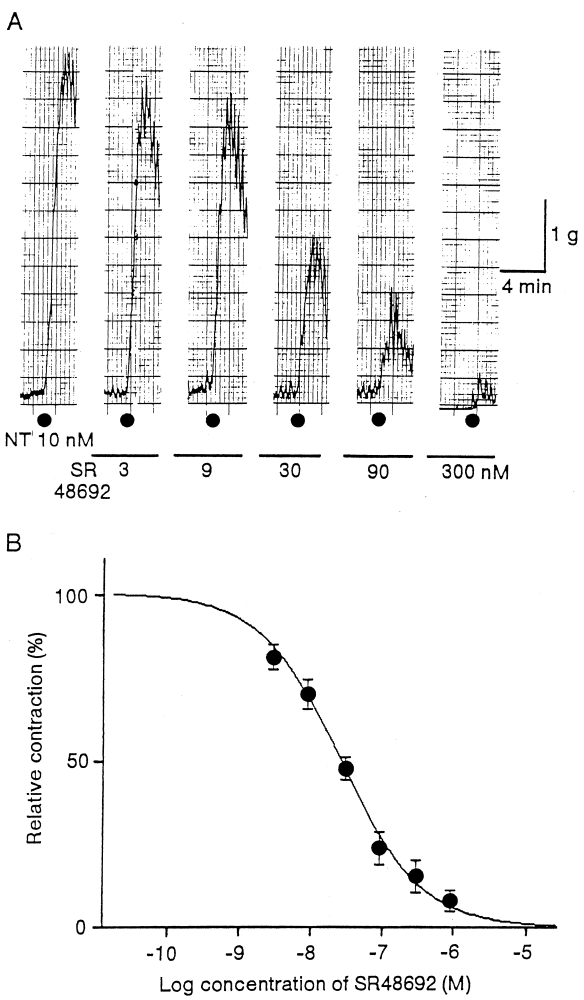

Fig. 4. The effect of SR 48692 on the contractile response induced by neurotensin (10 nM) in isolated chick rectums. SR 48692 was applied 15 min before neurotensin application and continued to be present. A, Selective and concentration-dependent inhibition of the neurotensin-induced contraction by SR 48692 in an isolated chick rectum. B, A mean concentration–inhibition curve. Ordinate, percent change in the size of the contractile responses. The size of the contractile response to 10 nM neurotensin in the absence of SR 48692 was taken as 100%. Abscissa, −log (concentration of SR 48692). Each point represents the mean ± S.E.M. (vertical line) of 6 measurements.

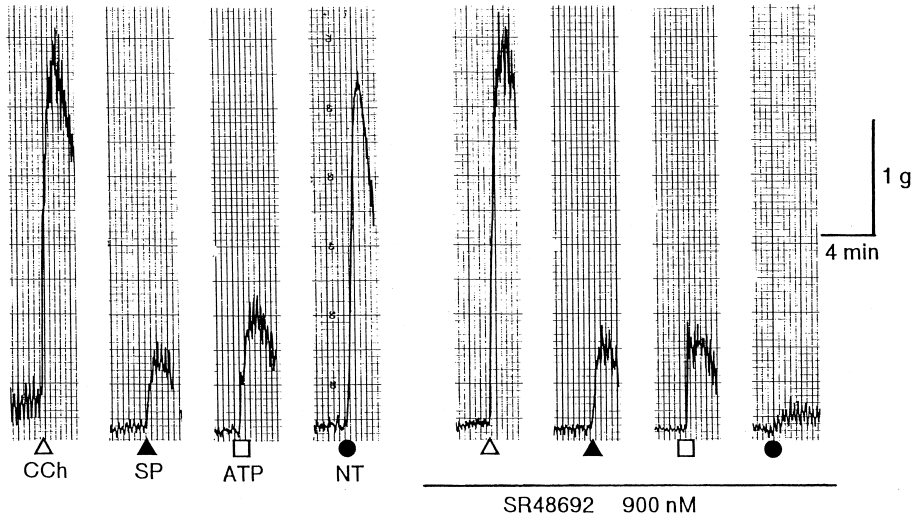

Fig. 5. The selective effect of SR 48692 on the contractile response to neurotensin in an isolated chick rectum. Contractile responses to carbachol (△, CCh 300 nM), substance P (▲, SP 5 nM), ATP (□, ATP 100 μM) and neurotensin (●, NT 10 nM) were obtained before (left panel) and 15 min or more after (right panel) application of SR 48692 (900 nM). See selective inhibition of the contractile effect of neurotensin with SR 48692.

48692 was 0.9 nM and concentrations more than 900 nM were required to block completely the neurotensin-induced contraction, as shown in Fig. 3. Six experiments gave mean values for Hill coefficient and IC_{50} as 0.90 ± 0.05 and 31.6 ± 9.5 nM, respectively. From IC_{50} in individual experiments, the mean K_b was calculated to be 3.2 ± 0.9 nM ($n = 6$) in the same way as used for inhibition of the relaxant response to neurotensin. The K_b value was significantly different from the corresponding value (0.9 ± 0.2 nM) derived in the relaxant response.

3.3. The effect of SR 48692 on contractile responses to neurotensin in isolated chick rectums

Neurotensin produced a concentration-dependent contraction in isolated chick rectums, as previously reported by Ohashi et al. (1994). Individual concentration–response curves obtained from six experiments gave a mean EC_{50} of 2.4 ± 0.4 nM ($n = 6$).

In experiments in which the inhibitory effect of SR 48692 on the contractile response to neurotensin was tested, the response to 10 nM neurotensin was chosen. The minimum concentration of SR 48692 required to inhibit the contractile response was 3 nM and the effect increased as the concentration was increased, as shown in Fig. 4. At 3 μM, it blocked the neurotensin (10 nM)-induced contraction completely. Seven experiments gave the mean Hill coefficient and IC_{50} as 1.0 ± 0.2 ($n = 7$) and 28.9 ± 6.1 nM ($n = 7$), respectively. SR 48692 was confirmed to inhibit selectively the contractile response induced by neurotensin, but did not affect contractile responses to carba-

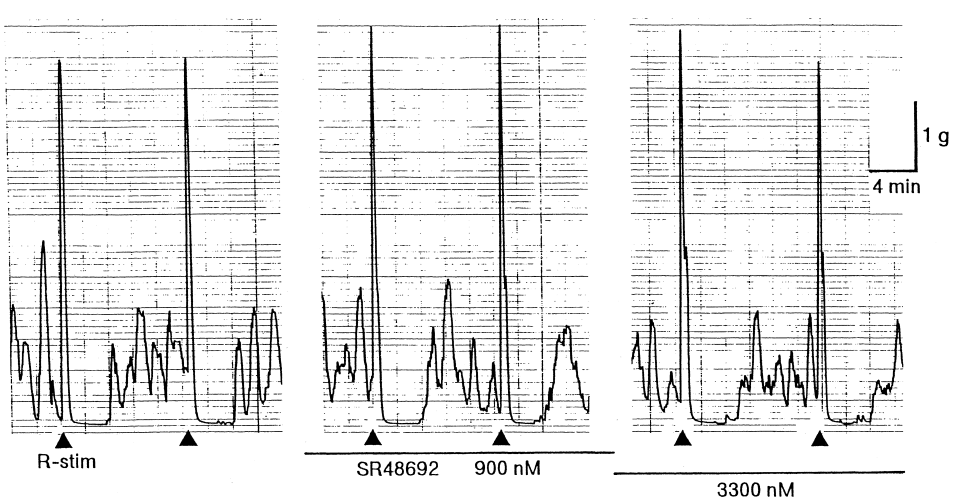

Fig. 6. The effect of SR 48692 on contractile responses to NANC nerve stimulation in an isolated chick rectum. Contractile responses to NANC nerve stimulation (▲, R-stim) with a train of 8 rectangular pulses (0.8 ms duration, 10 Hz, supramaximal intensity) were obtained before (left panel) and 15 min or more after (right panel) application of SR 48692 (3.3 μM). Guanethidine (2 μM) and atropine (1 μM) were used to block adrenergic and cholinergic pathways, respectively. See no change in the contractile responses.

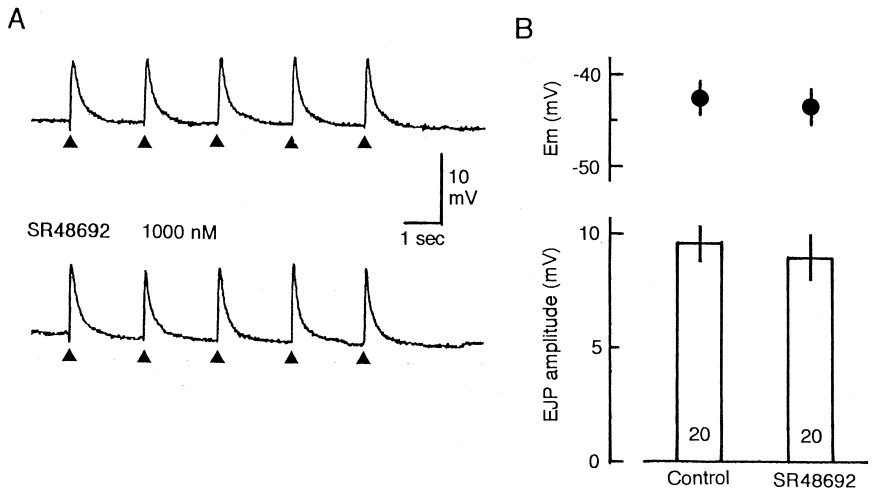

Fig. 7. The effect of SR 48692 on excitatory junction potentials (EJPs) evoked by NANC nerve stimulation in isolated chick rectums. EJPs were recorded intracellularly from smooth muscle cells after blockade of adrenergic and cholinergic pathways as described in Fig. 6. Furthermore, methoxyverapamil (D 600, 10 μM) and isoprenaline (1.3 μM) were used to prevent electrical and mechanical activities of the muscle preparation. NANC nerve stimulation (▲) was achieved by field stimulation of intramural nerves with single rectangular pulses (0.2 ms duration, supramaximal intensity). A, Control EJPs (upper panel) and EJPs in the presence of SR 48692 (1 μM). (lower panel). B, Mean resting membrane potential (Em) ± S.E.M. (vertical line) and mean EJP amplitude ± S.E.M. (vertical line) in the absence and presence of SR 48692 (1 μM) recorded from 20 cells in four different preparations. See no change in Em and EJP amplitude.

chol, substance P and ATP in isolated chick rectums, as shown in Fig. 5.

The mean K_b was calculated to be 5.4 ± 1.0 nM, which was not significantly different from that (3.2 ± 0.9 nM) for the contractile response to neurotensin, but significantly different from that (0.9 ± 0.2 nM) for the relaxant response to neurotensin, in ileal muscle strips.

3.4. The effect of SR 48692 on contractile responses to NANC nerve stimulation in isolated chick rectums

The rectum of the bird receives powerfully nonadrenergic, noncholinergic (NANC) excitatory innervation via Remak's nerve and the possibility has been proposed that neurotensin acts as an unidentified neurotransmitter of NANC nerves (Komori et al., 1986, 1992). NANC nerve stimulation elicited such a contraction that the tension peaked within 10 s after the onset of the nerve stimulation and declined with a similar time course to a lower level than its pre-stimulation one. In general, the lower tension persisted for some 60 s, as shown in Fig. 6. SR 48692 was applied at concentrations (up to 3.3 μM) at a time when the magnitude of contractile responses to NANC nerve stimulation at a 5 min interval was nearly constant, and 15 min later the nerve stimulation was repeated again. The highest concentration of the antagonist used was 500 times or more higher than IC_{50} in antagonizing the contractile response to neurotensin in isolated chick rectums. SR 48692 caused no detectable change in the responses. In fact, the magnitude of the responses after application of SR 48692 was $102.1 \pm 3.5\%$ of that of the control response (immediately before application of SR 48692) in three different preparations.

In some experiments, NANC excitatory junction potentials were evoked by electrical field stimulation of the intramural nerves with single pulses at a 2 s interval. Excitatory junction potentials were recorded from five cells in each of four different preparations and the resting membrane potential of each cell was measured 500 ms before the first excitatory junction potential. The mean resting membrane potential was -42.5 ± 1.5 mV ($n = 20$). The excitatory junction potentials increased in amplitude until the fifth stimulus, and then they were evoked with a constant amplitude, as previously reported (Komori and Ohashi, 1982). The mean amplitude of the fifth excitatory junction potentials was 9.6 ± 0.8 mV ($n = 20$), as shown in Fig. 7, which was not significantly different from 9.0 ± 0.9 mV ($n = 20$) measured after application of SR 48692 (1 μM). The mean of 43.2 ± 1.7 mV ($n = 20$) for the resting membrane potential was also not significantly different from the control.

4. Discussion

The nonpeptide neurotensin antagonist, SR 48692 antagonized both the relaxant and contractile effects of neurotensin in ileal muscle strips of the guinea-pig with different potencies (K_b values, 0.9 nM and 3.2 nM, respectively). The K_b values were distinct from each other. This can be readily explained by assuming that the relaxant and contractile effects are mediated via different subtypes of neurotensin receptor. The idea is not incompatible with the fact that neurotensin produces the two types of effect with different potencies (EC_{50}, 8.9 nM for the relaxation, 5.8 nM for the contraction) which correlates

with its binding affinity. The K_b value (3.2 nM) of SR 48692 in antagonizing the contractile response to neurotensin in guinea-pig ileal muscle strips is not significantly different from that (5.4 nM) in isolated chick rectums. The results suggest that SR 48692 exerts its inhibitory effect on the contractile responses to neurotensin in the two different types of preparation by its action on similar neurotensin receptors. Recently, Mule et al. (1996) characterized the contractile and relaxant responses to neurotensin in rat duodenum and colon and suggested that both responses are mediated via neurotensin receptors which are related but not identical to each other. However, K_b values derived in rat duodenum and colon are slightly higher than those in ileal muscle strips of guinea-pig. Therefore, SR 48692 might exert its antagonistic action with some species specificity, as suggested by Mule et al. (1996).

Neurotensin exerts a direct action to contract the longitudinal and circular muscles of guinea-pig small intestine (Ohashi et al., 1994), in addition to its well-documented indirect action brought about by the release of acetylcholine from neural elements (Kitabgi and Freychet, 1978, 1979; Kitabgi, 1982; Huidobro-Toro and Way, 1982). If so, since the Hill coefficient was close to 1, the two types of action may be mediated via the same type of neurotensin receptors. If different neurotensin receptors are involved, SR 48692 might not discriminate between them. The K_b (2.4 nM) was not significantly different from that (5.4 nM) for the inhibition of the contractile effect of neurotensin brought about by its direct action alone in chick rectums, suggesting that the contractile responses, independent of species, may be mediated via pharmacologically-indistinguishable neurotensin receptors.

Recently, Labbe-Jullie et al. (1994) reported that the K_b values of SR 48962 for the inhibition of the contractile and relaxant responses to neurotensin in ileal muscle strips and in isolated ileal segments of guinea-pig ileum, respectively, were 3.9 nM and 5.1 nM, and that these two K_b values were not statistically different from each other. The K_b value for antagonism against the contractile response to neurotensin was well comparable with the present value. However, the K_b value for the inhibition of the relaxant effect was about 2 times higher than the present value. The discrepancy may result from their use of isolated ileal segments in which, irrespective of intensity, both relaxant and contractile responses are produced by neurotensin in the longitudinal and circular muscle layers. The overall relaxant effect in the longitudinal axis is frequently complicated by such a simultaneous contraction of the circular muscle layer that it results in elongation of the segment (Ohashi et al., 1994).

In guinea-pig small intestine, neurotensin exerts both inhibitory and excitatory actions via its receptors sensitive to SR 48692 on the peristaltic activity (Ohashi et al., 1996). The excitatory action varies with an increasing gradient toward the terminal end of the small intestine, and the inhibitory action involves apamin-sensitive mechanism. Taken together with the present results, the regional variation of the neurotensin effect on the peristaltic activity may be attributable to difference of the distribution of neurotensin receptor subtypes.

The rectum of the bird receives NANC excitatory innervation from Remak's nerve which is the most conspicuous nerve sending many fibers to the digestive tract of the bird (Takewaki et al., 1977). Although the neurotransmitter has not been identified, neurotensin is proposed as a candidate for it since neurotensin could be isolated from the rectum (Iwabuchi et al., 1987) and exogenously-applied neurotensin produced mechanical and membrane responses similar to those to NANC nerve stimulation (Komori et al., 1986, 1992). SR 48692, which can antagonize the contractile response to neurotensin, however, was without effect on NANC contractile responses and EJPs, providing a negative evidence for the idea that neurotensin acts as an unidentified neurotransmitter of NANC nerves in the rectum of the bird. If there are neurotensin receptor subtypes insensitive to SR 48692, the idea can still persist.

In summary, SR 48692 antagonized the contractile and relaxant responses to neurotensin in intestinal smooth muscles and both inhibitory and excitatory effects of neurotensin are mediated via distinct neurotensin receptor subtypes. Physiological or pathophysiological functions of intestinal neurotensin are expected to be elucidated by use of SR 48692.

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