EFFECTS OF VARIOUS DRUGS AND EXTRINSIC DENERVATION ON THE NON-ADRENERGIC INHIBITORY NEURONS IN THE RAT STOMACH

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The effects of various drugs and extrinsic denervation on the non-adrenergic inhibitory nerves in the rat stomach were examined. Stimulation of the intramural nerves supplying the longitudinal smooth muscle strips taken from the rat stomach produced responses consisting initial contractions followed by relaxations in the presence of guanethidine in case of the tone of the preparation maintained with serotonin. After scopolamine treatment, transmural nerve stimulation (TMS) caused a relaxation or a relaxation followed by a transient after-contraction. All of these responses were abolished reversibly with tetrodotoxin. After cold storage of rat stomach strips for 5 d at 4°C, the TMS-induced response was not observed, but noradrenaline still relaxed these strips. The TMS-induced relaxation reached a maximum amplitude at 2–5 Hz, and was entirely independent of α - and β -blockers, or a combination of them. It was not affected by treatment of 6-hydroxydopamine and reserpine. These results strongly suggested that the relaxation was not myogenic, but neurogenic, and was not elicited by stimulation of adrenergic neurons. Strychnine and poly-L-lysine inhibited the relaxation elicited by TMS even in normal and transection-treated rats. Histochemical studies showed degenerative changes due to the dissection at the axons of a ganglion in the Auerbach's plexuses by transection. The effect of various drugs and extrinsic denervation on the response to TMS in relation to non-adrenergic inhibitory nerves in the rat stomach is discussed.

Keywords — non-adrenergic inhibitory nerve; denervation; strychnine; poly-L-lysine; rat stomach

INTRODUCTION

The existence of non-adrenergic, non-cholinergic neurons in the mammalian gastrointestinal tract was widely recognized.^{1–3)} It was satisfactory to consider that the cell bodies of these neurons were located in intramural plexuses (Auerbach's plexuses) and many were controlled by preganglionic parasympathetic neurons.^{1–4)} However, there was no direct evidence that the cell bodies on the non-adrenergic, non-cholinergic neurons were located in Auerbach's plexuses. So, we developed a new surgical denervation (transection) in order to make it clear.

The evidence that adenosine triphosphate might be the transmitter of these inhibitory neurons has been reviewed and the characteristics of purinergic nerves have been recently described.^{1,2)} On this subject, many investigators have been reported pharmacological studies of these inhibitory neurons in the stomach of several species.^{1–3,5–7)} However, there was no description about the effect of completely extrinsic denervation on these inhibitory neurons and details have not been reported on the non-adrenergic inhibitory neurons in the rat stomach.

The present experiments were carried out in

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order to study the effects of various drugs and extrinsic denervation on the non-adrenergic inhibitory neurons in the rat stomach.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley (SD), weighing between 250–350 g, were stunned and bled. The stomach was dissected out immediately. After two strips of about 2 mm width and 2.5 cm length were cut from the longitudinal smooth muscle of the forestomach, the mucosal layers were removed. The strip was placed vertically in an organ bath containing 30 ml of modified Krebs solution (mM: NaCl 120, CaCl₂ 2.2, MgCl₂ 1.2, KCl 4.7, NaHCO₃ 25, KH₂PO₄ 1.2, glucose 14; pH 7.4) equilibrated with a mixture of 95% O₂ and 5% CO₂ at 37°C. The mechanical activities of the preparations were recorded isometrically with mechano-electronic transducers (Nihon Kohden Model: SB-1T). After the preparations were allowed to equilibrate for 90 min before starting the experiment, it was loaded with a tension of 0.5 g. Transmural nerve stimulation (TMS) to the muscle was applied through two parallel platinum (2 × 2.5 mm, separated by 4 mm). An electronic stimulator was used to deliver the rectangular pulses. Responses were obtained with pulses of 50 volts strength and 0.1–1 ms duration. Trains of pulses were applied for 15 s.

For cold storage, the fundus strips were placed in 1000 ml of modified Krebs solution at 4°C for several days.

Transection and Transection (SGA)—Male SD rats weighing 250 and 350 g were used. Under ether anesthesia, a mid-line incision was made along the abdominal wall and the stomach was gently pulled to the outside by means of a stainless steel ring-pincette. Two small clamps were then gently applied 1–2 cm apart along the limiting ridge. An incision was made along the limiting ridge. Simultaneously with this incision, the omentum around the forestomach was also resected except the short gastric artery and vein. The rats were used for the experiments 3 d after this operation, which was termed a 'transec-

tion'. In addition to this transection, when the remaining short gastric artery and vein were resected, the rats were employed in the experiments 7 d after this resection, which was termed a 'transection (SGA)'. Our prepared rats have been kept in the laboratory for more than 6 months since these operations were performed.

Sympathectomy—Surgical sympathectomy was done mainly according to Kamata and Kasuya.⁸⁾

Vagotomy—Bilateral surgical vagotomy was done according to Lambert.⁹⁾ The nerves were isolated for a distance of about 15 mm. The vagus was then cut below the diaphragm, using fine straight scissors, and segment, roughly 10 mm long, was resected. Completeness of vagotomy could be assessed by means of electrical stimulation of the vagus after vagotomy. An absence of contraction of the stomach after electrical stimulation was taken to indicate that vagotomy was complete.

Chemical Sympathectomy—The 6-hydroxydopamine (6-OHDA) was diluted in saline containing 0.5% ascorbic acid in nitrogen atmosphere to a concentration to make 50 mg in 1 ml of the vehicle. 6-OHDA 150 mg/kg was administered intravenously. A second treatment with 250 mg/kg was made 1 week later. Equal numbers of control rats were treated at the same time with saline containing 0.5% ascorbic acid.

Reserpine Treatment—Reserpine was administered intraperitoneally. Rats were treated first with 3 mg/kg of reserpine and furthermore, twice with 1 mg/kg every 24 h. The stomach was dissected out 12 h after the final administration of reserpine.

Acetylcholinesterase Histochemistry—Frozen preparations (30 μm thickness) of the rat forestomach, fixed in glutaraldehyde-phosphate buffer (pH 7.3) for 90 min, were incubated in maleate-citrate buffer (pH 6.0) containing tetraisopropylpyrophosphoramidate (10⁻⁵M) for 90 min to inhibit pseudocholinesterase as described by Karnovsky and Roots.¹⁰⁾

Monoamine Fluorescence Histochemistry—Mucosa-free forestomach preparations

were prepared as described above. All tissues were placed in ice-cold modified Krebs solution. The forestomach was first frozen in isopentane cooled in liquid nitrogen and then freeze-dried in a Haake freeze dry at -30°C and 2×10^{-3} mm Hg for 3 d before treatment with gaseous paraformaldehyde (relative humidity 80%) at 80°C for 3 h. It was then vacuum embedded in paraffin wax and $7\text{ }\mu\text{m}$ sections prepared. The sections were placed on non-fluorescent microscope slides, deparaffinized by addition of xylene and mounted in Enteran (Merck) for fluorescence photomicroscopy according to the method described by Falck, Hillarp, Thieme and Torp.¹¹⁾

Electron Microscopic Specimens—After fixation in Karnovsky's 0.5–1.5% paraformaldehyde-glutaraldehyde (pH 7.4) solution, the stomach was quickly removed and opened along the greater curvature. 10 to 15 strips or pieces were cut from both the lesser and the greater curvature and along the middle axis from both anterior and posterior wall of the stomach, and then fixed in Karnovsky's 1–3% paraformaldehyde-glutaraldehyde solution (pH 7.4). Blocks of 2 to 3 mm wide and 3 to 4 mm long, including part of the mucosal layers, were trimmed. These blocks were postfixed in 2% OsO_4 for 2 h, then in 2% uranyl acetate solution for 30 min, dehydrated, and embedded in epoxy resin. To search for Auerbach's plexus, semithin sections were cut and stained with toluidine blue or colored pink with felt tip marker and ganglia were oriented under the stereomicroscope. Thin sections in the region of the ganglia were mounted on uncoated grids, stained with 2% uranyl acetate and lead citrate, and subsequently examined with a JEOL 100 CX electron microscope.

Drugs used—adenosine triphosphate disodium salt (Sigma), bicuculline (Sigma), chlorpheniramine maleate (Sankyo Co., Ltd), cimetidine (SK&F), compound 48/80 (Sigma), dibucaine (Teikoku Chem. Ind.), dilazep dihydrochloride (Kowa Company, Ltd), diphenhydramine (Kowa Company, Ltd), dipyrindamole (Boehringer Ingelheim), dopamine hydrochloride (Wako

Pure Chem. Ind.), γ -amino butyric acid (Wako Pure Chem. Ind.), glutamic acid (Wako Pure Chem. Ind.), glutaraldehyde (Polyscience), guanethidine sulfate (Ciba-Geigy), haloperidol (Dainippon Pharm. Co., Ltd), hemicholinium (Aldrich), hexamethonium bromide (Sigma), histamine dihydrochloride (Wako Pure Chem. Ind.), 6-hydroxydopamine hydrobromide (Calbiochem-Behring Corp.), kainic acid (generously supplied by Dr. Shinozaki, H., The Tokyo Metropol. Institute of Med. Sci.), leucine- and methionine-enkephalin (Calbiochem-Behring Corp.), morphine hydrochloride (Sankyo Co., Ltd), naloxone (generously supplied by Endo Laboratories, Inc.), *l*-noradrenaline bitartrate (Sigma), osmium tetroxide (OsO_4 , Merck), ouabain (Merck), paraformaldehyde (Wako Pure Chem. Ind.), phentolamine (Regitin: Ciba-Geigy), picrotoxin (Wako Pure Chem. Ind.), poly-L-lysine (Miles-Yeda, Ltd, M.W. = 2320), propranolol hydrobromide (Sigma), quisqualic acid (generously supplied by Dr. Shinozaki, H.), reserpine (Apoplon: Daiichi Seiyaku Co.), scopolamine hydrobromide (Wako Pure Chem. Ind.), sotalol hydrochloride, strychnine nitrate (Wako Pure Chem. Ind.), tetrodotoxin (Sankyo Co., Ltd), theophylline (Sigma), tolazoline (Imidalin: Yamanouchi Seiyaku Co.), uranyl acetate (Taab), yohimbine hydrochloride (Wako Pure Chem. Ind.), α -methyl-*p*-tyrosine (Sigma).

All other chemicals were reagent grade.

RESULTS

Response to Transmural Nerve Stimulation

The tone of the preparation was maintained with serotonin (10^{-5}M). Guanethidine was added in a sufficient amount ($5 \times 10^{-6}\text{M}$) to abolish responses to adrenergic nerve stimulation. Relationships between stimulation frequency and responses were investigated by applying stimuli for 5 s with pulses of 50 volts strength and 0.1–1 ms duration. Transmural nerve stimulation (TMS) caused only a relaxation at a low range of frequency (0.5–10 Hz) in the presence of scopolamine (10^{-5}M) and guanethidine ($5 \times 10^{-6}\text{M}$). However, when the frequency was

increased over 20 Hz, the response became somewhat variable in the absence or presence of scopolamine (10^{-5}M) to abolish responses to cholinergic nerve stimulation (Fig. 1).

The response was classified into the following types in the absence of scopolamine; initial rapid contraction followed by relaxation; relaxation followed by after-contraction; pure relaxation. Relaxation responses were also examined in the presence of scopolamine. TMS elicited a relaxation over the range of frequency from 0.5–50 Hz. The depth of relaxations increased with a maximum at about 5 Hz. Maximum relaxation sometimes occurred at 2 Hz.

We examined whether the TMS-induced relaxation described so far were neurogenic or myogenic responses. The following experiments were performed in the presence of scopolamine (10^{-5}M) and guanethidine ($5 \times 10^{-6}\text{M}$). Tetrodo-

toxin (TTX, $5 \times 10^{-7}\text{ g/ml}$), cocaine ($4.4 \times 10^{-4}\text{M}$) and dibucaine ($2.6 \times 10^{-5}\text{M}$) reversibly abolished the inhibitory responses by TMS, but noradrenaline ($3 \times 10^{-6}\text{M}$) still relaxed these preparations (Fig. 2A–C). As shown in Fig. 2D, the cold storage treatment for 5 d inhibited the TMS-induced contraction in the absence of scopolamine and also the TMS-elicited relaxation in the presence of scopolamine in case of the tone of the preparation maintained with serotonin, but the noradrenaline (10^{-7}M)-induced relaxation was still observed. These results show that the TMS-elicited responses are neurogenic.

Effects of Various Drugs on the TMS-induced Relaxation

All the responses described so far may be mediated by stimulation of the intramural nerve because TMS with a short pulse duration was ef-

FIG. 1. *Relationships between Frequency of Transmural Nerve Stimulation and Types of Responses in the Absence of Scopolamine, or in the Presence of Scopolamine and Guanethidine in the Longitudinal Smooth Muscle from the Rat Forestomach*

Transmural nerve stimulation was applied at various frequencies for 15 s (1 ms duration) in the absence of scopolamine (upper trace-(A)) and in the presence of scopolamine (10^{-5}M) and guanethidine ($5 \times 10^{-6}\text{M}$) (lower trace-(B)). The horizontal bar shows the scale for 1 min and the vertical bar the scale for 5 g.

fective. Therefore, experiments were carried out to study the effect of various drugs on the TMS-induced responses. The following experiments were performed in the presence of scopolamine (10^{-5}M) and guanethidine ($5 \times 10^{-6}\text{M}$) in case of the tone of the preparation maintained with

FIG. 2. Effects of Tetrodotoxin, Cocaine, Dibucaine and Cold Storage on Responses to Transmural Nerve Stimulation of the Longitudinal Smooth Muscle from the Rat Forestomach

(A): tetrodotoxin (TTX: 5×10^{-7} g/ml); (B): cocaine ($4.4 \times 10^{-4}\text{M}$); (C): dibucaine (2.6×10^{-6} and $2.6 \times 10^{-5}\text{M}$); (D): effect of cold storage for 5 d at 4°C on the transmural nerve stimulation-induced contraction (TM-contr.) in the absence of scopolamine and guanethidine and on the transmural nerve stimulation-induced relaxation (TM-relax.) in the presence of scopolamine (10^{-5}M) and guanethidine ($5 \times 10^{-6}\text{M}$) in case of the tone of the preparation maintained with serotonin (10^{-5}M). Transmural nerve stimulation was applied at 2, 5, 10 and 20 Hz (1 ms duration).

serotonin (10^{-5}M).

Nicotine (10^{-4}M) and dimethylphenylpiperazinium ($5 \times 10^{-5}\text{M}$) relaxed the preparation (data not shown). As shown in Table I, the inhibitory responses elicited by TMS were unaffected by hexamethonium (10^{-4} to 10^{-6}M), which completely abolished the relaxation induced by the ganglionic stimulants and the vagus nerve stimulation (data not shown).

α -, β -Adrenergic Blocking Agents and Adrenergic Neuron Blocking Agent

It is reported that several 2-substituted imidazolines such as phentolamine, tolazoline and yohimbine, selectively blocked the inhibitory response of the guinea-pig taenia coli to non-adrenergic nerve stimulation.¹²⁾

To make certain of this, the effect of these drugs on the inhibitory responses elicited by

TABLE I. *Effects of Various Drugs on the Relaxation to Transmural Nerve Stimulation of the Normal, Reserpine-treated, 6-Hydroxydopamine-treated and Transection (SGA)-treated Rat Forestomach in the Presence of Scopolamine (10^{-5}M) and Guanethidine ($5 \times 10^{-6}\text{M}$)*

Drugs tested		Normal	Reserpinized	6-OHDA	Transection (SGA)
Phentolamine	$4.4 \times 10^{-5}\text{M}$	(—)	(—)	(—)	(—)
Tolazoline	$2.8 \times 10^{-5}\text{M}$	(—)	(—)	(—)	(—)
Yohimbine	$5.0 \times 10^{-6}\text{M}$	(—)	(—)	(—)	(—)
Propranolol	10^{-5}M	(—)	(—)	(—)	(—)
Sotalol	10^{-4}M	(—)	(—)	(—)	(—)
Guanethidine	10^{-5}M	(—)	(—)	(—)	(—)
Chlorpheniramine	$5.0 \times 10^{-6}\text{M}$	(—)	(—)	(—)	(—)
Diphenhydramine	10^{-6}M	(—)	(—)	(—)	(—)
Cimetidine	$4.0 \times 10^{-6}\text{M}$	(—)	(—)	(—)	(—)
Haloperidol	10^{-5}M	(—)	(—)	(—)	(—)
Dipyridamole	10^{-5}M	(—)	(—)	(—)	(—)
Dilazep	$5.0 \times 10^{-6}\text{M}$	(—)	(—)	(—)	(—)
Kainic acid	$4.7 \times 10^{-5}\text{M}$	(—)	(—)	(—)	(—)
Quisqualic acid	$5.3 \times 10^{-5}\text{M}$	(—)	(—)	(—)	(—)
Hexamethonium	10^{-4}M	(—)	(—)	(—)	(—)
Morphine	$3.1 \times 10^{-5}\text{M}$	(—)	(—)	(—)	(—)
Naloxone	10^{-5}M	(—)	(—)	(—)	(—)
Compound 48/80	$3.0 \times 10^{-5}\text{g/ml}$	(—)			(—)
Glutamate	10^{-3}M	(—)		(—)	(—)
Bicuculline	10^{-6}M	(—)		(—)	(—)
Ouabain	$5.0 \times 10^{-6}\text{M}$	(—)			
Theophylline	10^{-5}M	(—)			(—)
Hemicholinium	10^{-4}M	(—)			
Picrotoxin	10^{-5}M	(—)		(—)	(—)
Dibucaine	$2.6 \times 10^{-5}\text{M}$	(+)			(+)
Cocaine	$4.4 \times 10^{-4}\text{M}$	(+)	(+)		(+)
Tetrodotoxin	$5.0 \times 10^{-7}\text{g/ml}$	(+)	(+)		(+)
Strychnine	10^{-4}M	(+)	(+)	(+)	(+)

The tone of the preparation was maintained with serotonin (10^{-5}M). (—) symbol indicates that the relaxation to transmural nerve stimulation was unaffected by various drugs, while (+) symbol indicates that the relaxation of transmural nerve stimulation was blocked by them. Experimental details are given in the text.

TMS was examined. Phentolamine (10^{-7} to $4.4 \times 10^{-5} \text{M}$) had little effect on the relaxation induced by TMS, but in concentrations higher than 10^{-4}M it relaxed the fundus strips. The relaxation in response to TMS was almost never inhibited by other α -adrenoceptor blocking agents (tolazoline 10^{-7} to $2.8 \times 10^{-5} \text{M}$ and yohimbine 10^{-7} to $5 \times 10^{-6} \text{M}$) and β -adrenoceptor blocking agents (propranolol 10^{-7} to 10^{-5}M and sotalol 10^{-6} to 10^{-4}M) or the combined use of α -blockers and β -blockers and an adrenergic neuron blocking agent (guanethidine 10^{-7} to 10^{-5}M) (Table I).

Histamine and Antihistamines (Chlorpheniramine,

Diphenhydramine and Cimetidine)

The effects of histamine and antihistamines on the inhibitory responses elicited by TMS were examined. Histamine only produced the small contraction in the fundus strips of the rat forestomach, and the relaxations induced by TMS were unaffected by histamine (10^{-4}M) and its antagonists, chlorpheniramine (10^{-8} to $5 \times 10^{-6} \text{M}$), diphenhydramine (10^{-6}M) and cimetidine (10^{-8} to $4 \times 10^{-6} \text{M}$) (Fig. 3A and Table I).

Dopamine and Haloperidol

Dopamine antagonist, haloperidol (10^{-5}M) had no significant effect on the relaxation

FIG. 3. Effects of Histamine, Compound 48/80, Morphine, Naloxone, Leucine-enkephalin, Glycine, γ -Aminobutyric Acid and α -Methyl-*p*-tyrosine-treatment on Inhibitory Responses to Transmural Nerve Stimulation of the Longitudinal Smooth Muscle from the Rat Forestomach in the Presence of Scopolamine (10^{-5}M) and Guanethidine ($5 \times 10^{-6} \text{M}$) in Case of the Tone of the Preparation maintained with Serotonin (10^{-5}M)

(A): histamine (10^{-4}M); (B): effect of haloperidol (10^{-5}M) on the relaxation to transmural nerve stimulation in α -methyl-*p*-tyrosine-treated preparations (200 mg/kg, i.p., 6 h); (C): compound 48/80 ($3 \times 10^{-5} \text{g/ml}$); (D): morphine ($3.1 \times 10^{-6} \text{M}$); (E): naloxone (10^{-4}M); (F): leucine-enkephalin (10^{-5}g/ml); (G): glycine (10^{-3}M); (H): γ -aminobutyric acid (GABA: $5 \times 10^{-3} \text{M}$).

The horizontal bar shows the scale for 1 min and the vertical bar the scale for 1 g.

caused by TMS. Dopamine like other sympathomimetic drugs produced relaxation only. Furthermore, in the fundus strips from α -methyl-*p*-tyrosine (200 mg/kg *i.p.* 6 h)-treated rats, TMS-induced relaxations were unaffected in the absence or presence of haloperidol (Fig. 3B and Table I).

Morphine, Naloxone, Compound 48/80, and Methionine- and Leucine-enkephalin

Compound 48/80 up to 3×10^{-5} g/ml, had no effect on the relaxation elicited by TMS (Fig. 3C

and Table I), but it relaxed the fundus strips gradually at concentrations higher than 10^{-4} g/ml. Morphine (3.1×10^{-5} M) unaffected the inhibitory responses to TMS in the stomach strip in frequency higher than 2 Hz (Fig. 3D and Table I). However, morphine, in frequency lower than 1 Hz, depressed slightly the inhibitory responses of the stomach strip to TMS in some preparations, an action of which could not be reversed by naloxone (10^{-5} M). In addition, naloxone (10^{-7} to 10^{-5} M) alone had no effect on

FIG. 4. *Effects of Strychnine, Kainic Acid, Quisqualic Acid, Tachyphylaxis to Adenosine Triphosphate, Poly-L-lysine and Ouabain on the Relaxation to Transmural Nerve Stimulation of the Longitudinal Smooth Muscle from the Rat Forestomach in the Presence of Scopolamine (10^{-5} M) and Guanethidine (5×10^{-6} M) in Case of the Tone of the Preparation maintained with Serotonin (10^{-5} M)*

(A): strychnine (10^{-4} and 3×10^{-4} M); (B): kainic acid (4.7×10^{-6} and 4.7×10^{-5} M) and quisqualic acid (5.3×10^{-6} and 5.3×10^{-5} M); (C): desensitization with adenosine triphosphate (ATP: 5×10^{-5} M); (D): poly-L-lysine (5×10^{-4} g/ml); (E): ouabain (5×10^{-6} M).

Transmural nerve stimulation was applied at 2 Hz (0.1 and 1 ms). The horizontal bar shows the scale for 1 min and the vertical bar the scale for 1 g.

the relaxation by TMS (Fig. 3E and Table I). Methionine- (data not shown) and leucine-enkephalin (10^{-7} to 10^{-5} g/ml) also did not modify the muscle tension and unaffected the non-adrenergic inhibitory responses to the stomach strip to TMS (Fig. 3F).

γ -Aminobutyric Acid (GABA), Glycine, Glutamate, Bicuculline, Picrotoxin, Strychnine, Kainic Acid and Quisqualic Acid

In all preparations, GABA (5×10^{-3} M), glycine (10^{-3} M) and glutamate (10^{-3} M) failed to give any direct response. In the above concentration, they did not affect the inhibitory response to TMS (Fig. 3G, 3H and Table I). Bicuculline (10^{-5} M) and picrotoxin (10^{-5} M), antagonists of GABA, had no effect on the inhibitory responses to TMS (Table I). Strychnine caused an increase in tension slightly and inhibited almost completely the relaxation induced by TMS, and noradrenaline (10^{-7} M) relaxed fully these preparations (Fig. 4A and Table I). Both kainic acid (4.7×10^{-6} to 4.7×10^{-5} M) and quisqualic acid (5.3×10^{-6} to 5.3×10^{-5} M) gave no direct response and unaffected the inhibitory response to TMS (Fig. 4B and Table I).

Dipyridamole, Dilazep, Theophylline and Tachyphylaxis to Adenosine Triphosphate (ATP)

Dipyridamole (10^{-7} to 10^{-5} M), dilazep (10^{-7} to 5×10^{-6} M) and theophylline (10^{-7} to 10^{-5} M) unaffected the relaxation to TMS (Table I) and to ATP (data not shown).

ATP, at concentrations over 10^{-6} M, mimicked the inhibitory response elicited by TMS, especially at a lower frequency and a short duration (Fig. 4C). The inhibitory responses began quickly and attained to a peak amplitude within the same time as the responses induced by TMS. In addition to a relaxation, a biphasic response (a relaxation followed by a slight contraction) was observed (Fig. 4C). Furthermore, if ATP should be the transmitter liberated from the endings of the non-adrenergic inhibitory nerves, exposure to high concentrations of ATP will desensitize the fundus strip to ATP. In addition, the inhibitory responses elicited by TMS must be abolished when the ATP-produced desensitization of the

preparation was completed.

We, therefore, investigated whether the rat fundus strip preparations could be desensitized to ATP and TMS at the same time. Desensitization induced by ATP could be quite easily attained in rat fundus strip by repeated administration of ATP (10^{-6} to 10^{-5} M) at short intervals without washing out the bath. When the desensitization caused by ATP of the preparations was achieved, the TMS still induced a relaxation without any appreciable modifications (Fig. 4C). By washing out the bath solution for about 60 min, the relaxation to ATP was recovered.

Hemicholinium

In order to elucidate the involvement of a cholinergic nerve in the non-adrenergic inhibitory transmission, the following experiment was performed using the cholinergic neuron blocking agent, hemicholinium.¹³⁾

In the absence of scopolamine, TMS caused a response consisting of a contraction that was scopolamine- and atropine-sensitive. TTX (5×10^{-7} g/ml) blocked this contraction induced by TMS. Following incubation with hemicholinium (10^{-4} M) over 3 h with intermittent stimulation (10 Hz, 0.5 ms, 15 s), the contractile response to TMS was gradually reduced and abolished eventually (data not shown). Under this condition, TMS produced only relaxations or relaxation followed a transient after-contraction in the presence of scopolamine and guanethidine in case of the tone of the preparation maintained with serotonin (Table I).

Poly-L-lysine and Ouabain

Effects of poly-L-lysine (PLL) and ouabain on the TMS-induced relaxation were investigated (Fig. 4D and 4E). PLL (5×10^{-4} g/ml) caused a transient relaxation followed by tonic after-contraction and depressed the relaxation elicited by TMS in the presence of scopolamine and guanethidine. The relaxation to PLL at concentrations of 5×10^{-4} and 10^{-3} g/ml were $49 \pm 6\%$ and $55 \pm 5\%$, respectively. These inhibitory responses were concentration-independent in the range between 5×10^{-4} and 10^{-3} g/ml. Lysine had no effect on the TMS-induced relaxation

and the relaxation to PLL was insensitive to TTX. We observed the increasing tension by the administration of ouabain ($5 \times 10^{-6}M$), but the relaxation to TMS was unaffected by that of ouabain ($5 \times 10^{-6}M$) in the presence of scopolamine and guanethidine.

Cholinesterase Histochemistry

In the rat forestomach muscle, fine fibers, ganglia and nerve bundles were present. However, the nerve plexuses (Auerbach's plexuses) were more abundant than catecholamine-positive neurons. Furthermore, cholinesterase-positive ganglion cells were always spherical. As shown in Fig. 5, in the transection (SGA)-treated forestomach, fine fibers, ganglia and nerve bundles were also present and almost intact. Continuous variations in cholinesterase activity from intense to very low (or negative) occurred in intramural ganglia of the transection (SGA)-treated forestomach.

Catecholamine Fluorescence Histochemistry

Monoamine fluorescence histochemistry revealed numerous greenish fluorescence nerve

cell bodies associated with nerve bundles and fibers were sparsely distributed throughout the muscle, but many were seen running parallel to and around blood vessels. Most fibers exhibited fluorescence throughout their length, but varicosities with higher intensities of fluorescence were occasionally seen. Furthermore, dense fluorescence adrenergic plexuses were present in the forestomach. Adrenergic nerves diminished following chemical sympathectomy with 6-hydroxydopamine and Auerbach's plexus was hardly affected. This observation suggests that there is no effect of chemical sympathectomy in the Auerbach's plexuses.

In the transection (SGA)-treated forestomach, ganglia and nerve bundles were also present and almost intact. However, it can be considered

FIG. 5. *Histochemical Localization of Acetylcholinesterase in Sectioned Preparations of the Rat Forestomach (1 Week after Transection (SGA))*

A dense network of acetylcholinesterase-positive structures can be observed throughout the muscle layers. Many ganglion cells which are attached to nerve fibers are stained for acetylcholinesterase. In some ganglion cells, acetylcholinesterase activity is very low or negative.

FIG. 6. *Longitudinal Muscle of the Rat Forestomach (3 Weeks after Transection (SGA))*

Marker: $1 \mu m$; SM: smooth muscle; Mt: mitochondria; N: nucleus.

that most of these nerve bundles were associated with blood vessels. The nerve fibers were sparsely distributed throughout the muscle, but many were seen running parallel to and around blood vessels (data not shown).

Ultrastructure of Smooth Muscle

The longitudinal smooth muscle cells were arranged in parallel bundles transverse to the longitudinal axis of the forestomach. In some cases, Golgi apparatus, mitochondria and rough endoplasmic reticulum were recognized in the vicinity of the two poles of the nucleus. The numerous myofilaments were filled in the cytoplasm and the dense body was recognized. The numerous caveola was formed near the surface of the smooth muscle cells and there existed a closely positional relation to the endoplasmic reticulum. The endoplasmic reticulum was

FIG. 7. Myenteric Ganglion of the Rat Forestomach

Note neurosecretory granule (arrow) seen in the Golgi apparatus.
Marker: 1 μ m; N: nucleus.

mainly the rough one and its form of the existence was closely connected with cytoplasm and/or recognized near the nucleus. Mitochondria were mainly observed in the vicinity of the two poles of the nucleus. A large difference between the normal and transection (SGA)-treated forestomach smooth muscle cells was not recognized (Fig. 6).

Ultrastructure of Nerves

Most of the axons and their varicosities were seen to run parallel and/or transverse to the

FIG. 8. Myenteric Ganglion of the Rat Forestomach (3 Weeks after Transection (SGA))

The arrow points to neurosecretory granules. The perikaryon of this ganglion shows degenerative changes after transection (SGA); dispersion, unfolding and swollen chambers.
Marker: 1 μ m; N: nucleus; NE: nerve ending; V: vacant or swollen chambers.

smooth muscle cell bundles. In the electron microscope, the intramural neurons showed the ultrastructural features common to most neurons. Most nerves were unmyelinated and partly or totally ensheathed by Schwann cells. Nerve cells showed a high diversity in their shape, structure

and size. It had a large, round or oval nucleus, often eccentrically placed, with a prominent nucleus. The nucleoplasm was finely granular. Ribosomes, smooth and rough endoplasmic reticulum, bundles of neurofilaments were well in evidence in the cytoplasm. Ribosomes were

FIG. 9. Effects of Various Drugs on Inhibitory Responses to Transmural Nerve Stimulation of the Longitudinal Smooth Muscle from the Transection (SGA)-treated and Sympathectomized Forestomach in the Presence of Scopolamine ($10^{-5} M$) and Guanethidine ($5 \times 10^{-6} M$) in Case of the Tone of the Preparation maintained with Serotonin ($10^{-5} M$)

(A): effects of morphine ($3.1 \times 10^{-5} M$), sotalol ($10^{-5} M$) and phentolamine ($4.4 \times 10^{-5} M$) on inhibitory responses to transmural nerve stimulation in sympathectomized tissues. The horizontal bar shows the scale for 1 min and the vertical bar the scale for 1 gram. (B): effects of morphine ($3.1 \times 10^{-5} M$), sotalol ($10^{-4} M$) and haloperidol ($5 \times 10^{-6} M$) on the response to transmural nerve stimulation in transection (SGA)-treated tissues. The horizontal bar shows the scale for 1 min and the vertical bar the scale for 100 mg.

Transmural nerve stimulation was applied at 0.5 Hz (0.1 ms duration), 1 Hz (0.1 ms) and 2 Hz (0.1 and 0.2 ms).

either attached to sacs of endoplasmic reticulum or arranged in rosettes and spirals (Fig. 7). Mitochondria were recognized uniformly in the cytoplasm. Microtubules and lysosomes were common. Near the Golgi apparatus, scattered in the cytoplasm, large dense-cored vesicles could be observed, but were generally scanty. In Auerbach's plexuses, structural differences between neurons were prominent in most cases. Each ganglion was surrounded by a basal lamina, around which bundles of collagen fibrils and processes of fibroblasts were visible.

Glial cells were numerous in the Auerbach's plexuses, which covered part of the surface of the ganglion cells and penetrated among various axonal and dendritic processes. In these cases, the cell surface was smooth and regular. Nuclei of glial cells were smaller than those of neurons. The cytoplasm of glial cells was rich in ribosomes, endoplasmic reticulum and bundles of gliofilaments. After transection (SGA), marked ultrastructural changes could be identified in some nerve cells. The perikaryon of this ganglion showed degenerative changes after transection (SGA); dispersion and unfolding of endoplasmic reticulum, then formation of dense bodies (which may be lysosomes). The axons which synapsed with this ganglion at two sites did not undergo degeneration (Fig. 8).

Effect of Extrinsic Denervation

Various drugs investigated had no effect on the relaxation elicited by TMS after transection (SGA) and sympathectomy as being analogous to the case of the normal rat forestomach as shown in Table I and Fig. 9. A similar tendency was shown in vagotomy and transection (data not shown). The relaxation to TMS after extrinsic denervation was blocked by TTX, cocaine, dibucaine, strychnine and poly-L-lysine. A large difference between control (normal) and extrinsic denervation in a frequency-relaxation curve was not recognized (data not shown).

DISCUSSION

The present experiments show that the inhibitory responses which are produced by stimula-

tion of the intramural nerves supplying the longitudinal smooth muscle strips taken from the rat forestomach are neurogenic and that the cell bodies of non-adrenergic inhibitory nerves are located in Auerbach's plexuses and ATP is probably not the transmitter substance in the non-adrenergic inhibitory nerves.

The inhibitory responses elicited by TMS were reversibly abolished by TTX, cocaine and dibucaine, but they were not significantly affected by hexamethonium. TMS and ganglionic stimulants in the presence of scopolamine and guanethidine consistently produced a relaxation or a relaxation followed by an after-contraction in case of the tone of the preparation maintained with serotonin. Furthermore, it becomes a serious problem whether the TMS-induced relaxation described so far were neurogenic or myogenic responses. Generally, it is well known that prolong cold storage caused degeneration of the peripheral neurons.^{14,15} After cold storage of the rat forestomach strips for 5 d at 4°C, the inhibitory responses elicited by TMS were not observed, but noradrenaline still caused a relaxation. The amplitude of relaxation induced by TMS was increased with an increase in frequency until it reached a maximum at 5 Hz. From these observations, it is suggested that the TMS-elicited relaxations are not myogenic, but neurogenic.

It becomes a matter of great concern that the TMS-elicited relaxations are caused by excitation of adrenergic neurons or the non-adrenergic inhibitory ones in the longitudinal smooth muscle preparations isolated from rat forestomach. They were insensitive to the adrenergic neuron blocking agent, and to α - and β -adrenoceptor blocking agents. The specific catecholamine fluorescence was decreased by administration of reserpine, whereas the TMS-induced relaxations were not affected by that of reserpine. In the 6-OHDA-treated tissues, they were also unaffected, but the relaxation elicited by perivascular nerve stimulation completely disappeared. The specific catecholamine fluorescence was reduced but did not disappear completely

(unpublished data). It is well known that 6-OHDA produces a destruction of the whole adrenergic neurons in newborn rats and provides a "permanent sympathectomy".¹⁶⁾ In achieving permanent sympathectomy, the TMS-caused relaxation still remained (unpublished data). In surgical denervated tissues, they were unaffected.

From these facts, it may be suggested that nerves responsible for producing the relaxation of the preparations are not adrenergic, but similar to the non-adrenergic inhibitory neurons, as reported for various other preparations.^{1,2)}

Phentolamine, Tolazoline and Yohimbine

It was reported that several 2-substituted imidazolines selectively blocked the inhibitory response of the guinea-pig taenia coli to non-adrenergic nerve stimulation and ATP.¹²⁾ Phentolamine had little effect on the relaxation elicited by TMS in the present preparations, but it in concentrations higher than 10^{-4}M relaxed the fundus strip in analogous to the case of the rabbit portal vein.¹⁷⁾ The inhibitory responses induced by TMS were substantially resistant to other α -blockers in the longitudinal smooth muscle preparations isolated from rat forestomach. Recently, it was reported that these drugs were not specific for ATP.²⁾ Hence, there could be no generalization that these α -adrenoceptor blocking agents selectively blocked the relaxation by stimulation of the non-adrenergic inhibitory nerves.

Histamine and Antihistamines (Chlorpheniramine, Diphenhydramine and Cimetidine)

Histamine has also been reported to cause relaxations of the guinea-pig fundus in the presence of hyoscine¹⁸⁾ and to inhibit the lower esophageal sphincter muscle in the opossum.¹⁹⁾ Histamine-induced relaxation was found to be inhibited in a dose-dependent manner by the addition of metiamide in the isolated rat stomach fundus strip.²⁰⁾ However, histamine only produced small contraction in the rat fundus strips, and the relaxations caused by TMS were unaffected by histamine and antihistamines. This discrepancy could be due to the difference of the

experimental condition; for instance, the tissues were subjected to 1–3 g initial tension and mepyramine (10^{-6}M) was contained in the superfusion medium in their study.

Dopamine and Haloperidol

Haloperidol selectively blocked the relaxations induced by TMS in the pyloric antrum strip of rat stomach.²¹⁾ It is assumed that the mechanism of the relaxation to TMS was due to dopamine release by stimulation of the dopaminergic neurons, and that dopamine was the neurotransmitter for receptive relaxations of the dog stomach.²²⁾ Dopamine caused the relaxation of the rat stomach fundus strip in the presence of scopolamine, and haloperidol did not block the relaxations elicited by TMS in the presence of scopolamine and guanethidine. In α -methyl-*p*-tyrosine-treated tissues, the relaxation to TMS could not be blocked. These facts indicated that the relaxations to TMS were not caused by stimulation of the dopaminergic neurons in the fundus strip of the rat stomach.

Morphine, Naloxone, Compound 48/80, and Methionine- and Leucine-enkephalin

Morphine has been found to depress the non-adrenergic inhibitory response of the taenia coli to TMS, an action of which could be reversed by naloxone.²³⁾ Compound 48/80 is well known to be the most potent liberator of histamine from mast cells.²⁴⁾ Recently, it has been suggested that compound 48/80 acts as a selective and competitive antagonist at opiate receptors located in the intramural cholinergic nerves of guinea-pig ileum.²⁵⁾ In addition, enkephalin-immunoreactive nerve terminals and cell bodies have been observed in the intestinal wall.²⁶⁾

However, these drugs except morphine had no effect on the inhibitory responses to TMS in the fundus strip. In some preparations, morphine slightly depressed the inhibitory responses of the stomach strip to TMS at frequency lower than 1 Hz, an action of which could not be reversed by naloxone. The observations of our experiments demonstrated that an inhibitory action of morphine, as is seen with taenia coli, could not be detected. Also, neither enkephalin nor naloxone

had any effect on the inhibitory responses to non-adrenergic intramural nerve stimulation in the fundus strip of the rat stomach. There may be a wide difference between the non-adrenergic inhibitory innervation of rat stomach and that of guinea-pig taenia coli.

GABA, Glycine, Glutamate, Bicuculline, Picrotoxin, Strychnine, Kainic Acid and Quisqualic Acid

There is evidence that GABA and glycine function as an inhibitory synaptic transmitter in most areas of the mammalian central nervous system, whereas glutamate may be the major excitatory transmitter in the central nervous system and at the crayfish neuromuscular junction.²⁷⁻²⁹⁾ In the isolated rat fundus strip, however, these amino acid unaffected the inhibitory response to TMS. Bicuculline and picrotoxin, antagonists of GABA, had no effect on the inhibitory response to TMS. It is reported that kainic acid enhanced the depolarizing action of L-glutamate²⁹⁾ and quisqualic acid produced similar effects to those of L-glutamic acid at the crayfish neuromuscular junction.³⁰⁾ Kainic acid and quisqualic acid unaffected the inhibitory response to TMS and gave no direct response in the isolated rat fundus strip. From these observations, it is impossible to consider that these amino acids will be a transmitter substance for the non-adrenergic inhibitory transmission. Surprisingly, strychnine, considered as the glycine antagonist, blocked the relaxation elicited by TMS in the isolated rat fundus strips. On this point, details will be reported at a later date.³¹⁾

Dipyridamole, Dilazep, Theophylline and Tachyphylaxis to ATP

Dipyridamole has been shown to selectively potentiate the inhibitory response of the guinea-pig taenia coli to non-adrenergic nerve stimulation as well as ATP by inhibiting the uptake of adenosine.³²⁾ In the present preparation, however, dipyridamole was not observed to be effective in potentiating the relaxation induced by TMS as shown previously.³³⁾ Accordingly, we have used dilazep, a more potent and selective nucleoside transport inhibitor.³⁴⁾ Dilazep did not potentiate the relaxation elicited by TMS. It has

also been reported that dipyridamole could not potentiate the relaxation caused by stimulation of non-adrenergic inhibitory nerves in the pig stomach,⁷⁾ in the guinea-pig taenia coli³⁵⁾ and in the rabbit duodenum.³⁶⁾

The possibility that ATP might be the transmitter liberated from the endings of the non-adrenergic inhibitory nerves has been proposed.^{1,2)} In the rabbit ileum, it has been reported that following desensitization to ATP, the inhibitory responses to TMS were also abolished, whereas the effects of adrenergic stimulation were unaffected.³⁷⁾ This result might be considered as evidence supporting the purinergic nerve hypothesis. In the same ATP-desensitized preparations, however, there was no change in the inhibitory responses to either transmural or adrenergic nerve stimulation when the detailed quantitative experiments were achieved.³⁸⁾ In rat stomach, desensitization to ATP had no effect on the relaxation to TMS in the presence of scopolamine and guanethidine. In guinea-pig stomach fundus,⁶⁾ following desensitization to ATP, the inhibitory responses to TMS were much reduced, but in pig stomach,⁷⁾ the relaxation to TMS could be evoked without any modification, following desensitization to ATP in the presence of atropine and guanethidine. Thus, the results obtained following desensitization to ATP were equivocal. These discrepancies could be due to the difference in species and in experimental methods.

Experiments on the effect of theophylline on the responses induced by adenosine nucleotides in a variety of biological system have shown that theophylline antagonizes the responses to ATP and TMS in the mammalian gastrointestinal tract.^{6,39)} In the fundus strip of guinea-pig, theophylline (50 μ M) almost fully antagonized the ATP- and TMS-induced relaxation,⁶⁾ whereas it did not inhibit either the relaxation of the smooth muscle cells or hyperpolarization of the cell membrane caused by ATP and TMS.^{40,41)} From the latter results, it has been suggested that theophylline is not the drug of choice to obtain evidence for the purinergic nerve hypothesis.⁴¹⁾

In the present preparation, theophylline also unaffected the relaxation to TMS and ATP in the presence of scopolamine and guanethidine. The existence of the theophylline-sensitive adenosine receptors in the rat stomach was not confirmed in the present study.

Hemicholinium

It is established that hemicholinium interferes with the synthesis of acetylcholine by competing for choline at the nerve terminal or at the ganglion.¹³⁾ In the unatropinized fundic strip of the guinea-pig stomach (but with guanethidine present), hemicholinium markedly reduced or abolished the response to field stimulation without affecting response to ATP or acetylcholine.⁴²⁾ It is suggested that this result supported a cholinergic origin for the endogenous mediator of non-adrenergic inhibitory transmission. In rat fundic strip, the relaxation to TMS in the presence of scopolamine and guanethidine in case of the tone of the preparation maintained with serotonin was unaffected, following incubation with hemicholinium for 4 h with intermittent stimulation. This observation suggests that the cholinergic nerves could not take part in the non-adrenergic inhibitory transmission.

Poly-L-lysine and Ouabain

Poly-L-lysine (PLL) might be interfering with Na transport by tubular cells and cause both permeability changes and inhibition of active Na uptake in trout gill.⁴³⁾ In consonance with these report, PLL increased ²²Na permeability only in liposomes made of phosphatidylserine.⁴⁴⁾ Furthermore, the effects of PLL on epithelia involves disruption of membrane integrity. In addition, it was recently observed that ouabain increases both the spontaneous and evoked transmitter release at the neuromuscular junction of the frog⁴⁵⁾ and facilitates the neuromuscular transmission in skeletal muscle.⁴⁶⁾ In the present preparation, the relaxation to TMS was unaffected by ouabain, whereas PLL caused a transient relaxation and depressed the relaxation elicited by TMS. This observation was not due to the inhibition of Na pump, resulting from the ineffectiveness of ouabain. Lysine had

no effect of the TMS-induced relaxation and the relaxation to PLL was insensitive to TTX. From these results, PLL might act directly on the smooth muscle cells. In this regard, further investigation will be necessary.

Effects of Surgical Denervations (Vagotomy, Sympathectomy, Transection and Transection (SGA))

There has been little study of the changes in ultrastructure for nervous control of gastric motility after surgical denervation.⁴⁷⁾ The Auerbach's plexuses were network-like structures with ganglia and strands of nerve fibers connecting them with silver impregnation and methylene blue methods after each extrinsic denervation (unpublished data). Acetylcholinesterase histochemistry did not reveal the markedly degenerative changes after each extrinsic denervation as being analogous to the case of silver impregnation. Monoamine fluorescence histochemistry revealed ganglia and nerve bundles even in the transection (SGA)-treated forestomach.

Accordingly, we have investigated the changes in ultrastructure of the rat forestomach after transection (SGA) as the markedly degenerative changes were little recognized by microscopical examination. A wide difference between the normal and transection (SGA)-treated forestomach smooth muscle cells was not always observed. Furthermore, some ganglia of the Auerbach's plexuses showed degenerative changes after transection (SGA); dispersion and unfolding of endoplasmic reticulum, then formation of dense bodies (which may be lysosome). These observations indicated the degenerative changes due to the dissection at the axons of these ganglia by transection (SGA). However, the great majority of the ganglia of the Auerbach's plexuses did not degenerate after extrinsic denervations of the rat forestomach as described previously in guinea-pig ileum.⁴⁸⁾

In the present preparations, various drugs investigated had no effect on the relaxation elicited by TMS after extrinsic denervation as being analogous to the case of the normal rat forestomach. The relaxation to TMS after extrinsic denervation

vation were blocked by TTX, cocaine, dibucaine, strychnine and PLL. A large difference in frequency-relaxation curves was not observed at the normal forestomach and at the transection (SGA)-treated one. Therefore, the extrinsic denervation, especially transection (SGA), could make the preparation consisting of the only intramural nerve in rat forestomach. In addition, transection (SGA) will become a very useful method to examine the effect of drugs on the intramural nerve transmission.

These results showed the direct evidence that the cell bodies of non-adrenergic inhibitory neurons were located in Auerbach's plexuses and suggested that there may be different types of non-adrenergic innervation in individual tissues. More detailed studies are now in progress regarding the mechanism of strychnine and PLL action in relation to the phenomenon outlined herein.

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