Isolation of Smooth Muscle Excitatory Substances from Chicken Rectum and Their Characterization

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Abstract—Acid-acetone extracts of the chicken rectum were subjected to chromatographic and electrophoretic separation, and two new smooth muscle-contracting substances close to purity were obtained. One of them showed chemical and biological characteristics similar to those of substance P, but it was clearly different from substance P on the basis of chromatographic and electrophoretic criteria. Thus, one could be a peptide belonging to the substance P-family. The other substance was also shown to be of peptide nature since its biological activity was destroyed by chymotrypsin and carboxypeptidase A. Parallel bioassay on the two tissues of the longitudinal muscle of the guinea-pig ileum and the isolated whole chick rectum revealed that none of the peptides such as substance P, physalaemin, kassinin, eledoisin, bradykinin and angiotensin II could be a candidate for the active substance. The biological activity was not antagonized by naloxone, suggesting that the substance was a peptide other than the opioid compounds. The molecular sizes estimated by gel filtration are 1300 for the substance P-like peptide and 1600 for the other substance. The possible physiological roles of the two substances as an excitatory non-adrenergic, non-cholinergic transmitter were discussed.

In the chicken rectum, electrical stimulation of the Remak nerve, an extrinsic nerve, elicits non-adrenergic, non-cholinergic (NANC) contraction and excitatory junction potentials (1-3).

Recently, two excitatory substances have been extracted from the Remak nerve, which are neither acetylcholine, histamine, serotonin, ATP and its related substances nor prostaglandins (4, 5). On the basis of their chemical and pharmacological properties, one could be a peptide belonging to the substance P-family. The second was also shown to be of peptide nature, but has not yet been characterized because of its limited amount. However, the latter substance seems to be a more promising candidate for the NANC neurotransmitter, at least in the chicken rectum, since it has a much higher potency than the substance P-like peptide in contracting the isolated whole chick rectum (WCR).

Assuming that the WCR-contracting substance is derived from NANC neurones in the Remak nerve, it should be also present in the rectum with a dense distribution of NANC nerve terminals. In the present study, we tried to extract the active substance from the chicken rectum and to characterize it.

Materials and Methods

Tissue samples: In earlier experiments, domestic fowls (Gallus domesticus) of either sex, more than 100 days old, were obtained from commercial sources. Birds were stunned and bled, and the whole rectum was removed and immersed in ice-cold Tyrode solution containing indomethacin (7.5 µg/ml) after its lumen was flushed with the same solution. In recent experiments, rectal regions
of the chicken intestine were obtained within 20 min after slaughter at a meat product company (Minokashiwa), washed out with ice-cold Tyrode solution containing indomethacin (7.5 μg/ml), immediately immersed in the same solution and brought to the laboratory. The isolated rectum was opened by dissecting along longitudinal axis of the organ, swirled around in ice-cold Tyrode solution to remove traces of blood and luminal content, immersed in fresh Tyrode solution and kept at about 4°C for a few hours until used for extraction.

**Extraction:** Some of the present extractions were conducted by the same procedure as that used for the nerve of Remak (4, 5). For most extractions, tissue samples of 100–800 g wet weight were minced and stirred for 60 min at room temperature with acetone-1 N HCl solution (100:3, V/V) in a volume of 5 ml/g wet weight of the tissue samples. The mixture was filtered under suction and the residue was again extracted with acetone-0.01 N HCl solution (80:20, V/V) in a volume of 2 ml/g wet weight of the tissue samples. The filtrates were pooled, and acetone in the solution was removed first by extracting 5–7 times with petroleum ether and then by evaporating under a reduced pressure at 40°C for several hours. The aqueous residue was centrifuged at 10000×g at 4°C for 20 min. The supernatant was defatted with ethyl acetate, lyophilized and stored in a deep freezer at -20°C.

**Gel filtration:** Lyophilized material was dissolved in 1–2 ml 0.1 M acetic acid and applied to a column (1.2×55 cm or 1.6×80 cm) of Sephadex G-25. Elution was performed with 0.1 M acetic acid at a flow rate of 21.4 ml/hr for the smaller column and 40 ml/hr for the larger column. Eluates in which smooth muscle-contracting activity was detected were pooled, lyophilized and stored at -20°C until required. The eluate was monitored by measuring UV absorption at 254 or 280 nm. The void volume of each column was determined with blue dextran.

**Cation exchange chromatography:** The lyophilized material obtained after gel filtration on Sephadex G-25 was dissolved in 50 ml of 0.1 M acetic acid, and its active substances were adsorbed onto a column of SP-Sephadex C-25 (0.8×10 cm) equilibrated with this acid solution. The column was washed with 20 ml of 0.05 M pyridine-acetate buffer (pH 5.5) and then eluted with a 360 ml linear gradient of the concentration of the column buffer ranging from 0.05 M to 2 M, at a flow rate of 20 ml/hr. This eluate was collected in 5 ml fractions. Aliquots of each of the fractions were lyophilized and dissolved in distilled water when required for bioassay.

**High voltage paper electrophoresis:** High voltage paper electrophoresis was performed on Whatman No. 3 MM paper at 80 volts per cm using an immersed strip type apparatus. The buffer solution was pH 1.9, formic acid-acetic acid-water (20:80:900). Serine (5 μg), substance P, neurotensin and angiotensin II (45 μg) were spotted on one side of the samples as appropriate standard amino acid and peptides, which were located by staining with 0.2% ninhydrin in aceticone. Strips (1 cm in width) of the sample side of the paper were each extracted with 0.1 M acetic acid, and the contracting activity was bioassayed.

**Bioassay:** The longitudinal muscle layer of the ileum of the guinea-pig (LMGPI) and the whole rectum isolated from young chicks (less than 2 weeks) (WCR) were used as assay preparations. Usually parallel assays on both preparations were carried out. The tissues were separately suspended in a 2.5 ml polypropylene organ bath filled with Tyrode solution composing of (mM) NaCl, 136.9; KCl, 2.7; NaH₂PO₄, 0.4; CaCl₂, 1.8; MgCl₂,
2.1: NaHCO₃, 11.9; and glucose, 5.6, bubbled with air and kept at 33°C. Atropine, pyrilamine and methysergide were added to Tyrode solution to give their respective concentrations of 0.2 µg/ml, 1 µg/ml and 1 µg/ml, in order to eliminate contractile effects of ACh, histamine and serotonin that may be possibly present in the assay samples. Isometric tension of the assay tissues was recorded by a force-displacement transducer (Nihon Kohden, TB-612T) and a potentiometric recorder (Hitachi, O56). Assay samples and drugs were injected into the bathing medium with a micropipette in a volume less than 0.02 ml (less than 0.8% of the bath volume), and they were washed away by replacing the bathing medium with fresh medium.

**Enzymatic studies:** Enzyme preparations used were pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4, Type II), α-chymotrypsin (Type II), carboxypeptidase A (EC 3.4.12.2, Type II), leucine aminopeptidase (EC 3.4.11.1, Type V) and pyroglutamate aminopeptidase (EC 3.4.11.8) which were all purchased from Sigma. Susceptibility of substances to the enzymes and to chicken serum was tested in the same way as that used for the active substances isolated from Remak nerve (5).

**Stability and solubility:** Stability in acid or alkali solution and solubility in organic solvents of the active substances obtained were tested by conducting the same procedures as those used for the active substances of Remak nerve (5).

**Intracellular recording of membrane potential:** Pieces of intestinal wall taken from the rectum of young chicks (less than 100 days old) were used for the experiments. The preparation was pinned out on a black rubber board and immersed in a 1 ml chamber perfused at 1 ml/min with oxygenated Tyrode solution warmed to 30°C. Intracellular recordings of membrane potential were made from cells lying in the longitudinal muscle layer by the use of glass microelectrodes (40 to 100 Mohms resistance) filled with 3 M KCl. Test solutions were applied to the muscle by injecting in a small volume (less than 0.02 ml) from a micropipette. During the application, perfusion of the chamber was usually interrupted. In most experiments, atropine (0.2 µg/ml), methysergide (1 µg/ml) and pyrilamine (1 µg/ml) were added to the perfusing solution.

**Drugs:** Drugs used were atropine sulphate (Nakarai), tetrodotoxin (Sankyo), indomethacin (Sigma), pyrilamine maleate (Sigma), methysergide hydrogen maleate (Sandoz) and naloxone hydrochloride (Endo Laboratories), substance P, [D-Pro², D-Trp⁷,⁸]-substance P, [D-Arg¹, D-Pro², D-Trp⁷,⁹, Leu¹¹]-substance P, physalaemins, kassinin, eleidoisin, bradykinin, leucine-enkephalin, vasoactive intestinal polypeptide (VIP), angiotensin II and neurotensin (all purchased from Peptide Institute Inc., Osaka).

**Results**

**Gel filtration and ion-exchange chromatography:** Acid-acetone extracts of the chicken rectum were submitted to gel filtration on a Sephadex G-50 column. Figure 1 gives a typical gel filtration profile showing contractile effects of the eluate on the longitudinal muscle of the guinea-pig ileum (LMGPI) and on the isolated whole chick rectum (WCR) previously treated with atropine (0.2 µg/ml), pyrilamine (1 µg/ml) and methysergide (1 µg/ml), and UV absorbance at 280 nm. It can be seen that the region of eluate from which LMGPI-contracting activity was detected is overlapped with that from which WCR-contracting activity was detected. LMGPI-contracting activity occurred with one peak in an elution volume of 2.3–2.5 times of the void volume (Vo). However, WCR-contracting activity appeared frequently with more than one peak or with a plateau-like peak so that the elution pattern was more complicated. The main peak activity, if measured, eluted in an elution volume of 2.1–2.5 times of Vo. The peaks of both activities invariably eluted before the main peak of UV absorbance at 280 nm. When active fractions of each extract were pooled and rechromatographed on a Sephadex G-25 column, the LMGPI- and WCR-contracting activities eluted again in similar regions. The mean ratio of elution volume of the peak of LMGPI-contracting activity (Ve) to Vo was 1.66±0.03 (±S.E., n=6) and the value for WCR-contracting activity was 1.69±0.05 (n=4).

The active sample obtained after gel
Fig. 1. Gel filtration profiles of the rectum extract on a Sephadex G-50 column. Sample, an acid-acetone extract from 200 g wet weight of tissue. The curves show UV absorption at 280 nm of the eluate (solid line) and its contractile effects on the longitudinal muscle of the guinea-pig ileum (○-○) and isolated whole chick rectum (●-●) pretreated with atropine (0.2 μg/ml), pyrilamine (1 μg/ml) and methysergide (1 μg/ml). Column size, 1.6×70 cm; fraction size, 4 ml; eluent, 0.1 M acetic acid; flow rate, 40 ml/hr. Fractions were lyophilized, then each residue was dissolved in 0.4 ml distilled water, and the contracting activity was measured.

Fig. 2. Ion exchange chromatography of active material on SP-Sephadex C-25. Sample, obtained after gel filtration on Sephadex G-25 and originated from the extract in Fig. 1. The curves show contracting activities of the eluate assayed on the longitudinal muscle of the guinea-pig ileum (○-○) and isolated whole chick rectum (●-●) pretreated with atropine (0.2 μg/ml), pyrilamine (1 μg/ml) and methysergide (1 μg/ml). The activity is expressed as percentage of the peak activity in each assay tissue. Elution was made with a 360 ml linear gradient concentration ranging from 0.05 M to 2 M of pyridine-acetate buffer (pH=5.5) (- - - - - -) Column size, 0.8×10 cm; fraction size, 5 ml; flow rate, 20 ml/hr. Fractions were lyophilized, then each residue was dissolved in 0.2 ml distilled water, and the contracting activity was measured.
filtration on Sephadex G-25 was subjected to cation-exchange chromatography on a SP-Sephadex C-25 column. The column was developed with pyridine-acetate buffer (pH 5.5) in which the molarity of pyridine-acetate was linearly increased from 0.05 M to 2 M. As shown in Fig. 2, the LMGPI-contracting activity was usually fractionated into two regions of the eluate, but sometimes the first component eluted at lower molarity of the column buffer was extremely small or absent. The WCR-contracting activity occurred with one main peak in the eluate region between those from which LMGPI-contracting activity was detected.

**High voltage paper electrophoresis:** The WCR region and the second LMGPI region of the eluate from the SP-Sephadex C-25 column were concentrated by lyophilization and subjected to high voltage paper electrophoresis. They were recovered from the paper at separate positions which were different from those for any of the standard peptides, such as substance P, angiotensin II and neurotensin, as shown in Fig. 3. The samples recovered from the paper, one producing LMGPI contraction (SI) and the other producing WCR contraction (Sw), were used for the following experiments.

**Characterization of the LMGPI-contracting substance (SI):** Table 1 summarizes the results of experiments in which stability in acid and alkali solutions and solubility in some organic solvents were tested. Susceptibility of the substance of SI to six proteolytic enzymes and to chicken serum was also examined, and the results are presented in Table 2. The excitatory activity was destroyed after incubation with α-chymotrypsin, trypsin and pepsin, but not with carboxypeptidase A, leucine aminopeptidase and pyroglutamate aminopeptidase. Preincubation with chicken serum had no effect on the LMGPI-contracting activity. Chicken serum was confirmed to be active in destroying the pharmacological activities of bradykinin.

![Fig. 3. High voltage paper electrophoresis of two active materials.](image)

Table 1. Stability and solubility of LMGPI-contracting substance and WCR-contracting substance isolated from the chicken rectum

|                      | LMGPI-contracting substance | WCR-contracting substance |
|----------------------|----------------------------|---------------------------|
| **Stability**        |                            |                           |
| Boiling in acid      | Stable                     | Stable                    |
| Boiling in alkali    | Labile                     | Labile                    |
| **Solubility**       |                            |                           |
| Methyl alcohol       | Soluble                    | Soluble                   |
| Ether                | Insoluble                  | Insoluble                 |
| Ethyl acetate        | Insoluble                  | Insoluble                 |
| Acetone              | Insoluble                  | Insoluble                 |

LMGPI: Longitudinal muscle of the guinea-pig ileum; WCR: Isolated whole chick rectum. The substances obtained after high voltage paper electrophoresis were used for the experiment.
leucine-enkephalin, or acetylcholine when it was mixed with these substances and incubated for 15 min.

The contracting activity of SI appeared to be a direct action on the smooth muscle as it was not blocked by tetrodotoxin (0.2 μg/ml). A substance P antagonist, [D-Pro², D-Trp⁷,⁸, Leu¹¹]-substance P, abolished or markedly reduced contractile effects on LMGPI of the extracted substance as well as substance P (Fig. 4). Similar results were obtained with another substance P antagonist, [D-Arg¹, D-Pro², D-Trp⁷,⁸, Leu¹¹]-substance P. Desensitization of LMGPI to substance P caused a marked reduction of responses to

| Enzymes            | LMGPI-contracting substance | WCR-contracting substance |
|--------------------|-----------------------------|---------------------------|
| Chymotrypsin       | Destroyed                   | Destroyed                 |
| Trypsin            | Destroyed                   | Destroyed                 |
| Pepsin             | Destroyed                   | Destroyed                 |
| Carboxypeptidase A | Not destroyed               | Destroyed                 |
| Pyroglutamate aminopeptidase | Not destroyed | Not destroyed |
| Leucine aminopeptidase | Not destroyed              | Not destroyed             |
| Chicken serum      | Not destroyed               | Not destroyed             |

LMGPI: Longitudinal muscle of the guinea-pig ileum; WCR: Isolated whole chick rectum. The substances obtained after high voltage paper electrophoresis were used for the experiment.

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| Enzymes            | LMGPI-contracting substance | WCR-contracting substance |
|--------------------|-----------------------------|---------------------------|
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| Trypsin            | Destroyed                   | Destroyed                 |
| Pepsin             | Destroyed                   | Destroyed                 |
| Carboxypeptidase A | Not destroyed               | Destroyed                 |
| Pyroglutamate aminopeptidase | Not destroyed | Not destroyed |
| Leucine aminopeptidase | Not destroyed              | Not destroyed             |
| Chicken serum      | Not destroyed               | Not destroyed             |

LMGPI: Longitudinal muscle of the guinea-pig ileum; WCR: Isolated whole chick rectum. The substances obtained after high voltage paper electrophoresis were used for the experiment.

Chemical and pharmacological properties of the WCR-contracting substance: Chemical properties of the substance producing WCR contraction (Sw) are also presented in Tables
1 and 2. Figure 5 shows how this substance differs from LMGPI-contracting substance (SI) with regards to pepsin and carboxypeptidase A susceptibility. The substance (Sw) caused a concentration-related contraction of WCR and relaxation of LMGPI (Fig. 6), which remained unchanged in the presence of tetrodotoxin (0.2 μg/ml). At this same stage of the purification, the WCR-contracting substance was more than 1000 times as active as the LMGPI-contracting substance in producing similar contractions of WCR. The excitatory response of WCR was highly reproducible and easily subsided on washing. In order to understand the membrane events that underlie the contractile effect of the substance, the membrane potential of the smooth muscle cells was intracellularly recorded. The resting membrane potential was measured to be $-52.0 \pm 0.6 \text{ mV}$ (mean±S.D., n=36), and in most preparations, no spontaneous spike activity was observed under the conditions used, as previously reported (6). Figure 7 shows that the substance (Sw) causes a dose-dependent depolarization up to more than 20 mV. Usually the depolarization led to initiation of spike discharge in quiescent muscle cells and to an increase in the frequency of spike discharge in spontaneously active cells.

Minimum concentrations of some active peptides required to produce a detectable rise in tension of WCR and LMGPI were measured and the results are presented in Table 3. It was found that only leucine-enkephalin had a much higher potency in contracting WCR than LMGPI. In this respect, the peptide resembled the WCR-contracting substance. However, naloxone antagonized completely the action of leucine-enkephalin on WCR without changing the action of the extracted substance, as shown in Fig. 8. Leucine-enkephalin lost its biological activity after incubation with leucine aminopeptidase or with chicken serum. From these properties, the extracted substance was clearly different from leucine-enkephalin.

**Estimation of molecular sizes of these active substances:** Samples of leucine-enkephalin (mol. wt. 556), angiotensin II (mol. wt. 1046), substance P (mol. wt. 1348) and vasoactive intestinal polypeptide (VIP)
Table 3. Minimum concentrations of active peptides required to produce a detectable rise in tension of WCR and LMGPI

|          | WCR        | LMGPI      |
|----------|------------|------------|
| Substance P | 100 (ng/ml) | 0.1 (ng/ml) |
| Eledoisin  | 800        | 4.0        |
| Physalaemin| 5200       | 0.5        |
| Kassinin   | 100        | 2.0        |
| Bradykinin | 800        | 2.0        |
| Angiotensin II | >4000 | 0.1        |
| Leu-enkephalin | 200     | >4000      |

WCR: Isolated whole chick rectum; LMGPI: Longitudinal muscle of the guinea-pig ileum.

Fig. 8. Effects of naloxone on contractile responses of the isolated whole chick rectum to leucine- enkephalin and to one highly purified substance (Sw). Sample (Sw), obtained after the high voltage paper electrophoresis. Naloxone, applied during the period (Nal. t ) at 8 μg/ml, abolished the contractile effect of leucine-enkephalin (L-En, ●) at 1 μg/ml, without changing the contractile effect of Sw (Sw, ○).

Fig. 9. Estimation of the molecular weight of two highly purified substances (Sw and SI). Samples (Sw and SI), obtained from the high voltage paper electrophoresis. Peptides of known molecular weights, leucine-enkephalin (Leu-Enk, 556), angiotensin II (A, 1046), substance P (SP, 1348) and VIP (3325), were applied to a column of Sephadex G-25; and the isolated substances, SI mediating contraction of the longitudinal muscle of the guinea-pig ileum and Sw mediating contraction of the isolated whole chick rectum, were applied to the same column. Column size, 1.2×55 cm; eluent, 0.1 M acetic acid; flow rate, 21.4 ml/hr. Ordinate, elution volume (Ve) to the void volume (Vo); abscissa, log molecular weight. Molecular weights of SI and Sw are estimated to be 1300 and 1600, respectively.

Discussion

Two substances of peptide nature were isolated from the chicken rectum. One substance exhibited biological activity similar to substance P, causing contractions of the longitudinal muscles of the guinea-pig ileum (LMGPI) and of the isolated whole rectum (WCR), but in WCR, it required concentrations of more than 1000 times as high as those used for LMGPI. However, it was distinguishable from substance P on the...
basis of chromatographic and electrophoretic criteria. Since the COOH-terminal hexapeptide of substance P has been shown to be the minimum structure necessary to elicit the biological activity (7), this substance seems likely to belong to a substance P-family (SP-like substance) with the common COOH-terminal structure. This notion was also supported by the observations that the activity of this substance was antagonized by the substance P analogues, [D-Pro², D-Trp⁷-⁹]-substance P and [D-Arg¹, D-Pro², D-Trp⁷-⁹, Leu¹¹]-substance P. The SP-like substance showed chemical characteristics similar to those reported by Komori et al. (4, 5) for the LMGPI-contracting substance isolated from the nerve of Remak from chickens. The biological and chemical similarities between the two substances prepared from the rectum and Remak nerve strongly suggest that they are identical compounds. Immunohistochemical studies indicate the existence of a substance P-like compound in the rectum of the chicken (8).

The other substance caused contraction of WCR, but relaxation of LMGPI, and all its properties examined were consistent with those of the WCR-contracting substance isolated from the Remak nerve, which suggests that both substances might be identical. However, at the present stage, the substance prepared from the Remak nerve is in insufficient amounts to perform experiments for further characterization.

The parallel bioassay on the two tissues of WCR and LMGPI used in this study showed that extremely different sensitivities to tachykinins (such as substance P, physalaemin, kassinin and eledoisin) and bradykinin and angiotensin II. This revealed that none of these peptides could be a candidate for the WCR-contracting substance. Leucine-enkephalin was found to have contractile effects on WCR. It has been demonstrated that opioid compounds such as leucine-enkephalin, methionine-enkephalin and endorphins have contractile effects on rat colon and rectum and on guinea-pig rectum, and the effects are completely antagonized by naloxone (9). The WCR-contracting substance caused contraction of WCR even in the presence of naloxone in concentrations high enough to block leucine-enkephalin-induced contractions of WCR. These results show that the contracting activity is not mediated by activation of naloxone-sensitive opioid receptors, suggesting that the WCR-contracting substance is not any of these opioid compounds. This view is also supported by the findings that the biological activity of leucine-enkephalin, unlike that of the isolated substance, was destroyed after incubation with leucine aminopeptidase or chicken serum and that this peptide had no inhibitory effects on LMGPI. Furthermore, the molecular size of 1600 of the WCR-contracting substance estimated by gel filtration is much larger than leucine-enkephalin (mol. wt., 556). From the present results, the excitatory substance could not be characterized as any of the known peptides examined yet. However, the characteristics that it has a potent excitatory effect on WCR but a potent inhibitory effect on LMGPI, it is cleaved by chymotrypsin or carboxypeptidase A, but not by pepsin, leucine aminopeptidase or pyroglutamate peptidase, and its molecular size is roughly 1600 can probably be used for eliminating many known peptides from the list of its candidates. Further investigation directed toward isolation and characterization of this peptide is in progress.

Minimum concentrations (mg wet tissue/ml) of the active substances required to produce a detectable rise in tension of WCR and LMGPI were obtained as presented in Table 4. If the WCR-contracting substances prepared from the rectum and Remak nerve are identical, it would be present in the rectum at a 15–20 times higher concentration than in the nerve of Remak. On the same assumption for the LMGPI-contracting substance, there would be a reverse relationship between the two tissues. The distribution of the LMGPI-contracting substance is estimated to be 22±5 ng/g wet tissue in the rectum (mean±S.E., n=7) and 490±49 ng/g wet tissue in the nerve of Remak (n=4), by matching the contractions produced by the substance with the contractions produced by known concentrations of substance P. The concentration in the rectum is consistent with
Table 4. Minimum effective concentration (ng tissue wet weight/ml) of the active substances isolated from the rectum and Remak nerve of the chicken

| Biological activity | Samples from rectum | Samples from Remak nerve |
|---------------------|---------------------|-------------------------|
| WCR-contracting     | 1–4                 | 20–50                   |
| LMGPI-contracting   | 4–10                | 0.3–0.5                 |
|                     | 22±5* (n=7)         | 490±49* (n=4)           |

Samples, obtained after the gel filtration on Sephadex G-25. *: mean±S.E. ng/g tissue wet weight of LMGPI-contracting substance, estimated by matching the sample-induced contractions with the contractions produced by known concentrations of substance P. WCR: The isolated whole chick rectum; LMGPI: The longitudinal muscle of the guinea-pig ileum.

that of substance P-like compound estimated by radioimmunoassay (8).

The ultimate aim of the present experiments is to elucidate the neurotransmitter of NANC nerves, which are distributed densely in the chicken rectum and the cell bodies of which are located in ganglia in the nerve of Remak (10). Electrical stimulation of the nerves elicits excitatory junction potentials in the rectal smooth muscle (2, 3). Thus, minimum requirements for the putative neurotransmitter(s) are to be present in the nerve of Remak and rectum, to have an excitatory action on the smooth muscle of the rectum, and to be neither histamine, serotonin, ATP and its related compound nor prostaglandins (11). All these requirements seem to be satisfied by the SP-like substance. However, its concentration in the chicken rectum (20 ng/g wet wt. tissue) is about twenty times lower than the substance P concentration in guinea-pig ileum in which this peptide seems likely to be the NANC neurotransmitter (480 ng/g wet wt. tissue) (12, 13). Substance P-like immunoreactivity has been shown to occur mainly in the submucosal plexuses of the chicken rectum (8). These observations, if this substance is responsible for the immunoreactivity and it functions as the NANC neurotransmitter, suggest a sparse innervation by NANC neurones of the chicken rectum as compared to the guinea-pig ileum. On the contrary, electrophysiological studies on NANC innervation in the chicken rectum provided evidence for the presence of a dense innervation like the adrenergic innervation in the vas deferens of the guinea-pig (2, 3): Nerve stimulation-evoked NANC e.j.p.s could be recorded from almost all of the cells in the longitudinal muscle layer, and nerve stimulation with single stimuli was effective in eliciting twitch-like contractions which might be associated with e.j.p.s and generation of action potentials. In the guinea-pig ileum, NANC nerve-mediated contractions can be produced only when high frequencies are used for stimulation (12), and NANC e.j.p.s are evoked only in some smooth muscle cells (14). The fact that the SP-like substance has a very low potency in contracting WCR, combined with the mismatch mentioned above, makes this substance an unlikely candidate for the neurotransmitter for the e.j.p. recorded in the muscle. The substance might be a neurotransmitter within submucosal plexuses, since substance P has been reported to function as a neurotransmitter or a modulator within the enteric plexuses in mammalian intestines (15, 16).

It is very interesting that the WCR-contracting substance isolated from the chicken rectum fulfills some of the criteria identifying it as the NANC transmitter. It is, therefore, of importance to determine whether or not this substance can be released from the chicken rectum following nerve stimulation.

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