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Pharmacological properties of mechanical responses of the rat oesophageal muscularis mucosae to vagal and field stimulation

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1 Electrical stimulation applied to vagal oesophageal branches of the isolated curarized oesophagus, or via field electrodes to the isolated tunica muscularis mucosae (TMM), increased longitudinal tension and intraluminal pressure in a frequency-dependent manner.

2 Differences between cervical and distal TMM segments were noted in frequency-response relationships, as well as in the pulse-width dependence of contractions to field stimulation.

3 Vagally- and field-stimulated contractions were eliminated by tetrodotoxin or hyoscine, indicating their mediation by cholinergic neurones. The field-stimulated postganglionic responses were resistant to hexamethonium or (+)-tubocurarine and weakly inhibited by morphine.

4 Vagally- and field-stimulated TMM contractions were mimicked by muscarinic agonists, augmented by acetylcholinesterase inhibitors, and inhibited more effectively by β-than by α_1-or α_2-adrenoceptor agonists.

5 5-Hydroxytryptamine (5-HT) exerted excitatory and/or inhibitory effects: TMM *in situ* responded with a hexamethonium-resistant, ketanserin-sensitive transient increase in tension comparable to that produced by field stimulation. In the isolated TMM, moderate excitatory responses were limited to the distal portion with inhibition predominating in the remaining proximal portion. 5-HT-induced inhibitory effects on field-stimulated tension responses were paralleled by relaxant effects on muscarinic agonist-induced tonic contractile responses, both of which were resistant to 5-HT-receptor antagonists including ketanserin, lysergic acid diethylamide (LSD), methysergide or methergoline.

6 Field stimulation at a low frequency and pulse durations > 1.0 ms produced a relaxation response in preparations exposed to tetrodotoxin or hyoscine, provided that active muscle tonus was present. The relaxation in response to field stimulation was insensitive to antagonists of 5-HT, catecholamines, histamine, or indomethacin, suggesting a non-neurogenic origin.

7 Histochemical examination of the isolated TMM preparation for cholinesterases revealed the presence of an extensive submucosal ganglionic plexus.

8 It is concluded that: (i) intrinsic cholinergic neurones of the submucosal plexus form the final common pathway for extrinsic vagal and local (myenteric) projections to the TMM; (ii) the neural basis, if any, of non-cholinergic non-adrenergic inhibitory mechanisms remains to be established; (iii) the TMM may assist in generating propulsive oesophageal motility.

Introduction

In rodents such as the guinea-pig and the rat, the tunica muscularis mucosae (TMM) forms an inner tube extending the entire length of the oesophagus. Since the muscularis propria in these two species contains only striated muscle, the TMM inner tube may be engaged in motor functions similar, or equivalent, to those of the smooth muscle of the distal oesophagus in primates and marsupials. As noted by Christensen (1975), the cholinergic motor innervation of the TMM resembles pharmacologically that of the longitudinal smooth muscle of the tunica propria. Based on investigations in the guinea-pig, Bartlett (1968b) and Kamikawa & Shimo (1979; 1983a,b) suggested that contractile responses of the oesophageal TMM to stimulation of extrinsic vagal and intramural nerves are effected by a two-neurone

parasympathetic pathway involving a preganglionic nicotinic-cholinergic and a postganglionic muscarinic-cholinergic synapse. Vagal stimulation produced only atropine-sensitive excitatory responses; however, inhibitory responses could be elicited by transmural electrical stimulation and were pharmacologically characterized as being mediated by adrenergic extravagal nerves.

Immunohistochemical findings described by Schultzberg *et al.* (1980) indicate the presence of a bewildering variety of other putative enteric neuromediators in both ganglionic plexuses of the guinea-pig and rat oesophagus, including *inter alia* vasoactive intestinal peptide, substance P, somatostatin and opioid peptides.

The purpose of the present work was to characterize the peripheral neural control of the TMM in the rat and to complement observations concerning the central organization of swallowing in this rodent (Bieger, 1981; 1984). In particular, attempts were made to uncover regional differences in responsiveness of oesophagomotor nerves to electrical and pharmacological stimuli with a view to obtaining clues to the organization of the oesophageal intramural neural plexus and the existence of intrinsic inhibitory processes.

Some parts of this investigation have been communicated in a preliminary form (Bieger & Triggle, 1981).

Methods

Preparation of tissues

Oesophagi with attached trachea were excised en bloc from male Sprague-Dawley rats (200–350 g; $n = 60$) killed by a blow on the head. Tissues were quickly transferred to a Sylgard-coated Petri dish filled with oxygenated Tyrode solution for dissection under a stereo-microscope. Vagus-oesophagus preparations were made from the proximal ($n = 10$) and distal ($n = 8$) half of the oesophagus. For the former, the left vagal trunk was severed above the origin of the recurrent nerve which was separated from its attachment to the tracheal wall. For the distal half, the largest right vagal branch to the body of the oesophagus was dissected free to create a nerve segment of suitable length for stimulation. The ends of the oesophageal tube were secured with silk threads to enable the preparation to be mounted in a jacketed organ bath for isometric recording of longitudinal tension or intraluminal pressure by means of force or pressure transducers. To facilitate dissection of the muscularis mucosae, the excised oesphagus was pulled over a stainless steel rod. The muscularis externa was split lengthwise and carefully cut away, leaving behind the inner smooth muscle tube. The latter was divided into three portions of 20 mm corresponding to the proximal cervical, middle intrathoracic and distal intra-abdominal segment of the oesophagus. The muscle tube was secured at both ends with silk thread by which the preparation was mounted on an organ holder provided with stimulation electrodes. In the majority of experiments, preparations were set up for isometric recording of longitudinal tension (Grass FT03 transducer; Grass or Beckman ink-writer); in some experiments, intraluminal pressure was recorded simultaneously or alone by the use of a PE catheter tied into the lumen and connected to a Gould Statham P23 ID transducer.

The preparations were bathed in Tyrode solution aerated with 95% O_2/5% CO_2 at 37°C and containing (mM): NaCl 137.0, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.88, NaH_2PO_4 0.36, $NaHCO_3$ 12.0, glucose 5.5. Tissues were equilibrated for 60 min to allow preload tension to be readjusted until the baseline reached a steady state.

Procedures for electrical stimulation

Vagus-oesophagus preparations were electrically stimulated through a bipolar silver wire electrode over which the nerve was placed and secured with a silk thread. Stimuli consisted of trains of monophasic pulses (0.5–1.0 ms) of supramaximal intensity. Isolated muscularis mucosae preparations were subjected to field stimulation with 40V rectangular pulses applied through two concentric platinum rings spaced 10 mm apart and surrounding the tissue. Standard stimulation procedures employed pulses of 0.5 ms width, 5 to 7.5 Hz, delivered in 10 or 30 s trains at rates of 1.5 to 1.0 min^{-1}.

Histochemical studies

Oesophagi were dissected as described above. Segments of the isolated smooth muscle tube were immersed for 2 min in a mixture of phosphate-buffered formaldehyde (4%) and glutaraldehyde (1%), pH 7.2, and rinsed with distilled water. The lumen was filled with enough reaction medium to extend fully the muscle wall, the muscle tube ligated at either end and placed in a 10 ml vial containing the thiocholine medium prepared according to Shute & Lewis (1967). Following incubation for 12–16 h at 4°C, the specimen was cut open, rinsed with distilled water, and staining developed by immersion in potassium ferricyanide (10%). The staining solution was thoroughly washed off, and the unstained epithelial layer removed before mounting the specimen on gelatine-coated glass slides. Preparations were air dried, dehydrated in ethanol, cleared in xylene, and a

coverslip applied for microscopic examination. In experiments where inhibition of the enzymes was studied, specimens were presoaked for 20 min in aqueous solutions of either *iso*-OMPA, a selective inhibitor of butyrylcholinesterase (Adie, 1953) or BW284C51, a selective inhibitor of acetylcholinesterase (Silver, 1974) and then reacted in the thiocholine medium containing the same concentration of inhibitor.

Drugs

Drugs were obtained from the following sources: acetylcholine bromide (ACh), acetylthiocholine iodide, butyrylthiocholine iodide, 1,5 *bis* (4-allyldimethyl-ammoniumphenyl) pentanone dibromide (BW284C51), α-bungarotoxin, 5-hydroxytryptamine oxalate (5-HT), indomethacin, (±)-isoprenaline hydrochloride, (−)-noradrenaline hydrochloride, *Naja naja* toxin, *iso*-OMPA (tetra-*iso*propylpyrophosphoramide), (±)-propranolol hydrochloride, yohimbine hydrochloride (Sigma); (±)-muscarine hydrochloride, hyoscine methyl bromide, tetrodotoxin (TTX), (+)-tubocurarine hydrochloride (Calbiochem); metiamide*, phenoxybenzamine hydrochloride* (Smith, Kline & French); methysergide dimaleate*, lysergic acid diethylamide tartrate (Sandoz); dimethylphenylpiperazinium iodide (DMPP; Aldrich); physostigmine salicylate (BDH chemicals); BHT920* and clonidine hydrochloride* (Boehringer); ketanserin tartrate* (Janssen Pharmaceutica); methergoline* (Farmitalia); prazosin hydrochloride* (Pfizer); fluoxetine hydrochloride* (Eli Lilly). Compounds marked with an asterisk were generously donated by the manufacturers. CD (*cis*-2-methyl-4-dimethylamino-methyl-1,3-dioxolane methiodide) was synthesized as previously described by Chang *et al* (1972) and was generously supplied by Dr D.J. Triggle of S.U.N.Y. at Buffalo, New York state. Drugs were dissolved in distilled water with the following exceptions: indomethacin (0.25 M $NaHCO_3$); methergoline (0.2 M ascorbic acid); metiamide and phenoxybenzamine (10% ethanol); TTX (0.01 N acetic acid); stock solutions (0.1 M) of noradrenaline, isoprenaline and 5-HT were acidified with 0.01 N HCl.

Unless stated otherwise, drug effects described below were replicated at least twice in three separate tissues. Values for half-maximally effective drug concentrations were obtained by averaging individual measurements determined by graphical interpolation. Student's *t* test for unpaired samples ($P<0.05$) was used to test the significance of observed regional differences in responses to drugs or electrical stimuli.

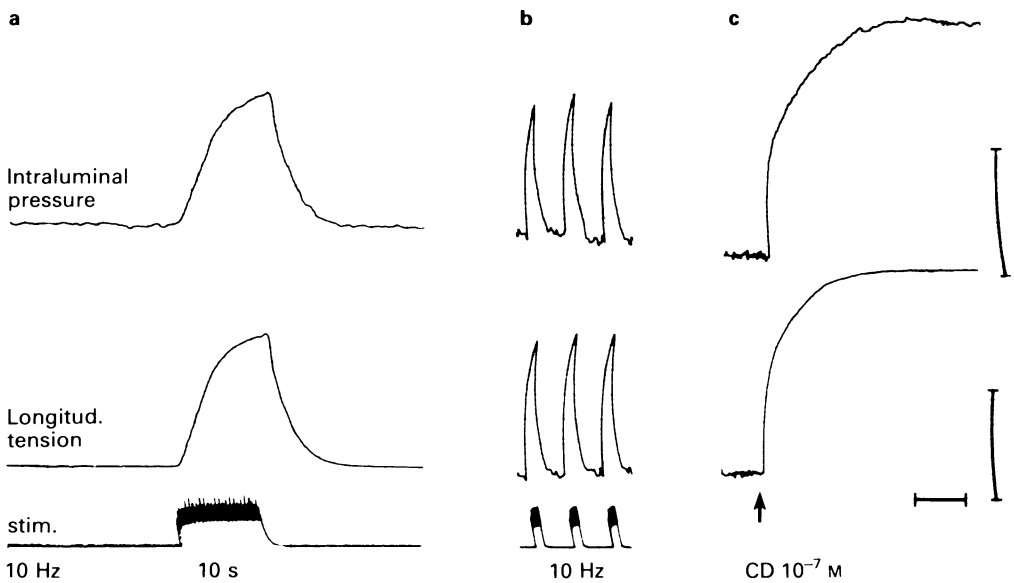

Figure 1 Mechanical responses of rat isolated muscularis mucosae oesophagi to electrical field and cholinergic stimulation. Intraluminal pressure (top traces) and longitudinal tension (middle traces) were registered simultaneously from the same preparation, and are shown at high (a) and at 10 times lower (b and c) speed. Note congruity between pressure and tension response evoked by submaximal field stimulation, and similarity with effect of muscarinic agonist, *cis*-2 methyl-4-dimethylaminomethyl 1-3 dioxolane (CD). Calibrations: vertical bars indicate respectively, 133 Pa (1 mmHg) and 9.8 mN (1 g); horizontal bar marks 1 min (b and c). Electrical stimulus is monitored in bottom trace.

Results

Contractile responses evoked by field stimulation

Muscularis mucosae preparations maintained under a preload of 2.9 mN (0.3 g) initially remained mechanically quiescent irrespective of the level of origin; however, after repeated exposure to pharmacological and electrical stimuli, low amplitude spontaneous activity was noted, particularly in the distal segment of the oesophagus. Longitudinal tension developed during field stimulation, reaching up to 40 mN per segment. Typically, there was an initial rapid rise in tension followed by a plateau, which was mimicked, with a slower time course, by the administration of muscarinic agonists (ACh, CD and muscarine). Likewise, intraluminal pressure increased, reaching a maximum of 200–250 Pa (1.5–1.8 mmHg). When both variables were simultaneously recorded from the same preparation, respective traces were nearly indistinguishable (Figure 1). At the end of field stimulation, tension rapidly decayed quasi-exponentially with half-maximal relaxation occurring within 3–5 s. Two types of after-response were observed: the first, a twitch-like and highly variable 'off'-contraction; the other, a delayed and slow after-contraction following stimulation at high frequency or long pulse durations.

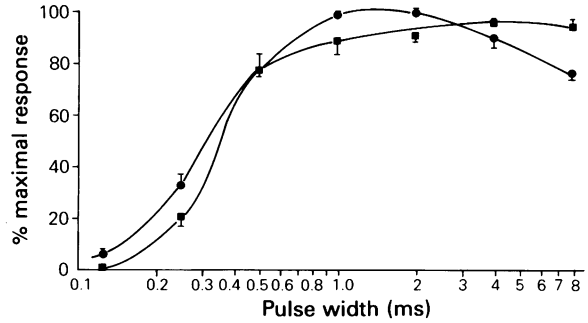

Figure 3 Influence of pulse width on the amplitude of the contraction of field-stimulated isolated tunica muscularis mucosae of proximal (■) and distal (●) rat oesophagus. Preparations were stimulated at 4 Hz. Each point represents the mean of 6 determinations from separate experiments, vertical lines show s.e.mean. Significant differences ($P<0.05$) between the segments are indicated by asterisks.

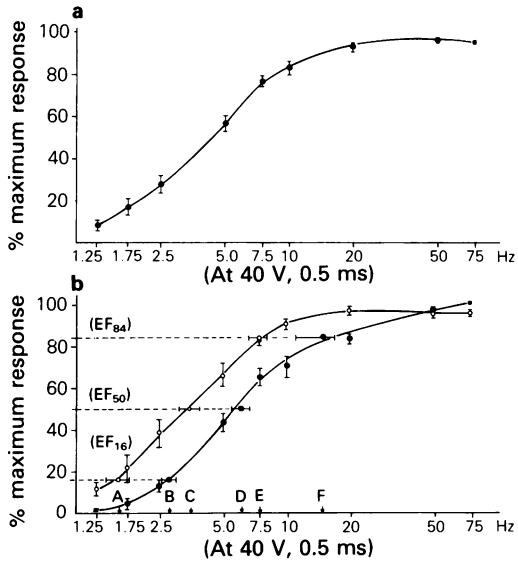

Figure 2 Frequency-response relationship of field stimulated isolated tunica muscularis mucosae of rat oesophagus. (a) Pooled data for proximal (cervical), middle (thoracic) and distal (intra-abdominal) segments. Each point represents the mean from 21 separate preparations and vertical lines show s.e.mean (b) Data for proximal (●) and distal (○) segments plotted separately. Lettered abscissal points denote calculated values for stimulus frequencies producing 16, 50 and 84%, respectively, of maximal contractions in both segments. Corresponding values (±s.e.mean) for distal segment (A,C,E) were EF_{16}:1.5±0.2; EF_{50}:3.5±0.4.; EF_{84}:7.4±0.7; and for proximal segment (B,D,F), EF_{16}:2.8±0.2; EF_{50}:6.1±0.6; EF_{84}:14.5±2.3 Vertical and horizontal lines represent s.e.mean. ($n=7$); significant differences ($P<0.05$) indicated by asterisks.

Following a prolonged period of inactivity, contractions were not maximal upon the first stimulation, but increased to a consistent level after 5–7 successive stimulation periods; in most cases (0.017 Hz train cycle, 30s train length, 5Hz pulse frequency) the response doubled.

Frequency and pulse width dependence of contractions evoked by field stimulation

Frequency-response relationships were determined for each proximal, middle and distal segment (Figure 2). When the data were analysed by region, a clear difference emerged between the distal and proximal segments as reflected by a nearly two fold difference in the half-maximally effective frequency (Figure 2b; Table 1). Low rate (<0.3 Hz) or single stimuli caused discrete mechanical responses no greater than 5% of the maximum tension, which were more readily obtained in the distal third of the oesophagus. In addition, distal segments showed a small diminution of the initial fast phase of tension responses at higher frequencies of stimulation.

The magnitude of the field-stimulated contraction also depended upon stimulus pulse width, revealing again significant differences between the distal and the proximal segment (Figure 3). With increasing

Table 1 Regional differences in rat oesophagus tunica muscularis musosae contractions due to electrical and pharmacological stimuli

	Segment	
	Proximal	*Distal*
EF_{50}* (Hz)	6.1±0.6 ($n=7$)§	3.5± 0.4 ($n=7$)
Excitatory effect of 5-HT	not observed	present at concentration $>10^{-6}$M
Inhibitory effect of 5-HT on field-stimulated contraction**	97 ±3%($n=8$)§	65 ± 6%($n=8$)
Inhibitory effect of 5-HT on CD-evoked tonic phase of contraction†	97.6±2 ($n=5$)§	50.8±10 ($n=5$)

*Frequency of field-stimulation (40 V, 0.5 ms) required to produce a half-maximal contraction.

**Mean values ± s.e. expressed as $\left(1-\frac{\text{amplitude of test response}}{\text{amplitude of control response}}\right)\times 100$; 5-hydroxtryptamine (5-HT) test concentration was 10^{-6}M.

†5-HT(10^{-6}M) was applied to tissue during the tonic phase of contraction induced by cis 2-methyl-4-dimethylamino methyl 1–3 dioxolane methiodide (CD; 10^{-7}M); values were expressed as $\left(\frac{\text{amplitude drop}}{\text{amplitude of CD response before 5-HT}}\right)\times 100$.

§Significantly different from distal segment; Student's t test ($P<0.05$)

pulse widths (>4 ms), there was an increased incidence of small after-contractions in both proximal and distal segments.

Pharmacological modification of contractions evoked by field stimulation

In the presence of BW284C51, 2×10^{-6}M, there was an initial small but variable enhancement of the response to field stimulation, followed by a progressive slowing of relaxation such that successive contractions fused with each other. Concomitantly, peak amplitudes of electrically-evoked contractions fell below 50% of pre-drug levels. By contrast, *iso*-OMPA, $0.4-1\times10^{-4}$M, was without a consistent effect.

The electrically-evoked contractions were unaffected by hexamethonium, $0.1-0.26\times10^{-3}$M, but abolished either by hyoscine or TTX, both at 10^{-7}M, and, depending upon the baseline tension, were replaced by a relaxation (Figure 4a and b).

This relaxation was present at all oesophageal levels and increased in amplitude with increasing pulse width to reach its full size at 2 to 4 ms. In most cases, field stimulation-evoked relaxation was not evident until active tension was generated, for instance, by muscarinic agonists such as CD (Figure 5b), high potassium (40 mM), or $BaCl_2$ (0.5nM). Attempts to inhibit these relaxations with a variety of receptor antagonists (eg.10^{-6} to 10^{-5}M phentolamine, phenoxybenzamine, yohimbine, propranolol, ketanserin, metiamide, naloxone, hexamethonium, and indomethacin, as a cyclo-oxygenase inhibitor) failed. Also no significant effect was obtained with fluoxetine, $10^{-6}-10^{-5}$M, a selective 5-HT uptake blocker (Wong *et al.*, 1975).

Contractions evoked by field stimulation were inhibited by 5-HT in the concentration range $5\times10^{-9}-10^{-5}$M, with the proximal segment being more sensitive than the distal (Figure 5a; Table 1). The inhibitory effect of 5-HT was, with the same differences in regional sensitivity and resistance to pharmacological interventions, evident in the form of a rapid relaxation induced in preparations precontracted with CD (Figure 6; Table 1). An excitatory effect of 5-HT was not observed below 10^{-6}M; contractions were variable and found only in the distal segments (Figure 5a), albeit not consistently. The excitatory effect of 5-HT was antagonized by ketanserin 10^{-6}M. The inhibitory effect was insensitive to this and other 5-HT antagonists (methergoline, methysergide, lysergic acid diethylamide LSD) and the same agents that failed to inhibit field-stimulated relaxation. On the contrary, after ketanserin (Figure 5b) or LSD, the inhibitory effect of 5-HT was enhanced in both proximal and distal segments.

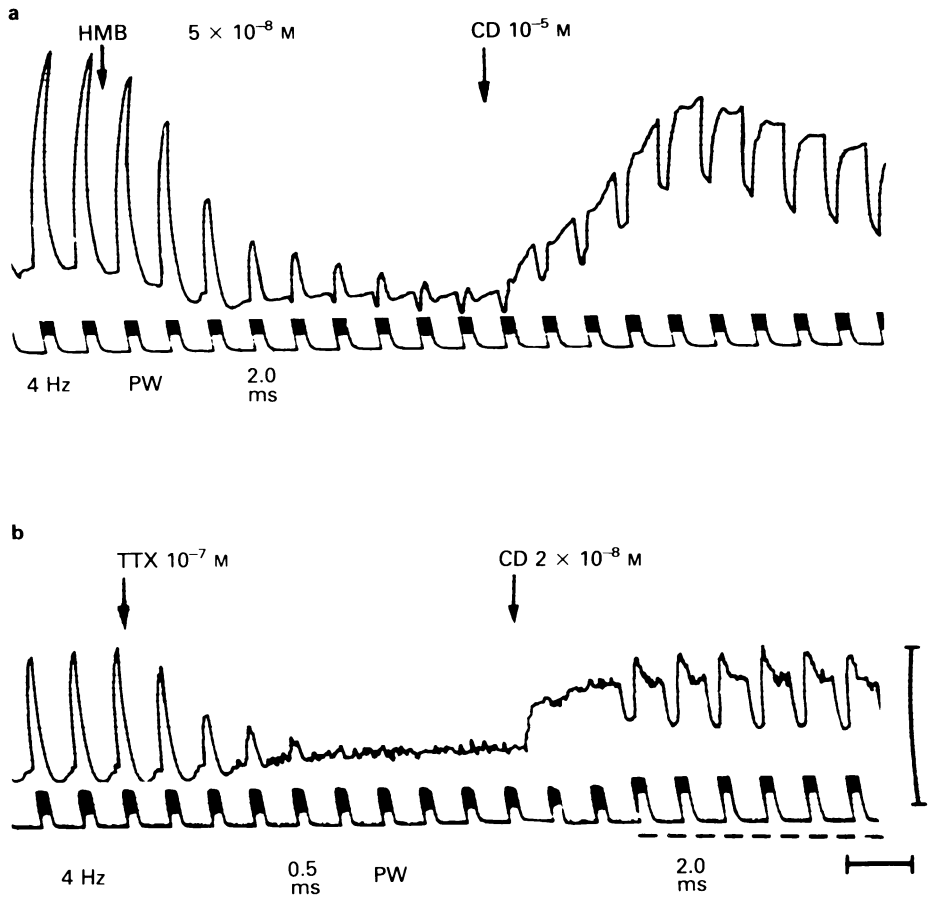

Figure 4 Blockade of field-stimulated contraction of rat isolated muscularis mucosae oesophagi by hyoscine methylbromide (HMB) or tetrodotoxin (TTX) and unmasking of relaxation. Recordings are from two different preparations taken from distal oesophagus with stimulus trains shown underneath each record. (a) Hyoscine converted the contraction into relaxation. After muscle tonus was raised by the muscarinic agonist *cis*-2 methyl-4-dimethylaminomethyl 1–3 dioxolane (CD), relaxation was enhanced. (b) Raising active tonus with CD in the tetrodotoxin-blocked preparation revealed a relaxation which was dependent upon the stimulus pulse width. Dashed line indicates change in pulse width (PW) from 0.5 to 2.0 ms. Calibrations: vertical bar 5 mN; horizontal bar 1 min.

During continued exposure to 5-HT, the electrically-evoked contractions partially escaped from inhibition; the extent of this apparent recovery diminished with increasing concentration and was less marked in the proximal segment (Figure 5).

All adrenoceptor agonists tested diminished electrically-induced contractions. On the basis of their effectiveness, agonists fell into two categories: those producing just subtotal to complete inhibition as typified by noradrenaline and isoprenaline, and those producing a partial inhibition, as typified by the α_2-selective agonists, clonidine and BHT 920. The inhibitory effects of all these agents were incompletely antagonized by 10^{-6}M phentolamine alone or in combination with 10^{-5}M yohimbine, 10^{-6}M prazosin and 5×10^{-6}M propranolol. Phenoxybenzamine 10^{-5}M abolished field-stimulated responses; however, at the same time caused a 50% reduction of contractions evoked by CD 5×10^{-7}M.

Morphine, 10^{-6}M, produced inhibitory effects of moderate intensity and gradual onset, reducing the amplitude of field-stimulated responses at 5–7.5 Hz by $28 \pm 8\%$ ($\pm$ s.e.mean, $n = 8$). The effects of higher concentrations were not studied.

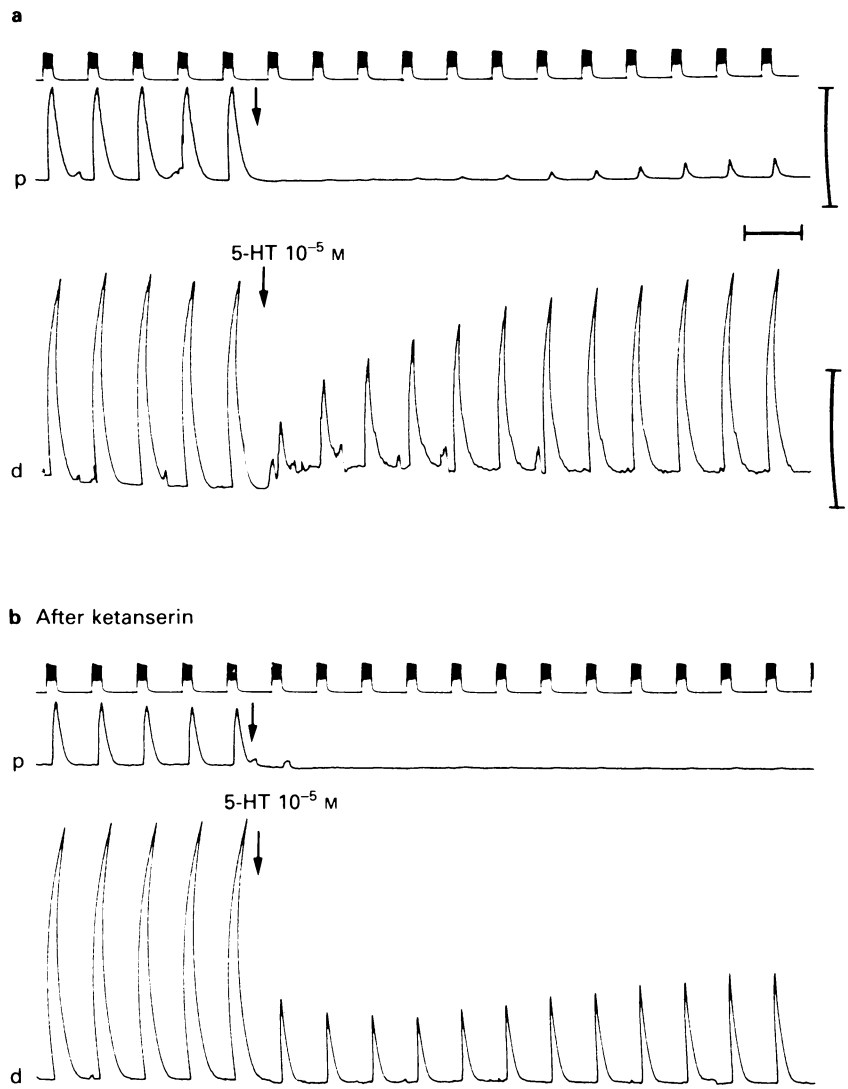

Figure 5 Effects of 5-hydroxytryptamine (5-HT) on the response to field stimulation tension of the isolated muscularis mucosae of the rat oesophagus. Recordings were obtained from proximal (p) and distal (d) segments of same oesophagus before (a) and 8 min after ketanserin 10^{-6}M (b). Trace at top of each panel represents 10 s stimulus trains consisting of rectangular pulses of 10 Hz, 0.5 ms duration, and 40V. In the proximal segment, the effect of 5-HT (applied at arrows) appeared to be only inhibitory; in the distal segment, a mixed effect was evident. Note absence of excitatory component and enhancement of inhibition after ketanserin. Calibrations: vertical bars 5 mN; horizontal bar 1 min.

Effects of cholinergic agonists on isolated tunica muscularis mucosae

In the concentration range 0.01 to 100 μM, ACh and the muscarinic receptor agonists (±)-muscarine and CD increased longitudinal tension in a graded manner. Maximal effects elicited by each agonist were of comparable magnitude and insensitive to hexamethonium 10^{-3}–10^{-4}M. EC_{50} values were 7×10^{-7}M for ACh, 5×10^{-7}M for muscarine, and 8×10^{-8}M for CD. DMPP, 5×10^{-5} or 10^{-4}M, produced non-parallel shifts to the right of the

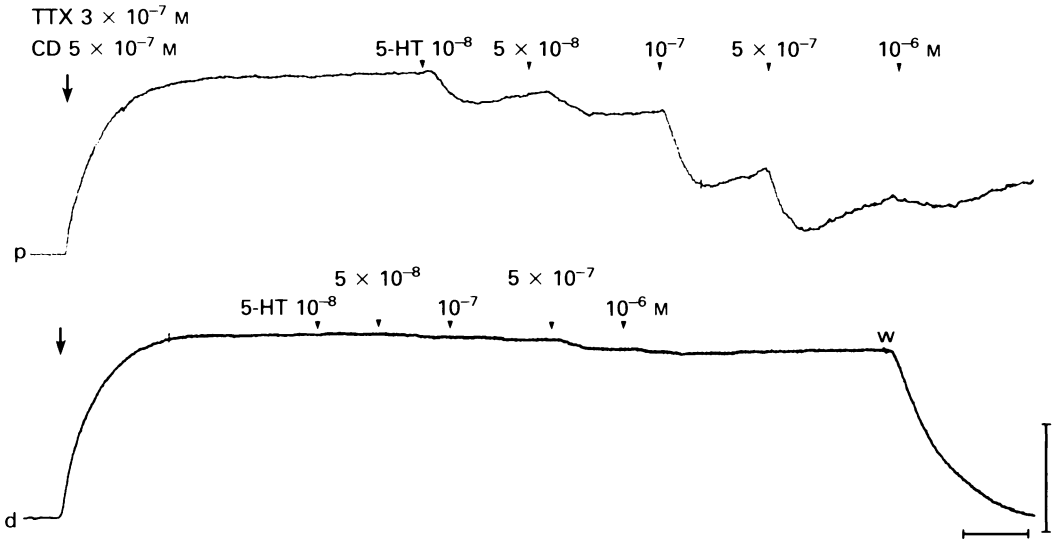

Figure 6 Relaxant effect of 5-hydroxytryptamine (5-HT) on rat isolated muscularis mucosae oesophagi contracted with *cis*-2 methyl-4-diethylamino-methyl 1–3 dioxolane (CD). Upper and lower trace are from proximal (p) and distal (d) segments, respectively, of same oesophagus. Tetrodotoxin (TTX) was given 5 min before CD (added to baths at arrow heads). Note difference in sensitivity between both segments with cumulative dosing of 5-HT. Calibrations: vertical bar 10 mN; horizontal bar 1 min.

concentration-response curves for ACh ($n=24$) and muscarine ($n=24$), associated with a significant depression of maximal responses at the higher concentration. DMPP caused only transient feeble increases in basal tonus. Morphine, 5×10^{-6} and 10^{-4}M, was without effect when added during the plateau phase of the tension response induced by muscarine, 10^{-5}M. In the presence of morphine, 5×10^{-6} or 10^{-5}M, ACh concentration-response curves ($n=10$) showed no change in threshold, but a steeper slope resulting from a 30% increase in maximal effects.

Contractions of the tunica muscularis mucosae in the isolated vagus-oesophagus preparation

Vagally-elicited contractions recorded from the proximal half of the oesophagus gave little indication of a smooth muscle component owing to the superimposed activity of the tunica propria. However, preparations obtained from the distal third of the oesophagus regularly displayed distinct low-amplitude wave-like responses lagging behind the faster initial twitch response (Figure 7). Both components were evident in recordings of either intraluminal pressure or longitudinal tension and, for convenience, will hereafter be referred to as vagally-evoked fast twitch (VFT) and vagally-evoked slow contraction (VSC).

As illustrated in Figure 7, it was possible to separate the VSC from the VFT with the help of neuromuscular blocking agents ((+)-tubocurarine 3 to 7×10^{-7}M, α-bungarotoxin 10^{-7}M, or *Naja* toxin 50 μg ml^{-1}). The mean frequency required to elicit a half-maximal VSC was 4.6±0.9Hz (±s.e.mean $n=8$) and thus agreed fairly closely with the corresponding value obtained in field-stimulated TMM preparations (Table 1).

Pharmacological modification of the vagally-evoked slow contraction

Like the field-stimulated TMM contractions, the VSC was readily abolished by hyoscine 10^{-7} M or by TTX 10^{-7}M. In the presence of the anticholinesterase agents, physostigmine or BW284C51, 2 to 4×10^{-6}M, the VSC showed a marked enhancement, as evidenced by more than 2 fold increases in amplitude and duration of contractions. On the other hand, the VSC was antagonized by hexamethonium 0.13–0.26 mM (Figure 7), i.e. at concentrations which did not affect contractile responses to muscarine, CD or ACh. Muscarinic agonists evoked contractions with a time course similar to that seen in TMM preparations.

5-HT produced mixed excitatory/inhibitory effects on the nerve-oesophagus preparation which generally resembled the biphasic effects observed in the isolated TMM, but showed a notable increase in the

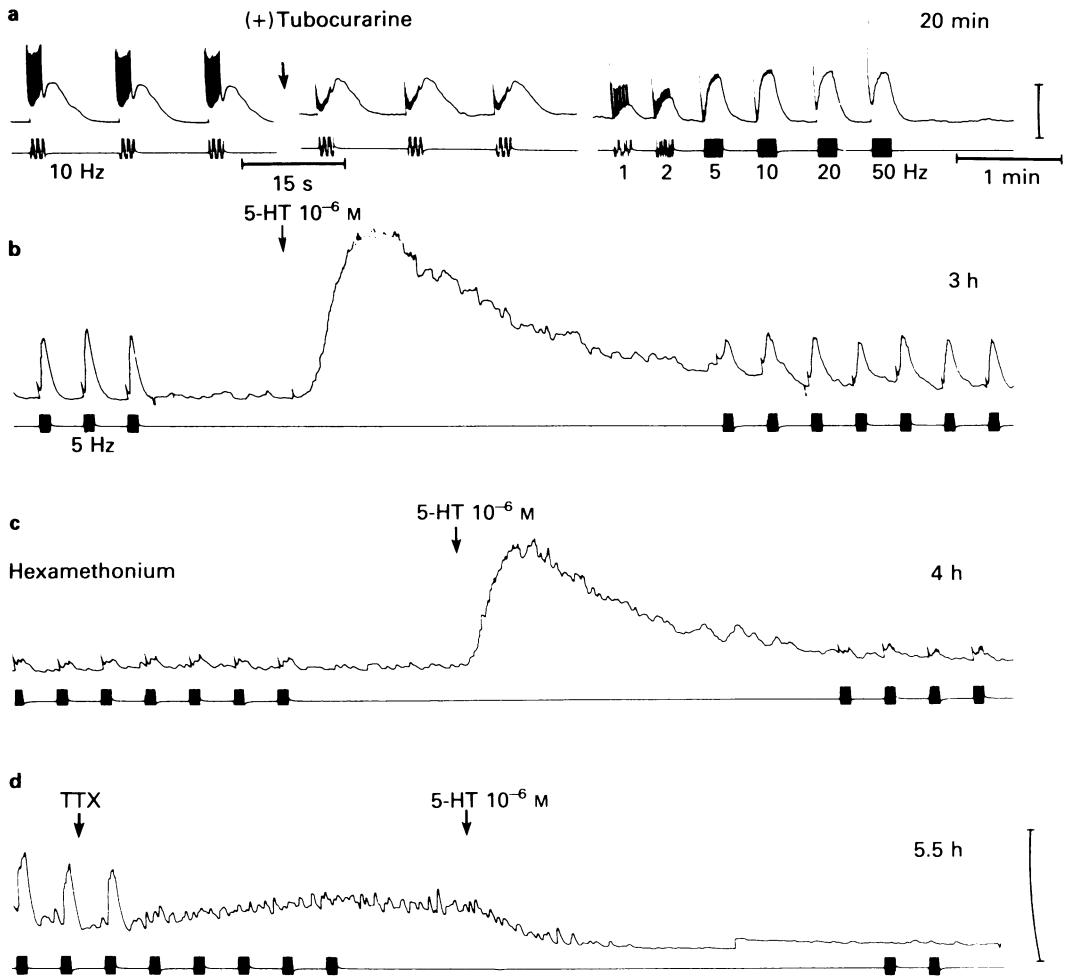

Figure 7 Muscularis mucosae contractions in the isolated vagus nerve-oesophagus preparation of the rat. All records are from a single experiment on a preparation dissected from the distal third of oesophagus; upper and lower trace in each pair represent, respectively, longitudinal tension and monitor signal for electrical stimulation applied to vagal nerve branch supplying distal oesophagus. Time intervals shown at right of each sequence refer to start of curarization. The preparation was kept curarized throughout; other agents were removed from bath fluid between each sequence. Initial deflections preceding slow contractile responses in panels (b) and (c) represent residual fast twitch of muscularis externa. (a) (+)-Tubocurarine (7.2×10^{-7} M for 10 min) antagonized vagally-evoked short-latency twitches, but left intact the slow contractile response, the frequency dependence of which is demonstrated in the sequence at the right side of panel. (b) 5-Hydroxytryptamine (5-HT) produced an initial stimulatory effect with a subsequent incomplete inhibition of the nerve-evoked response. (c) After hexamethonium (8×10^{-5} M, added 2 min before start of trace), there was a near-complete block of the vagally-driven slow contractile response, but only a weak inhibition of the 5-HT induced excitatory effect. (d) Tetrodotoxin (TTX) 10^{-7} M abolished both vagally-driven and 5-HT-induced slow contractile responses, unmasking the inhibitory effect of 5-HT. Calibrations: vertical bar 5 mN; horizontal bar 1 min.

stimulatory component between 5×10^{-8} and 10^{-6} M 5-HT (Figure 7 b and c). These contractions subsided completely within 3–4 min after their onset; concomitantly, the VSC was depressed to a variable degree. The excitatory effect was only weakly antagonized by hexamethonium, but completely and reversibly suppressed by both TTX and ketanserin, and, for the duration of the blockade, replaced by a

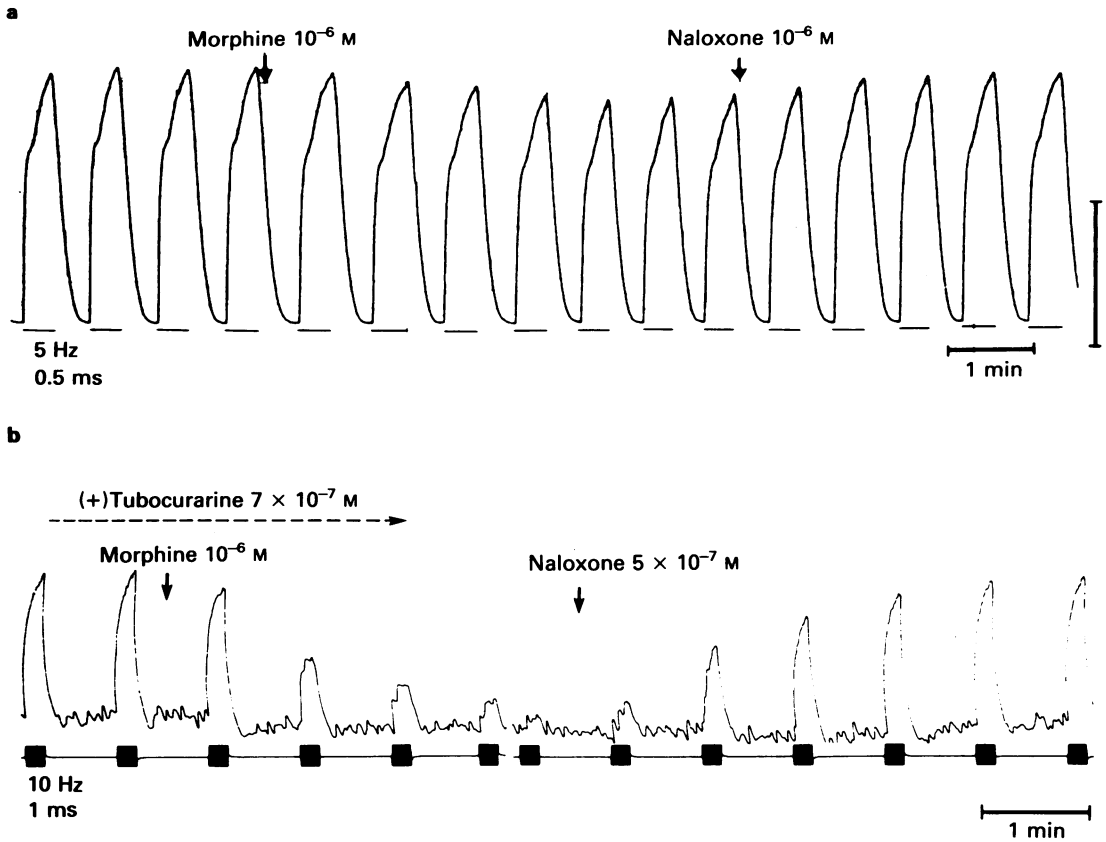

Figure 8 Inhibitory effects of morphine (10^{-6}M) on neurally-evoked longitudinal tension responses of rat oesophageal muscularis mucosae. (a) In distal segment of isolated smooth muscle tube, contraction due to field-stimulation (indicated by bars) was weakly antagonized by morphine, an effect reversed by naloxone (10^{-6}M). (b) A similar slow contraction was elicited by electrical stimulation of vagus in the curarized vagus-nerve-oesophagus preparation from distal segment. Note pronounced inhibitory effect of morphine 10^{-6}M and reversal of this effect by naloxone 5×10^{-7}M. A 2 min segment has been left out between the sixth and seventh stimulation period. Calibrations: vertical bars 5 mN; horizontal bar 1 min.

purely inhibitory effect on baseline tonus, when present (Figure 7d), and on the VSC, in the case of the 5-HT antagonist. A marked inhibition of the VSC was also observed with noradrenaline 10^{-6}–10^{-5}M. At 10^{-6}M, morphine reduced the amplitude of the VSC by $78 \pm 5\%$ mean ± s.e.mean, $n = 8$), and this inhibition was readily abolished by naloxone (Figure 8). Attempts to demonstrate a relaxation of the type obtained with the isolated field-stimulated TMM did not yield clear results.

Histochemical demonstration of submucosal nerve plexus

In mounts of the whole oesophageal inner tube preparation, histochemical visualization of cholinesterase activity by means of the thiocholine technique revealed the presence of an extensive submucosal nerve plexus adhering to the abluminal surface of the muscularis mucosae (Figure 9). Use of either acetylthiocholine (ASCh) or butyrylthiocholine (BSCh) as substrates resulted in the formation of a reaction product in both neural and muscular structures. ASCh-induced staining of smooth muscle cells, unlike that of nerve fibres, was completely suppressed by *iso*-OMPA (10^{-5}M), allowing visualization of the submucosal plexus against a background that was empty except for fine calibre nerve fibres outlining the contours of submucosal blood vessels. In the presence of BW284C51 (10^{-4}M), BSCh-induced staining persisted in muscle cells, but was attenuated in the ganglionic plexus. Small-sized ganglionic cell

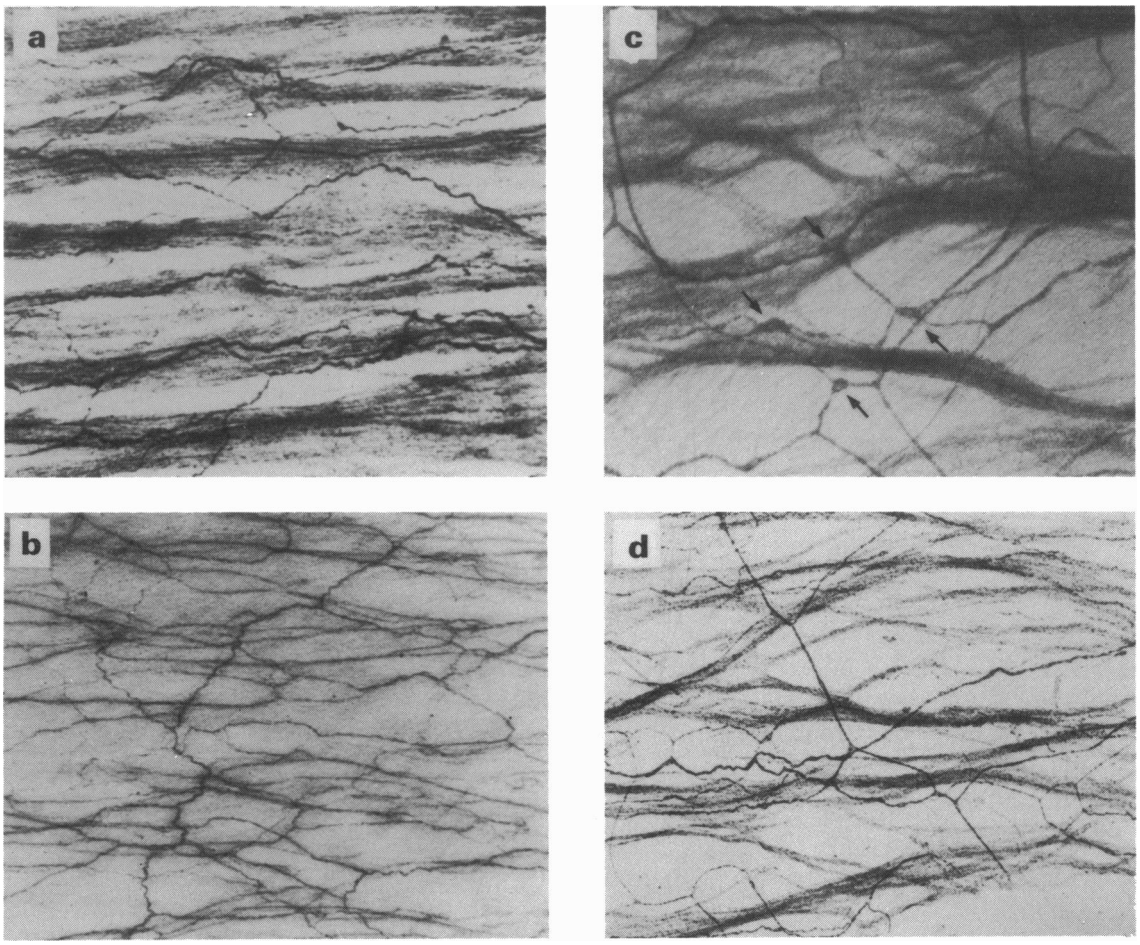

Figure 9 Submucosal nerve plexus of the tunica muscularis mucosae of the rat oesophagus as visualized by the thiocholine technique. Examples shown are light micrographs of whole-mounts of the inner smooth muscle tube from the mid-oesophagus. Tissue reacted with acetylthiocholine in the absence (a) or presence (b) of *iso*-OMPA shows extensive network of ganglionic plexus, with muscle staining suppressed by the butyrylcholinesterase inhibitor. In tissue reacted with butyrylthiocholine (c,d), several ganglia (arrows) embedded in internodal strands can be seen along with the meshwork of smooth muscle strands.

bodies were found scattered in groups of 2–3 along the internodal strands, which, together with preterminal axon bundles and preganglionic nerve branches, dominated the picture. No obvious regional differences were apparent in the density of plexus cells or fibres at proximal and distal levels.

Discussion

The role of the rodent oesophageal TMM in the production of deglutitory peristalsis remains to be adequately defined. Notwithstanding its anatomical name, the TMM would appear capable of assisting in the generation of propulsive motility of the oesophageal wall itself, rather then effecting localized movements of the mucosa. This concept is supported by the following evidence: (i) the TMM is richly innervated by an intrinsic nerve network receiving input from vagal oesophagomotor fibres; (ii) it is capable of generating intraluminal pressure; and (iii) it possesses a regionally differentiated responsiveness to intrinsic neural and exogenous chemical stimuli.

Neural control of the tunica muscularis mucosae

Cholinergic mechanisms The present study demonstrates that TMM contractions of similar frequency-response characteristics may be obtained *in situ* by electrical stimulation of preganglionic vagal efferents or direct stimulation of intrinsic ganglia in the isolated TMM tube. In both instances, motor effects were mediated via a common pathway of intramural cholinergic postganglionic fibres as they were eliminated by either TTX or hyoscine. Although field-stimulation of the TMM would be expected to activate pre- as well as post-ganglionic fibres, corresponding effects, given the ineffectiveness of hexamethonium, were probably overridden by post-ganglionic ones.

The present results extend to the rat and the guinea-pig (Bartlett, 1968 a,b; Kamikawa & Shimo, 1979; 1983; Kamikawa et al., 1982). However, the mode of operation of this parasympathetic cholinergic pathway reveals a striking species difference. In the rat, neurally-evoked motor responses were of a graded and tonic character, in contrast to the twitch-like phasic response in the guinea-pig. On the other hand, the rate of rise of TMM tension responses to muscarinic agonists appeared to be similar in both species. The effects of selective anti-cholinesterases on neurally-driven responses of the TMM support the view that acetylcholinesterase (AChE) is primarily responsible for inactivating ACh released from pre- and post-ganglionic oesophagomotor fibres. No evidence was obtained indicating a functional role for butyrylcholinesterase (BuChE).

Our histochemical observations indicate that the TMM isolated preparation as used in these pharmacological experiments contains what appears to be the submucosal ganglionic plexus in its entirety. Thus, this structure provides a morphological comparison for the nerve-mediated TMM responses described in this study. It is of interest that recent immunohistochemical work (Schultzberg *et al.*, 1980) has failed to reveal peptide-containing nerve cell bodies in the submucosal plexus.

Non-cholinergic mechanisms The complete blockade by hyoscine, of nerve-mediated contractions, either in the vagus-oesophagus preparation or the field-stimulated TMM tube, suggests that the observed inhibitory effects of the adrenoceptor agonists and 5-HT were exerted at non-junctional sites, and hence that the rat TMM lacks a significant innervation by nerves releasing catecholamines or 5-HT. In agreement with findings obtained in the guinea-pig (Kamikawa & Shimo, 1979; Kamikawa *et al.*, 1982), noradrenaline-induced inhibition appeared to result from a predominant action on β-adrenoceptors, with a minor α-adrenoceptor mediated component. However, noradrenaline-induced inhibition was only partially antagonized by various combinations of α-and β-adrenoceptor antagonists.

Unlike the guinea-pig TMM, (Bartlett, 1968b; Kamikawa & Shimo, 1983a), the rat TMM, both *in situ* and *in vitro*, exhibited not only excitatory, but also inhibitory responses to 5-HT. The TTX-sensitive excitatory responses differed from those described in the guinea-pig (Kamikawa & Shimo, 1983a) in that they displayed a lower threshold and were readily blocked by ketanserin, a selective 5-HT_2 antagonist (van Nueten *et al.*, 1981); moreover, they showed selectivity for the distal TMM and were markedly facilitated in whole oesophagus preparations in partial agreement with the observations of Bartlett (1968b), which suggested their complete dependence on neural structures outside the TMM. The inhibitory, unlike the excitatory, effects of 5-HT were not antagonized by either TTX or ketanserin and thus probably resulted from a direct action on the muscle. The observed proximo-distal gradient in 5-HT sensitivity would argue against a non-specific mechanism. Moreover, our results are inconsistent with a mediation via the release of endogenous catecholamines or prostaglandins. The mechanism underlying the 'fading' of the inhibitory 5-HT effects awaits further investigation.

The inhibitory effects of morphine on TMM responses to field stimulation were of a similar magnitude to those observed in the guinea-pig by Kamikawa & Shimo (1983b), who concluded that this opiate was acting as a partial agonist at presynaptic κ-receptors modulating ACh release from post-ganglionic fibres on submucosal nerve cells. However, in the present study, preganglionically-evoked responses of the rat TMM *in situ* were more susceptible than ganglionically-driven responses of the isolated TMM, suggesting that vagal efferents synapsing on submucosal ganglia represent an important site for the opiate-mediated inhibition.

The ability of the TTX-treated isolated TMM to relax in response to field stimulation invites comparison with similar inhibitory responses in certain arterial smooth muscle (Rooke *et al.*, 1982; Ebeigbe *et al.*, 1983). So far, our own results have failed to corroborate a mediatory role of a histamine-like endogenous factor.

The possible absence of an intrinsic inhibitory innervation in the rat TMM needs to be verified electrophysiologically, in view of its implications for the neural organization of oesophageal peristalsis. According to current hypotheses, underlying inhibitory mechanisms are thought to arise in neural structures located within the end organ (Roman, 1982; Miller, 1982).

Regional differences in responsiveness to neural and chemical stimuli

Neurally-evoked mechanical responses displayed regional differences indicative of a proximo-distal gradient in neuromuscular excitability of the TMM. Thus, the frequency-response curve obtained from the distal TMM was displaced to the left with respect to that from the cervical TMM. A similar aborally-directed gradient in the efficiency of oesophageal neuromyal transmission has already been uncovered in the smooth muscle tunica propria of the oppossum (Schulze *et al.*, 1978; Goyal & Gidda, 1981; Decktor & Ryan, 1982). The existence of such gradients has been interpreted as reflecting the presence of a locally organized mechanism for peristalsis.

Clearly, it would be important to determine if the above-noted regional differences in 5-HT sensitivity relate to neural inputs originating from the ganglia of the myenteric plexus. Whatever their basis, they strengthen the idea that the TMM participates in the production of oesophageal peristalsis. In keeping with this suggestion, oesophageal motility disturbances resulting from the administration of antimuscarinic agents (Hellemans, 1970; Roman, 1982) would at least in part be attributable to impaired function of the TMM.

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