Br. J. Pharmacol. (1988), **95**, 39–48

Presence of a substance P-like peptide in an acid extract of the intestinal bulb of the carp (*Cyprinus carpio*)

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1 The effect of an acid extract of the carp intestinal bulb (ECI) on guinea-pig ileum longitudinal smooth muscle (GPLM) and carp intestinal bulb longitudinal smooth muscle (CIBLM) was examined.

2 ECI caused a concentration-dependent contraction of GPLM and CIBLM. This ECI-induced response was reduced by atropine to 30–40% of the control, indicating that part of the contracting activity of ECI is attributable to acetylcholine. The atropine-resistant contracting activity of ECI was not mediated by histamine, 5-hydroxytryptamine, ATP, ADP, angiotensin II, neurotensin, vasoactive intestinal peptide or an opioid peptide.

3 The active material mediating the atropine-resistant contracting activity is probably a peptide, because the contraction in response to ECI was abolished on incubation with pepsin or α-chymotrypsin.

4 [D-Pro^2, D-Trp^{7,9}]-substance P, [D-Pro^4, D-Trp^{7,9}]-substance P (4–11) decreased the atropine-resistant contracting activity of ECI as did desensitization induced by substance P.

5 On a Sephadex G 25 column, the active material was eluted as one peak. The active fractions were pooled and then applied to another Sephadex G25 column to compare the Ve/Vo value for the active material with those for peptides of known molecular weights. The molecular weight of the active material was estimated to be 1200–1700 (1410 ± 70, $n = 6$).

6 The results indicate the presence of a substance P-like peptide in the carp intestinal bulb.

Introduction

It is well known that the contractile response following electrical stimulation of the mammalian intestinal tract is only partially inhibited by atropine or scopolamine. Indeed, the presence of a non-cholinergic, non-adrenergic (NCNA) nerve supply for intestinal smooth muscle has been postulated in mammals (Ambache & Freeman, 1968; Ambache *et al.*, 1970; Furness, 1971; Franco *et al.*, 1979a,b; Bauer & Kuriyama, 1982). The neurotransmitter of some NCNA nerves is considered to be ATP, 5-hydroxytryptamine or substance P. These substances were found in enteric nerves, released on depolarizing stimulation and caused a similar effect to that of NCNA nerve stimulation (Burnstock, 1972; Burnstock *et al.*, 1972; Costa & Furness, 1979; Franco *et al.*, 1979a,b; Leander *et al.*, 1981; Wali, 1985; Matušák & Bauer, 1986).

In a previous study, we demonstrated the excitatory innervation with non-cholinergic nerves in the carp intestinal bulb (Kitazawa *et al.*, 1986). The pharmacological properties of the non-cholinergic response of the carp intestinal bulb indicated that the neurotransmitter was not catecholamine, histamine, 5-hydroxytryptamine or an opioid peptide (Kitazawa *et al.*, 1987). ATP and related substances (ADP, AMP and adenosine) were unable to cause contraction of the carp intestinal bulb in the presence of atropine. Therefore, it is unlikely that ATP and related substances are neurotransmitters of NCNA nerve.

As neurotransmitters are synthesized in the nerve

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cell body, transported by axonal flow and stored in nerve terminals, one approach for identifying the neurotransmitter of NCNA nerves is to extract the bioactive material from the tissue which can cause a similar response to the NCNA response. Active peptides have been extracted from bovine spinal cord (Takahashi *et al.*, 1974), guinea-pig ileum (Franco *et al.*, 1979b) and chick Remak's nerve (Komori *et al.*, 1986), and the possibility of these peptides being the neurotransmitter of NCNA nerves has been discussed.

In the present study, we extracted a smooth muscle contracting material, other than acetylcholine, from the carp intestinal bulb, examined the pharmacological and biochemical properties of the active material and compared them with those of known bioactive materials.

Methods

Animals and extraction

Carp (*Cyprinus carpio*) of either sex, weighing from 800 to 1000 g, were killed by decapitation. After the abdominal cavity had been opened by means of a midline incision, the intestinal bulb was isolated and immediately immersed in an ice-cold modified Krebs solution containing physostigmine ($30\,\mu g\,ml^{-1}$). A segment of the carp intestinal bulb (about 3 g) was blotted with filter paper, weighed, minced with fine scissors and then placed in a glass tube containing 10 volumes of 1 N acetic acid: 0.02 N HCl mixture, followed by boiling in a water bath (100°C) for 10 min, cooling and finally homogenization. The homogenate was centrifuged (4°C, 10000 *g* for 30 min), and the supernatant was collected, fat removed by washing with petroleum ether (3–4 times) and then lyophilized. The dried material was stored in a deep freezer (−20°C) until used.

Bioassay

In order to determine the smooth muscle contracting activity of the lyophilized extract of the carp intestinal bulb, guinea-pig ileum longitudinal smooth muscle (GPLM) and, if needed, carp intestinal bulb longitudinal smooth muscle (CIBLM) were used. Both preparations (30 mm length) were suspended vertically in an organ bath (4 ml) containing Tyrode solution (35°C for GPLM) or a modified Krebs solution (20°C for CIBLM), equilibrated with a 95% O_2 + 5% CO_2 gas mixture. Tyrode solution and the modified Krebs solution used had the following compositions (mM); Tyrode solution: NaCl 136.9, KCl 2.7, NaH_2PO_4 0.4, $CaCl_2$ 1.8, $MgCl_2$ 2.1, $NaHCO_3$ 11.9 and glucose 5.6; modified Krebs solution: NaCl 100.8, KCl 3.35, $CaCl_2$ 1.5, KH_2PO_4 1.17, $MgSO_4$ 1.17, $NaHCO_3$ 25 and glucose 11.1. The mechanical activity of each preparation was measured with an isotonic transducer (Nihon Kohden, TD-112S) or a force displacement transducer (Nihon Kohden, TB-612T), and recorded with an inkwriting oscillograph. Each preparation was initially loaded at 1.5 g tension and then allowed to equilibrate for 60 min before starting the experiment. The dried material (lyophilized extract) was dissolved in distilled water to a concentration equivalent to 500 mg tissue wet weight per ml, followed by centrifugation at 10,000 *g* for 20 min at 4°C to remove insoluble materials. The drugs and the reconstituted extract tested were usually added to the organ bath directly with a micropippete in a volume of less than 1% (40 μl) of the bath volume. The extract and drugs were washed out from the organ bath by replacing the bathing solution with fresh nutrient medium.

Stability in acid and alkali solutions

Both the extract and drug solutions (ATP and ADP) were mixed with an equal volume of 2 N HCl or 2 N NaOH and then incubated in boiling water for 5 min. After neutralization, the smooth muscle contracting activity of these solutions toward GPLM was examined.

Gel filtration

The lyophilized extract was dissolved in 0.1 N acetic acid (500 mg tissue wet weight per ml) and then applied to a Sephadex G-25 column (2 × 66 cm) pre-equilibrated with 0.1 N acetic acid solution. Elution was performed with the same acid solution at a flow rate of $30\,ml\,h^{-1}$ in a cold room (4°C). Elution was monitored with a u.v. monitor at 280 nm. Fractions of 4 ml were collected at 4°C and then lyophilized. The lyophilized fractions were dissolved in 0.4 ml of distilled water and then their smooth muscle contracting activity was bioassayed on GPLM and CIBLM. The active fractions were collected, lyophilized and applied to another Sephadex G-25 column (1.2 × 60 cm) to estimate the molecular weight of the excitatory material. Elution was performed with 0.1 N acetic acid solution. Three ml fractions were collected and their contracting activity was bioassayed on GPLM. The void volume (Vo) of each column was determined with bovine serum albumin, and the elution volumes (Ve) of leucine-enkephalin, angiotensin II, substance P and motilin were determined by monitoring the peak u.v. absorbance at 280 nm or 220 nm.

Drugs

The following drugs were used: acetylcholine chloride (Wako), atropine sulphate (Tokyo Kasei), ATP (Sigma), ADP (P-L Biochemical Inc.), AMP (Wako), adenosine (Wako), 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP, Aldrich Chemical Company Inc.), histamine hydrochloride (Wako), 5-hydroxytryptamine creatinine sulphate complex (Sigma), pyrilamine hydrochloride (Sigma), methysergide hydrogen maleate (Sandoz), tetrodotoxin (Sankyo), angiotensin II, [Sar^1, Ala^8]-angiotensin II leucine-enkephalin, methionine-enkephalin, motilin, neurotensin, substance P, [D-Pro^2, D-Trp^{7,9}]-substance P, [D-Pro^4, D-Trp^{7,9}]-substance P (4–11) and vasoactive intestinal peptide (Peptide Institute Inc.). All drugs were dissolved in distilled water to the desired concentrations and added to the organ bath.

Statistical analysis

All values are expressed as means and s.e. The statistical significance of differences was determined by Student's t test. A 0.05 level of probability was accepted as being significant.

Results

Smooth muscle contracting activity of the extract

The extract of the carp intestinal bulb caused contraction of guinea-pig ileum longitudinal smooth muscle (GPLM). The contraction was rapid in onset and maximum tension was reached within 10 s after application of the extract. The contractile response to the extract was reproducible on repeated 2 min exposures with 15 min intervals. The extract at a concentration of 0.1 mg tissue wet weight per ml was effective in causing detectable contraction and the amplitude of the contraction increased in a concentration-dependent manner up to 5 mg tissue wet weight per ml (Figure 1). Tetrodotoxin (500 ng ml^{-1}), a concentration sufficient to abolish the response to DMPP (3.2 μg ml^{-1}), a stimulant of nicotinic cholinoceptors primarily on cholinergic nerves (Brownlee & Johnson, 1963), did not affect the contractile response to the extract (Figure 1), indicating that the extract acted directly on smooth muscle cells. The excitatory action of the extract was significantly reduced, to 30–40% of the control by atropine 200 ng ml^{-1}, which was sufficient to abolish the contraction due to acetylcholine up to 200 ng ml^{-1} (Figure 1). These results indicate that a part of the smooth muscle contracting activity of the extract is attributable to acetylcholine. In the presence of atropine, the extract-induced contraction

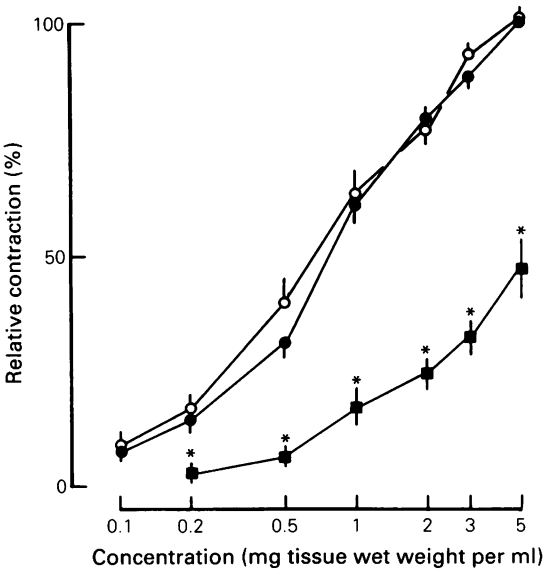

Figure 1 Relationship between the contractile response and the concentration of the extract in guinea-pig ileum longitudinal smooth muscle. Concentration-response curves for the extract in normal Tyrode solution (●), in the presence of tetrodotoxin (500 ng ml^{-1}) (○) and in the presence of tetrodotoxin (500 ng ml^{-1}) plus atropine (200 ng ml^{-1}) (■). Ordinate scale: relative amplitude of contraction expressed as a percentage of that induced by 5 mg tissue wet weight per ml. Abscissa scale: log extract concentration (mg tissue wet weight per ml). Each point is the mean of six experiments with s.e.mean shown by vertical lines. *Significant difference from the control response ($P < 0.01$).

was frequently preceded by a brief relaxation.

The acetylcholine concentration of the extract was examined on frog abdominal rectus muscle treated with physostigmine. This preparation has higher selectivity for acetylcholine compared with GPLM. The concentration of acetylcholine in the extract was estimated to be 870 ± 50 ng g^{-1} tissue wet weight ($n = 16$). The acetylcholine- and extract-induced contractions of the skeletal muscle were completely abolished by (+)-tubocurarine (1 μg ml^{-1}).

Comparison of the extract-induced contraction with that caused by histamine, 5-hydroxytryptamine, and ATP and related substances

Histamine Histamine caused a concentration-dependent contraction of GPLM (1–500 ng ml^{-1}) in the presence of atropine. The contractile response to histamine (15 ng ml^{-1}) was completely abolished by pyrilamine (1 μg ml^{-1}). However, the contractile

response to the extract of the same amplitude was unaffected by pyrilamine (Table 1).

5-Hydroxytryptamine 5-Hydroxytryptamine (5-HT) exhibited an excitatory action on GPLM in the concentration range of 1 ng ml^{-1} to 3 μg ml^{-1}. Methysergide (1 μg ml^{-1}) markedly decreased the contractile response to 5-HT (900 ng ml^{-1}) but had no effect on the extract-induced contraction (Table 1).

ATP and related substances ATP and ADP caused contraction of GPLM at concentrations higher than 10 μg ml^{-1} but the response decreased in spite of the continued presence of ATP and ADP in the bath. Neither AMP nor adenosine produced contraction, even at 300 μg ml^{-1}. The stabilities of the contractile activities of the extract and ATP in an acid or alkali solution were compared. The contractile activity of the extract was abolished after boiling in 1 N NaOH (for 5 min), but that of ATP was retained fairly well after this treatment. On the other hand, boiling in 1 N HCl for 5 min markedly decreased the contractile activity of ATP but not that of the extract (Table 1). Similarly, in the case of ATP, the contractile activity of ADP was also abolished after boiling in 1 N HCl (for 5 min).

Effects of pepsin and α-chymotrypsin pretreatment on the extract-induced contraction

Pepsin and α-chymotrypsin (Sigma Chemical Co.) were used to investigate the sensitivity of the active material to proteolytic enzymes.

Pepsin was dissolved in 0.1 N HCl at the desired concentration. The lyophilized extract was also dissolved in the same acid solution. Pepsin was added to aliquots of the extract to a final concentration of 50 μg ml^{-1}. Pepsin boiled for 30 min was used as inactivated pepsin. Each reaction mixture (extract plus enzyme) was incubated at 37°C in a water bath for 30 min. The contractile activity of the extract was markedly decreased on incubation with active pepsin but not with inactivated pepsin (Table 1).

In further experiments instead of pepsin, α-chymotrypsin was used. Both the lyophilized extract and α-chymotrypsin were dissolved in 0.05 M Tris-HCl buffer solution (pH 7.4). α-Chymotrypsin was added to the extract to a final concentration of 50 μg ml^{-1}, and then the reaction mixture was incubated at 37°C for 30 min. As in the case of pepsin, the contractile activity of the extract was abolished by α-chymotrypsin treatment (Table 1). These results suggest that the activity of the extract, which is resistant to atropine, methysergide and pyrilamine, is due to a peptide.

Comparison of the extract-induced contraction with that caused by some bioactive peptides

Angiotensin II, neurotensin and substance P caused the contraction of GPLM in the presence of atropine, pyrilamine and methysergide. However, methionine-enkephalin and leucine-enkephalin were ineffective on GPLM.

Angiotensin II The contractile response to angiotensin II (2.1 ng ml^{-1}) showed a slow and prolonged time course quite different from that of the extract-induced contraction. [Sar^1, Ala^8]-angiotensin II (1.9 μg ml^{-1}), a specific antagonist of the angiotensin II receptor, abolished the contractile response to angiotensin II (2.1 ng ml^{-1}), but had little effect on the response to the extract (Table 1).

Neurotensin Neurotensin (100 ng ml^{-1}) caused a transient contraction of GPLM, which was usually preceded by brief relaxation, with a slow time course. With the continued presence of neurotensin in the organ bath (100 ng ml^{-1}, for 8 min), the responsiveness of GPLM to subsequently applied neurotensin (100 ng ml^{-1}) was obviously decreased (Table 1), indicating that exposure to neurotensin for long periods caused the desensitization of the receptor for neurotensin. However, the extract-induced contraction was unaffected in this desensitized preparation (Table 1).

Substance P Substance P caused contraction of GPLM, the time course being similar to that produced by the extract. The contractile activity of substance P was abolished by pretreatment with pepsin (50 μg ml^{-1} in 0.1 N HCl) and α-chymotrypsin (50 μg ml^{-1} in 0.05 M Tris-HCl buffer) (Table 2). [D-Pro^2, D-Trp^{7,9}]-substance P (15.1 μg ml^{-1}) or [D-Pro^4, D-Trp^{7,9}]-substance P (4–11) (11.3 μg ml^{-1}) pretreatment (for 5 min) markedly decreased the contractile responses to substance P (3 ng ml^{-1}) and the extract (5 mg tissue wet weight per ml) (Figure 2a). However, the contractile response to acetylcholine (2 ng ml^{-1}) was unaffected by both antagonists (Table 2). The inhibition due to [D-Pro^2, D-Trp^{7,9}]-substance P (15.1 μg ml^{-1} for 5 min) was 65.3 ± 2.9% (n = 5) for the extract (5 mg tissue wet weight per ml) and 73 ± 4.1% (n = 6) for substance P (3 ng ml^{-1}) and that due to [D-Pro^4, D-Trp^{7,9}]-substance P (4–11) (11.3 μg ml^{-1}) was 68 ± 2.6% (n = 7) for the former and 72 ± 4.1% (n = 6) for the latter, respectively. Exposure of GPLM to a high concentration of substance P (650 ng ml^{-1}) caused a large but only transient contraction, and in the continued presence of such a high concentration of sub-

Table 1 Pharmacological and biochemical properties of the atropine-resistant smooth muscle contracting activity of the extract of the carp intestinal bulb

	Relative contraction[1]			
	Pyrilamine ($1\,\mu g\,ml^{-1}$)	*Methysergide* ($1\,\mu g\,ml^{-1}$)	[*Sar*[1], *Ala*[8]]-*angiotensin II* ($1.9\,\mu g\,ml^{-1}$)	*Neurotensin desensitization*[2] ($100\,ng\,ml^{-1}$ *for* 8 min)
Pharmacological properties				
Extract (5 mg tissue wet weight per ml)	102 ± 2 (7)[3]	93 ± 5 (5)	111 ± 6 (6)	103 ± 3 (4)
Histamine ($15\,ng\,ml^{-1}$)	0 ± 0 (7)	105 ± 3 (5)	109 ± 5 (5)	122 ± 7 (5)
5-Hydroxytryptamine ($900\,ng\,ml^{-1}$)	104 ± 8 (5)	29 ± 6 (6)	105 ± 14 (5)	105 ± 16 (5)
Angiotensin II ($2.1\,ng\,ml^{-1}$)	105 ± 8 (5)	95 ± 8 (5)	0 ± 0 (6)	103 ± 8 (5)
Neurotensin ($100\,ng\,ml^{-1}$)	98 ± 6 (4)	96 ± 6 (4)	93 ± 5 (5)	8 ± 2 (5)
Biochemical properties				
Acid and alkali treatment		*Acid treatment*		*Alkali treatment*
Extract (3 mg tissue wet weight per ml)		99 ± 4 (5)		9 ± 4 (5)
ATP ($100\,\mu g\,ml^{-1}$)		29 ± 8 (5)		99 ± 3 (4)
Enzyme pretreatment	*Pepsin* ($50\,\mu g\,ml^{-1}$)	*Inactivated pepsin* ($50\,\mu g\,ml^{-1}$)	*α-Chymotrypsin* ($50\,\mu g\,ml^{-1}$)	*Inactivated α-chymotrypsin* ($50\,\mu g\,ml^{-1}$)
Extract (5 mg tissue wet weight per ml)	11 ± 3 (6)	103 ± 2 (6)	15 ± 2 (4)	104 ± 6 (4)

The experiment was performed on guinea-pig ileum longitudinal smooth muscle treated with atropine ($200\,ng\,ml^{-1}$). Values are means ± s.e.

[1] Relative contraction is expressed as a percentage of the response in the absence of pharmacological or biochemical treatments.

[2] The preparation was incubated with a high concentration of neurotensin ($100\,ng\,ml^{-1}$) for 8 min and then the responsiveness of the extract and drugs was tested.

[3] Number of preparations examined.

Table 2 Comparison of the pharmacological and biochemical properties of the smooth muscle contracting activity of the gel filtrated fraction of the extract of the carp intestinal bulb with those of substance P and acetylcholine

	Relative contraction[1]		
	Active fraction	*Substance P* ($3\,ng\,ml^{-1}$)	*Acetylcholine* ($2\,ng\,ml^{-1}$)
Pharmacological properties			
Atropine ($200\,ng\,ml^{-1}$)	93 ± 4.1 (5)[2]	95 ± 5.0 (7)	0 ± 0 (6)
Pyrilamine ($1\,\mu g\,ml^{-1}$)	106 ± 5.6 (5)	104 ± 7.5 (6)	94 ± 1.8 (6)
Methysergide ($1\,\mu g\,ml^{-1}$)	104 ± 4.2 (5)	96 ± 2.5 (4)	100 ± 3.5 (4)
[Sar[1], Ala[8]]-angiotensin II ($1.9\,\mu g\,ml^{-1}$)	93 ± 3.7 (5)	96 ± 4.5 (7)	106 ± 4.1 (6)
[D-Pro[2], D-Trp[7,9]]-substance P[3] ($15.1\,\mu g\,ml^{-1}$)	24 ± 5.0 (6)	27 ± 4.1 (6)	96 ± 1.8 (5)
[D-Pro[4], D-Trp[7,9]]-substance P (4–11) ($11.3\,\mu g\,ml^{-1}$)	14 ± 3.3 (5)	28 ± 4.1 (6)	104 ± 2.7 (6)
Substance P desensitization[4]	7 ± 4.3 (4)	3 ± 2.1 (7)	ND
Biochemical properties			
Pepsin pretreatment ($50\,\mu g\,ml^{-1}$)	0 ± 0 (4)	1 ± 1 (5)	101 ± 1.5 (5)
α-Chymotrypsin pretreatment ($50\,\mu g\,ml^{-1}$)	6 ± 2.5 (5)	2 ± 1.5 (5)	99 ± 2.7 (5)

The experiment was performed on guinea-pig ileum longitudinal smooth muscle.
Values are means ± s.e. ND; Not determined.
[1] Relative contraction is expressed as a percentage of the control response obtained in the normal Tyrode solution.
[2] Number of preparations examined.
[3] To inhibit the contractile response to [D-Pro[2], D-Trp[7,9]]-substance P itself, the preparation was pretreated with pyrilamine ($1\,\mu g\,ml^{-1}$).
[4] Atropine-treated preparations were incubated with a high concentration of substance P ($650\,ng\,ml^{-1}$) for 10 min and then the responsiveness of the fraction and substance P was tested.

stance P, the contractile response to subsequently applied substance P ($3\,ng\,ml^{-1}$) was completely abolished. Under these conditions, the extract-induced contraction was also markedly decreased (Figure 2b).

Effect of the extract on carp intestinal bulb smooth muscle

In normal Krebs solution, the extract caused contraction of carp intestinal bulb longitudinal smooth muscle (CIBLM) in a concentration-dependent manner (0.1 mg–5 mg tissue wet weight per ml). Acetylcholine ($10\,ng\,ml^{-1}$), 5-hydroxytryptamine ($260\,ng\,ml^{-1}$) and histamine ($55\,\mu g\,ml^{-1}$) also contracted the CIBLM, producing a response of similar amplitude to the extract (5 mg tissue wet weight per ml). In the presence of atropine ($500\,ng\,ml^{-1}$), pyrilamine ($1\,\mu g\,ml^{-1}$) and methysergide ($1\,\mu g\,ml^{-1}$), the acetylcholine-, 5-hydroxytryptamine- and histamine-induced contractions were completely abolished but the extract-induced contraction remained at about 50% of the initial response, indicating that a material other than acetylcholine, histamine and 5-hydroxytryptamine contributes to the excitatory action of the extract on CIBLM. After pretreatment with pepsin or α-chymotrypsin, the contractile activity of the extract was completely abolished. Angiotensin II, neurotensin and vasoactive intestinal peptide were ineffective on CIBLM at $1\,\mu g\,ml^{-1}$. Substance P caused a concentration-dependent contraction of CIBLM, that was unaffected by substance P antagonists (Kitazawa *et al.*, 1988). To examine the contribution of a substance P-like material to the extract-induced contraction, a preparation that had been desensitized to substance P was used. Continued exposure to a high concentration of substance P ($1.3\,\mu g\,ml^{-1}$) decreased the responsiveness of CIBLM for this peptide. The extract-induced contraction was markedly reduced by the desensitizing pretreatment for substance P (data not shown).

Estimation of the molecular weight of the active material by gel filtration

We used two Sephadex G-25 columns (large, 2 × 66 cm; and small, 1.2 × 60 cm). The crude extract was applied to the large Sephadex G-25 column (flow rate, $30\,ml\,h^{-1}$; 4 ml fractions). The smooth muscle (GPLM) contracting material was eluted as one peak, as shown in Figure 3. When the contractile activity was assayed on CIBLM, the peak of contractile activity was coincident with that for GPLM (Figure 3). The fractions showing smooth muscle contracting activity were pooled, frozen and then lyophilized. The lyophilized fraction was dissolved in distilled water and then its pharmacol-

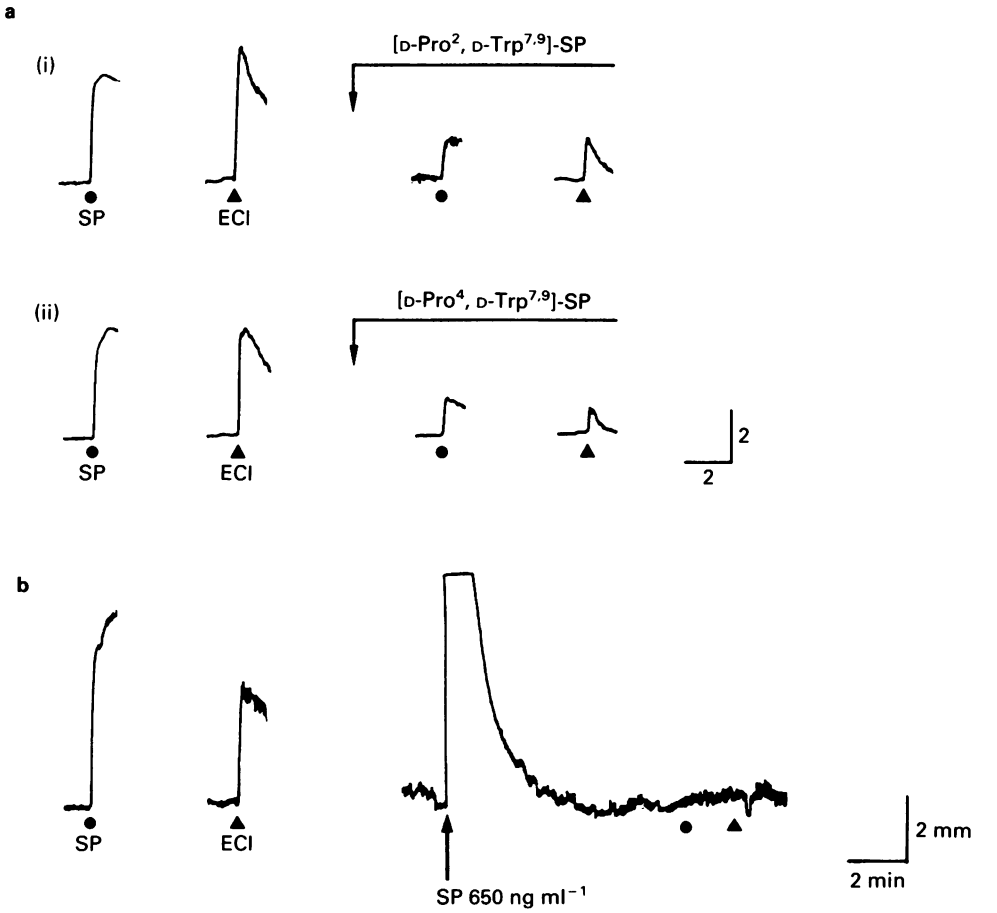

Figure 2 Inhibitory effects of substance P antagonists and substance P-induced desensitization on the contractile responses to substance P and the extract in guinea-pig ileum longitudinal smooth muscle. (a) [D-Pro^2, D-Trp^{7,9}]-substance P ((i), 15.1 μg ml^{-1}) or [D-Pro^4, D-Trp^{7,9}]-substance P (4–11) ((ii) 11.3 μg ml^{-1}) treatment (for 5 min) inhibited the contractile responses to substance P (SP, 3 ng ml^{-1}, ●) and the extract (ECI, 5 mg tissue wet weight per ml, ▲). (b) Substance P-induced desensitization (650 ng ml^{-1} for 10 min, ↑) completely abolished the contractile responses to substance P (3 ng ml^{-1}, ●) and the extract (ECI, 5 mg tissue wet weight per ml, ▲). All experiments (a, b) were performed on a muscle preparation treated with atropine (200 ng ml^{-1}), pyrilamine (1 μg ml^{-1}) and methysergide (1 μg ml^{-1}).

ogical properties were examined. Atropine, pyrilamine, methysergide and [Sar^1, Ala^8]-angiotensin II did not affect the contracting activity of the fraction. [D-Pro^2, D-Trp^{7,9}]-substance P, [D-Pro^4, D-Trp^{7,9}]-substance P (4–11) and substance P-induced desensitization decreased the contractile activity of the fraction and substance P equally (Table 2). This lyophilized fraction was dissolved in 0.1 N HCl or 0.05 M Tris-HCl buffer (pH. 7.4), and then the effect of pepsin (50 μg ml^{-1}) or α-chymotrypsin (50 μg ml^{-1}) pretreatment on its contractile activity was examined. The contractile activity of the fraction was almost completely abolished by both the pepsin and the α-chymotrypsin treatment (Table 2). These data indicated that the pharmacological and biochemical properties of the active fraction were consistent with those of substance P.

The lyophilized active fraction was dissolved in 0.1 N acetic acid solution and then applied to the small Sephadex G-25 column (flow rate, 15 ml h^{-1}; 3 ml fractions). The peak of smooth muscle contractile activity on GPLM appeared at Ve/Vo 1.66–1.90. Four peptides of known molecular weight (leucine-enkephalin, 556; angiotensin II, 1046; substance P, 1348; and motilin, 2700) were also run on the same column, and the relationship between the logarithm of the molecular weight and the ratio of Ve/Vo was plotted in Figure 4. From these data,

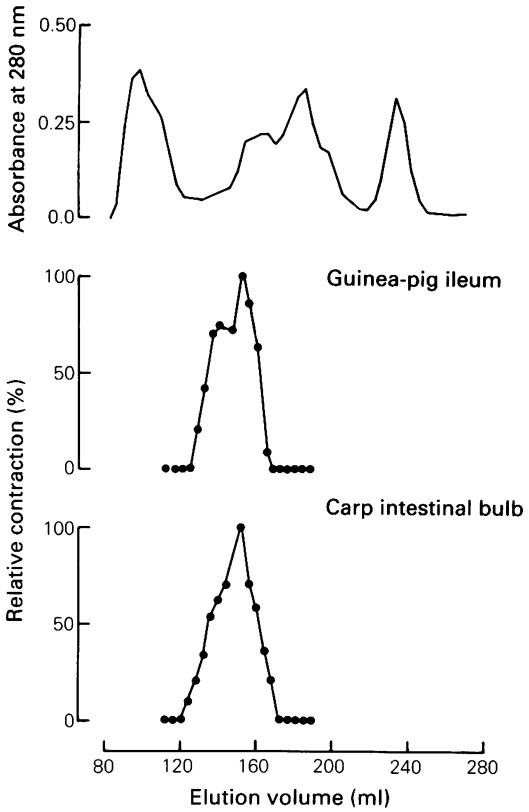

Figure 3 Gel filtration of the extract of the carp intestinal bulb on a Sephadex G-25 column. The crude extract (3 ml, 500 mg tissue wet weight per ml) was applied to a Sephadex G-25 column (2 × 66 cm), which was eluted with 0.1 N acetic acid at 30 ml h^{-1}. Four ml fractions were collected, monitoring the u.v. absorbance at 280 nm, lyophilized and then dissolved in 0.4 ml of distilled water. The smooth muscle contracting activity of each fraction was bioassayed on guinea-pig ileum longitudinal smooth muscle or carp intestinal bulb longitudinal smooth muscle strip in the presence of atropine (200 ng ml^{-1}), pyrilamine (1 μg ml^{-1}) and methysergide (1 μg ml^{-1}). Relative contraction was expressed as the percentage of each peak activity.

the molecular weight of the smooth muscle contracting material present in the carp intestinal bulb extract was estimated to be 1200–1700 (1410 ± 70, $n = 6$).

Discussion

An acid extract of the carp intestinal bulb exhibited smooth muscle contracting activity toward both guinea-pig ileum longitudinal smooth muscle (GPLM) and carp intestinal bulb longitudinal smooth muscle (CIBLM). As atropine decreased the contractile response to the extract to 30–40% of the initial response, the extract contained acetylcholine. In the gastrointestinal tract, acetylcholine is mainly present in cholinergic nerves (Johnson, 1963; Paton & Aboo Zar, 1968). Therefore, these results suggest the cholinergic innervation of the carp intestinal bulb. The presence of cholinergic nerves in the carp intestinal bulb has already been demonstrated in a physiological study, because the contractile response to transmural stimulation was potentiated by physostigmine and decreased by atropine (Kitazawa *et al.*, 1986). The acetylcholine content of the carp intestinal bulb was estimated to be 870 ± 50 ng g^{-1} tissue wet weight ($n = 16$), which was very much lower than that of GPLM (28 μg g^{-1} tissue; Paton & Aboo Zar, 1968) or chick intestine (9.7 μg g^{-1} tissue; Takewaki *et al.*, 1975). This result may reflect the sparse cholinergic innervation of the carp intestinal bulb compared with mammals and birds.

Histamine and 5-hydroxytryptamine are bioactive materials that cause the contraction of GPLM in the presence of atropine at low concentrations through the action on specific receptors. Pyrilamine and methysergide had no effect on the extract-induced contraction, indicating that it was unlikely that histamine or 5-hydroxytryptamine mediated the atropine-resistant contractile activity of the extract.

ATP and related nucleotides are proposed to be neurotransmitter substances released from NCNA nerves supplying smooth muscle preparations from a number of vertebrate classes (Burnstock, 1972; Burnstock *et al.*, 1972). ATP and ADP, but not AMP or adenosine, caused contraction of GPLM in the presence of atropine. The contractile activity of the extract was stable in a boiling acid solution but abolished in a boiling alkali solution, whereas the activity of ATP and ADP was abolished in a boiling acid solution but stable in a boiling alkali solution. Thus, the stability of the activity of the extract in an acid or alkali solution was quite different from that of ATP and ADP. This suggests that the method of extraction used in the present study involving a boiling acid solution prevented the presence of unchanged ATP and ADP in the extract. Consequently, the excitatory material present in the extract seemed unlikely to be ATP or a related substance. This conclusion was further supported by the fact that the extract exhibited an excitatory action on CIBLM, which was insensitive to ATP and ADP.

The contractile activity of the extract of the carp intestinal bulb decreased on incubation with the proteolytic enzymes, pepsin and α-chymotrypsin. Therefore, the active material mediating the atropine-resistant contraction in the extract was thought to be a peptide. On gel filtration on a Sephadex G-25 column, the fractions containing the

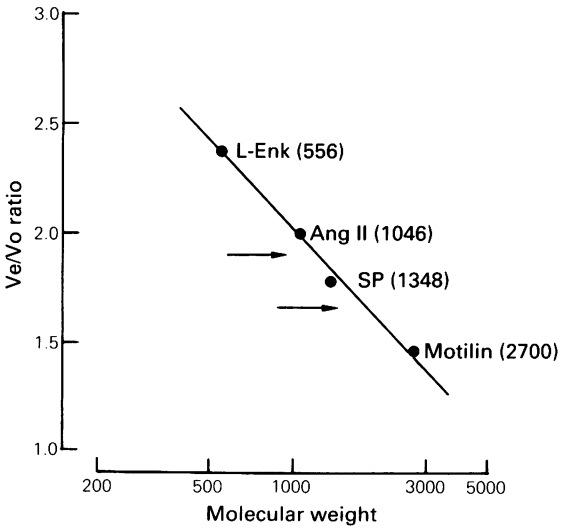

Figure 4 Estimation of the molecular weight of the active material in the extract of the carp intestinal bulb. The active fractions obtained by gel filtration on the large Sephadex G-25 column (2 × 66 cm) were pooled and then applied to another Sephadex G-25 column (1.2 × 60 cm). Elution was performed with 0.1 N acetic acid solution at 15 ml h^{-1}. Three ml fractions were collected and their contractile activity on guinea-pig ileum longitudinal smooth muscle was bioassayed in the presence of atropine (200 ng ml^{-1}), pyrilamine (1 μg ml^{-1}) and methysergide (1 μg ml^{-1}). Peptides of known molecular weights, leucine-enkephalin (L-Enk, 556), angiotensin II (Ang II, 1046), substance P (SP, 1348) and motilin (2700), were also applied to the same column and their elution volumes were determined by monitoring the u.v. absorbance at 280 nm or 220 nm. Ordinate scale; ratio of the elution volume to the void volume (Ve/Vo). Abscissa scale; molecular weight in logarithmic scale. The Ve/Vo values for the active material lie between 1.66 and 1.90 (between the two arrows).

active peptide for GPLM were approximately coincident with those for CIBLM. In other words, the active peptide in the extract causing the contraction of GPLM is the same as that causing the contraction of CIBLM. The smooth muscle excitatory peptide in the carp intestinal bulb extract was not angiotensin II, neurotensin, an opioid peptide or vasoactive intestinal peptide. Evidence for this is: (1) [Sar^1, Ala^8]-angiotensin II and neurotensin-induced desensitization had no effect on the extract-induced contraction, and (2) CIBLM was insensitive to angiotensin II, neurotensin and vasoactive intestinal peptide, and GPLM was insensitive to opioid peptides. Among the bioactive peptides examined in the present study, only substance P caused the contraction of both GPLM and CIBLM at a relatively low concentration (ng ml^{-1} order), even in the presence of atropine, pyrilamine and methysergide. The excitatory action of substance P on GPLM was selectively inhibited by substance P antagonists ([D-Pro^2, D-Trp^{7,9}]-substance P and [D-Pro^4, D-Trp^{7,9}]-substance P (4–11)) without a decrease in the responsiveness of the smooth muscle to acetylcholine and histamine (Leander *et al.*, 1981; Björkroth *et al.*, 1982; Mizrahi *et al.*, 1982). The contractile response to the extract was also decreased by these substance P antagonists. Substance P-induced desensitization has been used to reduce the response to substance P *in vitro* (Franco *et al.*, 1979a); treatment with 200 fold higher concentrations of substance P decreased equally the responses to substance P and the extract. The molecular weight of the active peptide estimated by Sephadex G-25 gel filtration was 1410 ± 70 (n = 6). This value was approximately the same as that for substance P (1348). These results strongly suggest that the active peptide present in the extract of the carp intestinal bulb is substance P or a closely-related peptide. Substance P is an undecapeptide whose amino acid sequence is Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (Chang *et al.*, 1971). However, the results of recent studies suggested the dissimilarity in the amino acid sequence of substance P between mammals and chick (Brodin *et al.*, 1981; Komori *et al.*, 1986). Therefore, it is probable that the amino acid sequence of the substance P-like peptide in the carp intestinal bulb is different from that in mammals. To clarify the dissimilarity, determination of the amino acid sequence of the carp substance P-like peptide is necessary.

An immunohistochemical study on the carp intestinal bulb has not been performed yet but nerve fibres exhibiting substance P-like immunoreactivity have been observed in the gastrointestinal tract of several other teleost fishes (Holmgren *et al.*, 1982; Holmgren, 1985; Jensen & Holmgren, 1985). These and the present results suggest the presence of substance P-like peptide containing neurones in the carp intestinal bulb. Substance P has excitatory action on CIBLM at a low concentration in the presence of atropine (Kitazawa *et al.*, 1988). Therefore, the substance P-like peptide is proposed as a candidate for the neurotransmitter mediating the excitatory NCNA response in the carp intestinal bulb, as established in the case of the mammalian gastrointestinal tract (Franco *et al.*, 1979a; Leander *et al.*, 1981; Wali, 1985; Matůšak & Bauer, 1986).

This study was supported by a Grant-in-Aid for Scientific Research, 62760259, from the Ministry of Education, Science and Culture, Japan.

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(Received September 9, 1987
Revised January 20, 1988
Accepted March 31, 1988)