A Smooth Muscle Excitatory Material from the Nerve of Remak of the Chicken Rectum

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Abstract—The chicken rectum receives a powerful excitatory innervation of non-adrenergic, non-cholinergic (NANC) nerves via the Remak nerve which is a ganglionated nerve trunk in the fowl viscera. To extract a smooth muscle excitatory material from the Remak nerve, tissue samples were boiled in 0.01 N HCl at 100°C for 5–7 min, homogenized, and centrifuged. Aliquots of supernatant were defatted with petroleum ether and lyophilized. The lyophilized residue dissolved in water (ERN) was bioassayed for contracting activity on the longitudinal muscle of the guinea-pig ileum and, if needed, the isolated whole chick rectum. Approximately half of the contacting activity of ERN was attributable to acetylcholine. The remainder was found to be mediated by neither histamine, serotonin, angiotensin II nor prostaglandins (E₁, E₂ and F₂α). The ERN activity was abolished by 2–3 min boiling in alkali and 30 min incubation at 37°C with pepsin, but sustained after boiling in acid, indicating that the mediator of the contracting activity is probably a peptide. Active fractions were obtained with one peak after gel filtration with Sephadex G-50. They were pooled and applied to a Sephadex G-25 column. The $V_o/V_o$ values for the active material ranged from 1.69 to 1.85, indicating that it has a molecular weight of 1000–1300 by comparison with $V_o/V_o$ values for peptides of known molecular weights applied to the same column.

The intestine in a variety of species of vertebrate animals possesses an excitatory innervation of non-adrenergic, non-cholinergic (NANC) nerves whose neurotransmitter is unknown (1–5). Adenosine 5-triphosphate (ATP) and substance P have been considered as excitatory neurotransmitters in some of the NANC nerves (4, 5, 6–8).

The rectum of the chicken receives a powerful excitatory innervation of NANC nerves via the Remak nerve which is a unique autonomic ganglionated nerve trunk in the fowl viscera (3, 9–11). Recently, the neuromuscular transmission has been studied on excitatory junction potentials (12). Evidence from pharmacological and electrophysiological studies indicates that cell bodies of NANC neurones innervating the rectum lie in Remak's ganglia (13). If NANC neurotransmitter is biosynthesized in cell bodies and delivered by an axonal transport to the axon terminals where it exerts its biological effects (14–16), one would expect the presence in the Remak nerve of a substance which serves as NANC neurotransmitter.

In the present study, we have attempted to extract from Remak nerve a smooth muscle contracting material which differs from acetylcholine. Some of these results have previously been reported to the Pharmacological Society in Japan (17).

Materials and Methods

Domestic fowls (Gallus domesticus) of either sex, aged more than 100 days, were obtained from commercial sources. The precise genetic background of these birds was not known, though they represented...
hybrids of White Leghorn and other strains. Birds were stunned and bled, and the whole rectum together with the Remak nerve was removed and immediately immersed in ice-cold Tyrode solution containing indomethacin (7.5 × 10⁻⁶ g/ml). Samples of the Remak nerve were dissected using fine scissors from the isolated organs under a binocular microscope.

**Extraction:** Tissue samples of one hundred Remak nerves were blotted dry, weighed (2.8–3.5 g), placed in a glass tube containing 30–60 ml of ice-cold 0.01 N HCl solution, boiled at 100°C for 5–7 min, and homogenized. After centrifugation at 10,000×g at 4°C for 20 min, aliquots of supernatant were lyophilized and stored in a deep freezer at -20°C. In some cases, aliquots of supernatant were defatted by washing 3 times with petroleum ether or ethyl acetate.

**Bioassay:** Lyophilized extracts were dissolved in distilled water to give a concentration equivalent to 0.3–0.5 g wet weight of fresh tissue per ml, centrifuged at 10,000×g for 10 min to remove insoluble materials, and then used. Activity of the reconstituted extract to contract intestinal smooth muscles was tested on the longitudinal muscle layer of the guinea-pig ileum, and, if needed, the whole rectum isolated from young chick (less than 2 weeks). Both were suspended in a 2.5 ml polypropylene organ bath, and isometric tension changes were recorded by a force-displacement transducer (Nihon Khoden, TB-612T) and a potentiometric recorder (Hitachi, 056). Tyrode solution, composed of (mM) NaCl, 136.9; KCl, 2.7; NaH₂PO₄, 0.4; CaCl₂, 1.8; MgCl₂, 2.1; NaHCO₃, 11.9; and glucose, 5.6, aerated and kept at 33°C, was used for the bathing solution. An abdominal rectus of the frog, eserinized overnight in Ringer solution for amphibia containing physostigmine (5 × 10⁻⁸ g/ml) at 4°C, was used to test activity of the extract to contract skeletal muscle. This muscle was suspended in a 2.0 ml organ bath filled with frog Ringer solution aerated at room temperature, and its isotonic tension was recorded on smoked paper with a writing lever (x×10). The extract and drugs were usually injected using a micropipette into the bathing solution in a volume less than 0.02 ml, i.e., less than 1% of the bath volume.

**Drugs:** Drugs used were propranolol hydrochloride (Sigma), phentolamine mesylate (Ciba Geigy), acetylcholine chloride (Tokyo Kasei), d-tubocurarine chloride (Merck), atropine sulphate (Nakarai), tetrodotoxin (Sankyo), serotonin creatinine sulphate (Wako), physostigmine salicylate (Sigma), ATP (Wako), ADP (Sigma), AMP (Sigma), adenosine (Wako), indomethacin (Sigma), SC 19220 (D.G. Searle & Co.), prostaglandins (E₁, E₂, and F₂α) (Sigma), histamine dihydrochloride (Wako), pyrilarmine maleate (Sigma), angiotensin II (Peptide Institute Inc.), [Sar¹, Ala⁸]-angiotensin II (Peptide Institute, Inc.), methyserydine hydrogen maleate (Sandoz), tryptamine hydrochloride (Sigma), Leu-enkephalin (Peptide Institute Inc.), VIP (Peptide Institute Inc.) and substance P (Peptide Institute Inc.). Indomethacin and prostaglandins were dissolved in 0.05 ml of 95% ethanol and then diluted with distilled water to the desired concentrations. All other drugs were dissolved in distilled water to the desired concentrations. Drugs were removed by replacing the bathing medium with the fresh solution. The final concentrations of their salts in the bathing solution are indicated in the text.

**Gel filtration:** The lyophilized extract was dissolved in 0.1 M acetic acid solution and applied to a Sephadex G-50 column (1.6×70 cm) or a Sephadex G-25 column (1.2×55 cm), pre-equilibrated with 0.1 M acetic acid solution. Elution was performed with the same acid solution at a flow rate of 30 ml/hr for the former column and 21.4 ml/hr for the latter column at room temperature. Fractions of 2 or 4 ml were collected and lyophilized. Each fraction was dissolved in 0.2 or 0.4 ml distilled water, and its activity to contract the longitudinal muscle layer of the guinea-pig ileum was assayed. Elution was monitored by a UV monitor at 254 or 280 nm. The void volume (V₀) of the columns was determined with blue dextran, and the elution volumes (Vₑ) of some synthetic peptides were determined by monitoring the peak UV absorbance at 280 nm.
Results

1. Contracting activity of the extract

The extract caused contraction of the longitudinal muscle layer of the guinea-pig ileum (LMGPI), as shown in Fig. 1. The contraction started within 2 sec after application of the extract, reached its maximal tension in 5–10 sec, and decayed immediately after removal of the extract by replacing the bathing solution with fresh Tyrode solution. When successive applications were made at an interval of 3–4 min, responses of the same magnitude were obtained. The extract in a concentration of 0.1–0.3 mg wet weight tissue per ml was effective in producing a detectable rise in tension of the muscle, and the effect increased in a concentration-dependent manner up to 4 mg wet weight tissue per ml (the maximal concentration examined). Frequently, the contractile response was preceded by a brief relaxation. The excitatory action of the extract was unaffected by tetrodotoxin (2×10⁻⁷ g/ml), indicating its direct action on the smooth muscle of LMGPI. Figure 1 also shows that the extract-induced contraction was reduced to about half of its initial magnitude by 2×10⁻⁷ g/ml atropine which was high enough to abolish responses to acetylcholine at concentrations up to 10⁻⁷ g/ml. The extract produced contraction of eserinized abdominal rectus of the frog, but the effect was completely blocked by d-tubocurarine (5×10⁻⁷ g/ml). In the presence of d-tubocurarine, it had no effect on the skeletal muscle even when its concentration was increased up to 20 mg wet weight tissue per ml, which was 100 times or so higher than the minimum effective concentration for LMGPI. These results indicate that only part of the smooth muscle contracting activity is attributable to acetylcholine, but all skeletal muscle contracting activity is attributable to acetylcholine. Thus, using the skeletal muscle, the acetylcholine concentration of the extract was estimated to be 3.5±0.7 μg/g wet weight tissue (n=3).

2. Comparison of some properties of the active substance and putative transmitters:

Histamine, serotonin, prostaglandins (E₁, E₂ and F₂ₐ), ATP, substance P and angiotensin II caused contractions of LMGPI.

**Histamine:** The contraction produced by histamine developed at a rate similar to that of the contraction produced by the extract.

![Figure 1. Smooth muscle contracting activity of extracts from Remak nerve. A, Contractions produced by extracts of Remak nerve (ERN, ●), 4 mg tissue wet weight/ml, and acetylcholine (ACh, ○), 2×10⁻⁸ g/ml, of the longitudinal muscle of the guinea-pig ileum (LMGPI). Atropine (atro, ▲), 2×10⁻⁷ g/ml, abolished ACh-induced contraction, but reduced only partially (about 50%) extract-induced contraction. B, A concentration-response curve showing the effect of extract of Remak nerve on the tension of LMGPI treated with atropine, 2×10⁻⁷ g/ml. Ordinate: Increase in tension as a percentage of the contraction produced by extract of Remak nerve, 4 mg tissue wet weight/ml. Abscissa: log extract concentration (mg tissue wet weight/ml). Each point is the mean of three determinations in one preparation.
Pyrilamine (10^{-6} g/ml), an antagonist of histamine, blocked the contractile response to histamine, but had little effect on the contractile response to the extract (Fig. 2).

**Serotonin:** The contractile response to concentrations of serotonin (up to 8 \times 10^{-7} g/ml) was markedly reduced by tryptamine (2 \times 10^{-6} g/ml) and abolished by methysergide (10^{-6} g/ml). Tryptamine itself caused a small but appreciable increase in tone and spontaneous activity of the muscle. Neither tryptamine nor methysergide reduced the extract-induced contraction. Actually tryptamine had a tendency to slightly enhance the extract-induced contraction.

**Prostaglandins (PGs):** An experiment with PGs (E_1, E_2 and F_2\alpha) is illustrated in Fig. 3. An antagonist of PGs, SC 19220 (5 \times 10^{-6} g/ml), reduced partially but specifically contractile responses to prostaglandins (E_1, \bigcirc, 8 \times 10^{-8} g/ml; E_2, \triangle, 8 \times 10^{-8} g/ml; and F_2\alpha, \Box, 2 \times 10^{-7} g/ml), but had little effect on the response to extract (ERN, •), 4 mg tissue wet weight/ml. The experiment was performed on the muscle treated with atropine (10^{-6} g/ml), pyrilamine (5 \times 10^{-6} g/ml) and tryptamine (2 \times 10^{-6} g/ml).}

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**Substance P:** The contraction produced by substance P resembled the contraction produced by the extract in time course. The concentrated solution of substance P was diluted with 0.05 M Tris-HCl buffer solution (pH=7.8). A small amount of the lyophilized extract was dissolved with 0.05 M Tris-HCl buffer solution (pH 7.8) to make a solution with about the same potency as the substance P solution. They were incubated with active this extraction procedure. Contracting activities of both lyophilized materials were compared on the same muscle. There was no detectable difference in the contracting activity between them, this indicating no loss of excitatory material after extraction with ethyl acetate.

**ATP and related substances:** ATP and ADP, but not AMP and adenosine, had an excitatory effect on LMGPI in concentrations higher than 2 \times 10^{-5} g/ml. ATP lost its contracting activity after a brief boiling in 1 N HCl solution (2–3 min), but the extract retained its activity fairly well after boiling in acid. By contrast, boiling with 1 N NaOH solution for 2–3 min resulted in complete loss of the contracting activity of the extract, but not of ATP (Fig. 4).
or inactivated (boiled at 100°C for 1 hr) chymotrypsin \(2 \times 10^{-6} \text{ g/ml}\) for 30 min at 37°C. Substance P was destroyed when incubated with the active enzyme, but the extract remained almost unchanged, as shown in Fig. 5. When 0.01 N HCl solution instead of 0.05 M Tris-HCl buffer solution and pepsin instead of chymotrypsin were used, the activities in both solutions were destroyed.

Angiotensin II: Angiotensin II was also an excitatory peptide on the muscle. The angiotensin-induced contraction was characterized by a slow and prolonged time course, and from this aspect, it was quite different from the extract-induced contraction. The effect of angiotensin was completely antagonized by \([\text{Sar}^1, \text{Ala}^8]\)-angiotensin II. The antagonist alone and its combination with any of the antagonists used above had no effect on the contracting activity of the extract.

Assuming that all contracting activity of the extract in the presence of atropine is due to each of these excitatory substances, the amount of each substance in the Remak nerve was estimated by matching the contractions produced by the extract with the contractions produced by known concentrations of the substance and presented in Table 1.

3. Effect of the extract on isolated chick rectum

Figure 6 shows contractile responses of the chick rectum to the extract (4 mg wet weight tissue per ml) in the presence of atropine \(5 \times 10^{-7} \text{ g/ml}\) which blocked

| Table 1. Estimated concentrations of the excitatory substances in Remak nerve (g/g wet weight tissue) assuming that all excitatory activity of the extract is due to each of them |
|---------------------------------------------------------------|
| Substance                  | Estimated Concentration |
|--------------------------|------------------------|
| ATP                      | \(4 \times 10^{-4} - 2 \times 10^{-4}\) |
| Substance P              | \(2.5 \times 10^{-6} - 10^{-4}\) |
| Angiotensin II           | \(10^{-5} - 5 \times 10^{-5}\) |
| Histamine                | \(2 \times 10^{-2} - 10^{-1}\) |
| Serotonin                | \(7 \times 10^{-7} - 10^{-6}\) |
| Prostaglandins \((E_1, E_2, \text{and } F_2)\) | \(5 \times 10^{-6} - 10^{-6}\) |
responses of the tissue to acetylcholine in concentrations up to $8 \times 10^{-6}$ g/ml. Minimum effective concentrations of the extract ranged from 0.2 to 0.4 mg wet weight tissue per ml, which were similar to those in LMGPI. It was confirmed that the excitatory action of the extract on this tissue was not attributable to histamine, serotonin, angiotensin II and prostaglandins. Figure 6 also shows a typical experimental result when the extract (4 mg wet wt. tissue/ml) was compared with histamine ($4 \times 10^{-7}$ g/ml) and serotonin ($8 \times 10^{-7}$ g/ml). Pyrilamine ($5 \times 10^{-6}$ g/ml) blocked completely the contractile response to histamine, but did not change that to the extract. With serotonin, a biphasic response was produced, consisting of an initial relaxation and a later contraction, which was clearly different in pattern from that to the extract.

4. Effect of extract from the sympathetic chains

The sympathetic chains as well as the Remak nerve were isolated from ten chickens, and extracts from both tissues were prepared by the same procedures. Activity to contract LMGPI was compared between both extracts in the presence of atropine ($2.5 \times 10^{-7}$ g/ml). The extract from the Remak nerve was about ten times more effective in contracting the muscle than the extract from the sympathetic chains, when used at the same wet weight tissue per ml. This relationship was held in additional presence of pyrilamine ($10^{-6}$ g/ml), methysergide ($10^{-6}$ g/ml), propranolol ($10^{-6}$ g/ml) and phentolamine ($10^{-6}$ g/ml). This provided evidence that the unknown active substance is present in the Remak nerve at a much higher concentration than that in the sympathetic chains, assuming that the activity of both extracts is due to a common active substance.

5. Gel filtration of the extract from Remak nerve

We used Sephadex G-50 and Sephadex G-25 columns. With a Sephadex G-50 column, the material with excitatory activity eluted with one peak in the eluate at the elution volume ($V_e$) of 2.4 times the void volume ($V_0$) ($V_e/V_0=2.4$), as illustrated in Fig. 7. Active eluates were pooled, lyophilized, dissolved in 0.1 M acetic acid solution, and then applied to a Sephadex G-25 column. In this case, the peak of excitatory activity appeared at $V_e/V_0=1.69-1.85$ (Fig. 8). Peptides whose molecular weights are known, leu-enkephalin (556), angiotensin II (1046) and vasoactive intestinal polypeptide (VIP) (3325), were run under the same conditions as the extract and their $V_e/V_0$ values were obtained (see Materials and
Methods). When these values were plotted against log molecular weight, there was a linear relationship, as shown in Fig. 9. From the value of $V_e/V_0$ for the contracting activity, the molecular weight of the active substance present in the extract was estimated to be 1000–1300 (mean±S.E.: 1150±75, n=4).

When the contracting activity was detected using the isolated chick rectum instead of LMGPI, the peak contracting activity appeared invariably at a slightly larger elution volume, as shown also in Fig. 7.
Fig. 9. Estimation of the molecular weight of active material in extract from Remak nerve. Peptides of known molecular weights, leu-enkephalin (556) angiotensin II (1046) and VIP (3325), were applied to the same column as in Fig. 8 and procedures of gel chromatography were also the same as in Fig. 8. Ordinate: ratio of elution volume (Ve) to the void volume (Vo). Abscissa: log molecular weight. Ve/Vo values for the active material present in extract of Remak nerve lie between the two dotted lines (n=4), indicating that it has a molecular weight of 1000-1300.

Discussion

Crude extracts obtained from the rectal region of the Remak nerve of the chicken showed activity to contract the longitudinal muscle of the guinea-pig ileum (LMGPI) and the chicken rectum. Acetylcholine was present in the extract, but it only accounted for approximately half of the activity since the contractile response to the extract was reduced to 50% in magnitude by atropine. The atropine-resistant contracting effect is mediated by some substance other than histamine, serotonin and angiotensin II, as shown by the failure to suppress the contracting effect by pyrilamine, methysergide and [Sar1, Ala8]-angiotensin II. Furthermore, it is nearly impossible to assume that histamine, serotonin and angiotensin II are present in the Remak nerve at such high concentrations as presented in Table 1.

Though fresh tissues, before extraction, were quickly immersed in Tyrode solution containing indomethacin in a concentration high enough to block biosynthesis of prostaglandins (PGs), PGs stored in these tissues, if any, can possibly appear in the extract. A PGs antagonist, SC 19220, inhibited contractions produced by PGs (E1, E2 and F2a), but did not alter the contracting effect of the extract. This excluded PGs from the possible mediators of the contracting effect. When the extract was defatted by partitioning at about pH 2.0 with ethyl acetate, the activity remained in the aqueous phase. This also provided evidence that the active substance differs from lipid substances of PGs.

Burnstock (6) proposed adenine nucleotides, especially ATP, as the neurotransmitter of NANC nerves. Indeed, ATP has an excitatory effect on both LMGPI and chicken rectum (2). However, the methods of tissue extraction used in the present experiments would not allow ATP to remain unchanged, and the excitatory compound present in the extract seemed unlikely to be this adenine nucleotide. This was confirmed by the observations that ATP was destroyed by an brief boiling in 1 N HCl solution, but sustained after a brief boiling in 1 N NaOH solution, whereas the LMGPI contracting activity of the extract remained almost unchanged after boiling in acid, but it was destroyed after boiling in alkali.

Peptidergic nerves have been suggested to be present in the gut (5, 18–20). In the guinea-pig ileum, it has been suggested that substance P serves as an excitatory neurotransmitter (4, 8). A similar role for substance P in NANC nerve-mediated contraction has been suggested in the chicken caecum (7). The active substance in the extract was destroyed by pepsin, indicating that the mediator of the contracting effect is probably a peptide. No susceptibility of the active material to chymotrypsin is a less convincing result, since the lyophilized extract was found to contain adenylic acid by monitoring UV absorption during gel chromatography and by testing biological activities of eluates. When lyophilized extract was dissolved in such a small volume (0.2 ml) of 0.05 M Tris-HCl buffer solution as in the present experiments, it would be possible for this adenylic acid to overcome the buffer capacity and reduce the pH to a pH at which chymotrypsin could not show its activity.
An Excitatory Material from Remak Nerve

possibility, therefore, is not ruled out that the mediator is substance P or its related compound. However, it should be pointed out that the concentration of substance P-like activity in the extract was estimated to be more than 700 ng/g wet weight tissue (Table 1), but a histochemical study with the indirect immunofluorescence technique failed to demonstrate substance P-positive nerve cells and fibers in the Remak nerve (Ikeda, personal communication), and that the molecular weight of this material determined by gel filtration is slightly smaller than substance P. Brodin et al. (7) reported that when applied to the Sephadex G-25 column, substance P-like compounds in extracts of chicken proventriculus and duodenum eluted slightly after synthetic bovine substance P, and they suggested the disimilarity in the amino acid sequence between them.

An interesting finding is that a slight difference was seen in the elution volume of peak contracting activity between LMGPI and chick rectum. This could mean that the extract contained two excitatory substances: one had a much higher contracting potency in LMGPI than in the smooth muscle of the chick rectum, and the other had a reverse relation in the contracting potency. To determine whether the material in question is substance P or its related compound, or a combination of two excitatory substances, further studies on purification and identification are needed.

The material has a high potency in contracting the smooth muscle of the chick rectum which receives a dense innervation of NANC nerves via the Remak nerve, and it is present at a much higher level in the Remak nerve than in the sympathetic chains. These results may mean that the material is one of the possible candidates for the transmitter of NANC nerves, at least in the chicken rectum.

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