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EVIDENCE THAT CELL BODIES OF NON-CHOLINERGIC, EXCITATORY NEURONES WHICH SUPPLY THE SMOOTH MUSCLE OF THE CHICKEN RECTUM ARE LOCATED IN THE GANGLIA OF REMAK'S NERVE

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1 A pharmacological investigation of the distribution of non-cholinergic excitatory nerve cell bodies was performed on the chicken's isolated perfused rectum with attached Remak's nerve supply.

2 Electrical stimulation of Remak's nerve trunk produced a contraction and a discharge of action potentials in the nerve branches which supply the smooth muscle of the rectum. Both responses were virtually blocked by hexamethonium when applied via the caudal mesenteric artery.

3 The contractile effect following stimulation of the nerve trunk was inhibited more effectively by hexamethonium when application was restricted to the trunk rather than to the intestine.

4 The contractile effect of stimulating the nerve branches was unaffected by hexamethonium.

5 It is concluded that ganglionic transmission, which is mediated by nicotinic receptors, occurs in the ganglia of Remak's nerve but not in ganglia of the enteric plexuses. Therefore, cell bodies of the postganglionic neurones which are considered to be non-cholinergic are located in Remak's nerve ganglia.

Introduction

It has been suggested that neurones which are non-adrenergic and non-cholinergic (NANC) may be involved in the excitatory innervation to avian and mammalian gastrointestinal tracts (Ambache & Freeman, 1968a, b; Hassan, 1969; Ambache, Verney & Zar, 1970; Bartlet & Hassan, 1971; Bartlet, 1974; Ohashi, Naito, Takewaki & Okada, 1977; Takewaki, Ohashi & Okada, 1977; Takewaki & Ohashi, 1977; Ahmad, Singh & Garg, 1978; Franco, Costa & Furness, 1979). The chicken rectum receives a dense NANC excitatory innervation from Remak's nerve. The excitatory nerve fibres arise from different parts of Remak's nerve and reach the rectal wall largely via caudal nerve branches running from the trunk to the intestine through the mesentery. These nerve fibres are particularly dense in the dorso-caudal region (Takewaki *et al.*, 1977). Excitatory junction potentials (e.j.ps) evoked by stimulation of Remak's nerve or by field stimulation have been recorded both extracellularly and intracellularly from cells of the longitudinal and circular muscle layers (Ohashi *et al.*, 1977; Takewaki & Ohashi, 1977).

Ganglion blocking agents have been used to test for the presence of ganglionic transmission in the NANC excitatory pathways before these nerves reach the intestinal smooth muscle cells (Bartlet & Hassan, 1971; Takewaki *et al.*, 1977). There was a large variation in the sensitivity of different preparations to these drugs.

This paper describes the effects of hexamethonium on the mechanical contractions of the isolated perfused rectum of the chicken and on the firing, in the rectal nerve branches, elicited by stimulation of the trunk of Remak's nerve. Ganglionic transmission, mediated by nicotinic receptors, occurs in the excitatory nerve pathways; the non-cholinergic neurones interconnect with preganglionic fibres in ganglia in the trunk of Remak's nerve and supply the smooth muscle of the rectum.

Methods

Preparation

The isolated rectum of the chicken and its extrinsic nerve supply (Remak's nerve) were prepared as described previously (Takewaki *et al.*, 1977). Each preparation was set up in a 60 ml organ bath of Tyrode solution (composition mM: NaCl 137.0, KCl 2.7, NaH_2PO_4 0.4, $NaHCO_3$ 12.0, $MgCl_2$ 2.0, $CaCl_2$ 1.8 and glucose 5.6) maintained at 30°C, and perfused at

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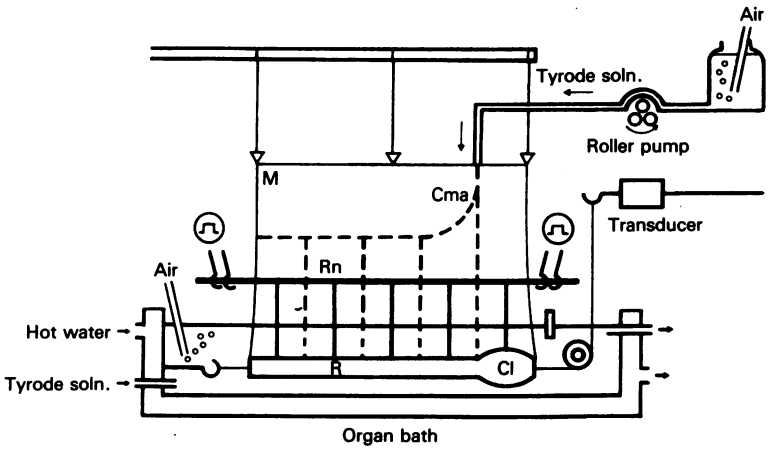

Figure 1 Diagrammatic sketch of the apparatus used: Rn, Remak's nerve trunk; R, rectum; Cl, cloaca; Cma, caudal mesenteric artery; M, mesentery.

a rate of 10 ml/min by means of a roller pump. The bathing solution was bubbled with air (Figure 1). The trunk of Remak's nerve was suspended above the surface of the bathing solution by holding up the mesentery at several points. The trunk was kept moist by covering it with cotton wool soaked in Tyrode solution. Both ends of the intestine were usually kept open, but were closed in those experiments in which the intestine was perfused with Tyrode solution via its caudal mesenteric artery at a rate of 3 ml/min by means of a roller pump. Effluent from the open caudal mesenteric vein was removed by overflow.

Measurement of mechanical activity

The mechanical activity of the rectum in its longitudinal direction was measured isometrically from the caudal end by a force-displacement transducer (Nihon Khoden, SB-1T) and recorded. The cut ends of the trunk of Remak's nerve and the branch running to the intestine were dissected free from the surrounding fascia in some experiments. Nerves were stimulated (Nihon Khoden, MSE-3) via electrodes of the type used by Burn & Rand (1960) by supramaximal square pulses of 0.5 ms duration at 20 Hz. Trains of 200 pulses were delivered at an interval of 4 min.

Recording of action potentials in the rectal nerve branches

The isolated preparation described above was immersed in a 100 ml organ bath containing Krebs-Henseleit solution (composition mM: NaCl 118.9, KCl 4.56, $CaCl_2$ 2.52, KH_2PO_4 1.19, $MgSO_4$ 2.44, $NaHCO_3$ 25.0 and glucose 11.10) kept at 25°C and bubbled with a mixture of 95% O_2 and 5% CO_2. This solution was also used for perfusion of the preparation via the vessels. Two nerve branches supplying the rectum which originated in different ganglia in Remak's nerve were dissected free from the surrounding tissues under a binocular microscope and the action potentials produced in response to stimulation of Remak's nerve trunk were recorded. Suction electrodes were used for recording purposes. The electrical responses were amplified by an RC-coupled amplifier with a time constant of 300 ms, displayed on an oscilloscope and photographed on 35 mm X-ray film.

Application of hexamethonium

Hexamethonium bromide was used. A stock solution of this drug was dissolved in distilled water and final dilutions were made in Tyrode or Krebs-Henseleit solution. In experiments in which the caudal mesenteric artery was perfused, the drug was usually added to the perfusing solution. When the drug was applied locally to the trunk of Remak's nerve or to the intestine, a layer of liquid paraffin was first made on the surface of the bathing solution. The drug was then infiltrated into the cotton wool covering the trunk of Remak's nerve and diffused into the trunk. Alternatively, the drug was injected into the perfusate bathing the preparation. A fine syringe of 0.5 ml capacity was used for the local application of the drug. The doses in the text and figures refer to those of the base in the bathing and perfusing solutions.

Results

Effects of hexamethonium

Electrical stimulation of either cut end of the trunk of

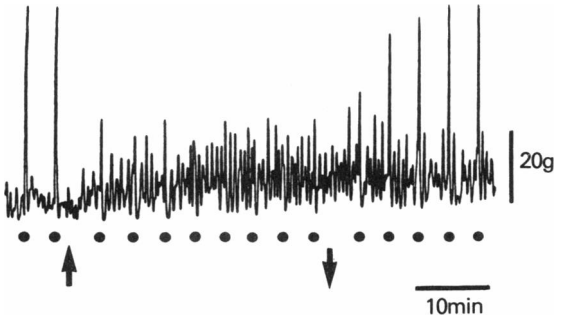

Figure 2 The reversible inhibitory effects of hexamethonium (↑, 500 μg/ml), added to the solution perfusing the caudal mesenteric artery, on the contractile response of the chicken rectum to stimulation of the anal cut end of Remak's nerve trunk (●). The nerve was stimulated every 4 min by square wave pulses of 0.5 ms duration at 20 Hz for 10 s. The preparation was washed at the arrow (↓).

Remak's nerve produced a contraction usually followed by relaxation of the rectum as observed by Takewaki *et al.* (1977). Hexamethonium (10 to 500 μg/ml) added to the solution perfusing the caudal mesenteric artery reduced the contractile responses in a concentration-dependent manner, irrespective of whether the anal cut end or the oral cut end of the trunk of Remak's nerve was stimulated. At 500 μg/ml hexamethonium, the contractile response was completely blocked in 10 out of 16 experiments and, in the remainder, it was reduced by more than 75%. The inhibitory effect appeared within 2 to 4 min, reached a maximum 4 to 10 min after application, and was completely reversed 15 to 30 min after replacing the perfusion solution with fresh Tyrode solution (Figure 2). These results indicate the ease with which the drug diffuses to the ganglion cells after application via the blood vessels and that transmission in the ganglia in the excitatory nerve pathways is cholinergic and mediated by nicotinic receptors.

To determine the site of ganglionic transmission, hexamethonium was applied locally to the trunk of Remak's nerve or to the intestine (see Methods). Applied to the intestine in this way, hexamethonium (500 μg/ml) did not depress the contractile response of the intestine to stimulation of Remak's nerve trunk except in 6 experiments (out of 22) where a small reduction (up to 30%) was obtained. In the majority of the remaining 16 experiments, the drug enhanced the nerve motor response by less than 20%. When applied to the nerve trunk, hexamethonium inhibited the contractile response. The degree of inhibition varied in different experiments (Figure 3), but the drug was more effective after local application to the nerve trunk than to the intestine. The time required to reach the maximum inhibitory effect following application to the nerve trunk was 10 to 15 min, significantly longer than with application via the caudal mesenteric artery. The inhibitory effect following application to the nerve trunk persisted after removal of the drug and was virtually irreversible, whereas inhibitory effects observed following application through the blood vessels were reversible.

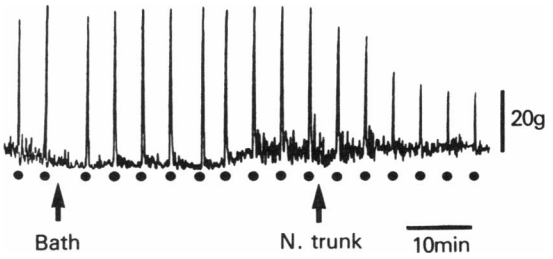

Figure 3 Effects of hexamethonium (500 μg/ml), applied first locally to the intestine (↑, Bath) and then to Remak's nerve trunk (↑, N. trunk), on the contractile response of the chicken rectum to electrical stimulation of the anal cut end of Remak's nerve trunk (●). The nerve was stimulated every 4 min by square wave pulses of 0.5 ms duration at 20 Hz for 10 s.

In three of six experiments in which the local application of hexamethonium to the intestine was ineffective, additional application of the drug to the nerve trunk produced a 65, 35 and 30% reduction respectively of the contractile response. Additional perfusion of the drug through the artery in the remaining 3 experiments caused a marked or complete suppression of the contractile responses (Figure 4).

These results suggest that ganglionic transmission may take place in the ganglia in Remak's nerve trunk rather than in those of the enteric plexuses.

The effect of stimulation of nerve branches to the intestine

The effect of hexamethonium on the contractile response of the rectum to stimulation of nerve branches which run from different ganglia of Remak's nerve to the rectum was investigated in seven preparations. In comparison with that elicited by stimulation of the trunk, the contractile response to stimulation of a nerve branch, though relatively small, had a similar pattern, consisting of an initial, brief contraction followed by a prolonged relaxation. The magnitude of the initial, contractile response was related to the level of Remak's nerve trunk from which the branch arose; the more caudal the branch, the larger the contractile component. The contractile responses were unaffected by hexamethonium, whereas those elicited by stimulation of the trunk were completely blocked (Figure 5). Thus, the response to stimulation of the

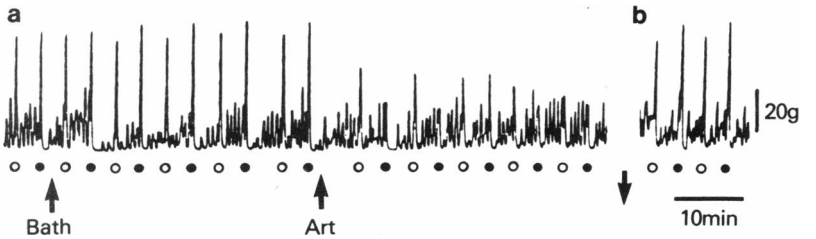

Figure 4 (a) Effects of hexamethonium (500 μg/ml), applied locally to the intestine first (↑, Bath) and then perfused through the artery (↑, Art) on the contractile responses of the chicken rectum to stimulation (square pulses of 0.5 ms duration at 20 Hz for 10 s) of either anal (●) or oral (○) cut end of Remak's nerve trunk. The drug was washed out at the arrow (↓). (b), recovery from the inhibitory effect 20 min after replacing the solution perfusing the artery with fresh Tyrode solution.

nerve branches appears to be due to stimulation of postganglionic fibres in the excitatory pathways.

This result supports the view that the ganglionic transmission occurs in Remak's nerve trunk but not in the enteric plexuses.

The electrical discharge of the branch to stimulation of Remak's nerve trunk

Electrical stimulation of the trunk with single supramaximal square wave pulses (0.5 ms duration) produced a discharge of action potentials in the branches which supply the smooth muscles of the rectum. The discharge was composed of two or more components with different sizes. Figure 6 shows the discharges in two branches. Here, the lower tracings are records from the branch originating in Remak's nerve ganglion nearer to the stimulating site. The electrical responses were abolished almost completely by hexamethonium, 100 μg/ml and presumably represent firing in the postganglionic axons. The inhibitory effect of the drug was reversible.

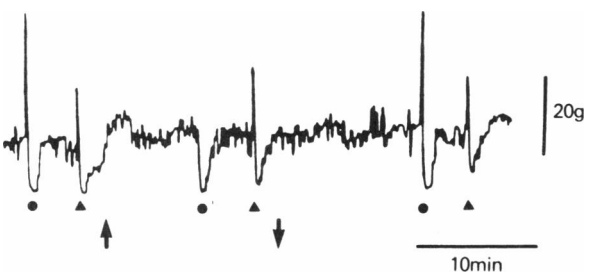

Figure 5 Effects of hexamethonium (100 μg/ml), added to the solution perfusing the caudal mesenteric artery at the arrow (↑) and washed out at the arrow (↓), on the contractile responses of the chicken rectum to stimulation of the anal cut end of Remak's nerve trunk (●) or of the peripheral cut end of nerve branches running to the rectum (▲). The nerves were stimulated by square wave pulses of 0.5 ms duration at 20 Hz for 10 s. Hexamethonium reduced the response to stimulation of the nerve trunk, indicating the presence of ganglia in this pathway but failed to affect the contractile response to nerve branch stimulation.

Discussion

The present results provide evidence for the presence of a nicotinic ganglionic synapse in the excitatory nerve pathways which innervate the smooth muscle cells of the chicken rectum. Differences in the route of application and hence of access of the drug could account for the differences between the present and earlier results (Bartlet & Hassan, 1971; Takewaki *et al.*, 1977). In the method employed earlier, the ganglion blocking agents, including hexamethonium, were added to the bathing solution in which the chicken isolated rectum with Remak's nerve supply was immersed. The ganglion which is presumably surrounded by a connective tissue sheath (Paton, 1954) would present a barrier to drugs applied topically. The ease with which hexamethonium, applied through the mesenteric artery, reversibly blocked the response to stimulation of the trunk of Remak's nerve, in contrast to the variability in the response which followed local application, supports this view.

The site of ganglionic transmission lies in the trunk of Remak's nerve, but not in the enteric plexuses. This conclusion depends on the following findings: first, the contractile effect following stimulation of the trunk was inhibited more effectively by hexamethonium when application was restricted to the trunk than to the intestine. Secondly, the drug failed to suppress the contractile effect of stimulating the branches, whereas it abolished the contractile effect of stimulating the trunk. Thirdly, the drug blocked the discharge of the branches evoked by stimulation of the trunk. Since the contraction of the rectum and the evoked discharge of the branches elicited by stimulation of the trunk virtually disappear after application of hexamethonium, a large proportion, if not all, of the branch is made up of postganglionic fibres.

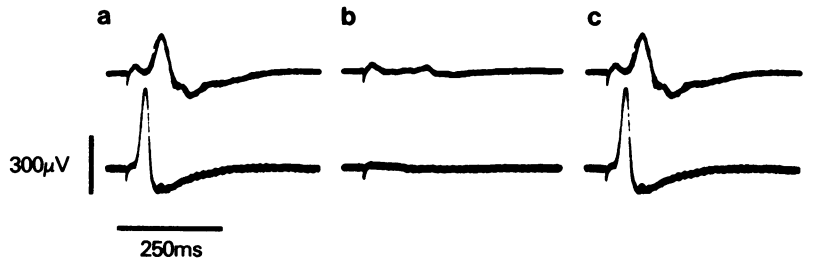

Figure 6 Effects of hexamethonium, 100 μg/ml, added to the solution perfusing the caudal mesenteric artery, on the electrical discharge in two nerve branches running to the rectum evoked by stimulation of Remak's nerve trunk. (a) Control; (b) during perfusion of the drug; (c) 40 min after removal of the drug. The lower tracings are records from the nerve branch originating in Remak's nerve ganglion nearer to the stimulating site than the nerve branch used for the upper tracings.

There is evidence that these excitatory fibres are non-cholinergic. The contractile effect of stimulating Remak's nerve is resistant to atropine and hyoscine (Bartlet & Hassan, 1971; Takewaki *et al.*, 1977). The excitatory junction potential is not suppressed, but rather potentiated, by atropine (Ohashi *et al.*, 1977; Takewaki & Ohashi, 1977) and, compared with the potential recorded at other cholinergic smooth muscle junctions of the digestive tract (Bennett, 1966; Ohashi & Ohga, 1967; Bennett, 1969; Ohashi, 1971), the junction potential in the chicken rectum differs considerably in time course, and in latency. The latency to onset of the junction potential in the chicken rectum is about 5 to 10 times shorter than at cholinergic junctions.

The present and our previous results (Takewaki *et al.*, 1977; Ohashi *et al.*, 1977; Takewaki & Ohashi, 1977) suggest that the non-cholinergic excitatory nerve pathways have their cell bodies in ganglia in Remak's nerve. These neurones receive synaptic connections from cholinergic preganglionic fibres ascending or descending in Remak's nerve trunk with the former predominating. Ganglionic transmission is mediated by acetylcholine through stimulation of nicotinic receptors. The axons of the post-ganglionic neurones leave the trunk and run, particularly in the caudal branches, to the rectum. Possibly they terminate shortly after entering the wall of the rectum and their terminals form synapses in the rectal smooth muscle. Thus, the smooth muscles in the dorsal-caudal area of the rectal wall receive a more dense excitatory innervation. The non-cholinergic excitatory transmitter from the nerve terminals to the smooth muscles is as yet unknown.

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References

AHMAD, A., SINGH, R.C.P. & GARG, B.D. (1978). Evidence of non-cholinergic excitatory nervous transmission in chick ileum. *Life Sci.*, **22**, 1049–1058.

AMBACHE, N. & FREEMAN, M.A. (1968a). Atropine resistant spasms due to excitation of non-cholinergic neurones in guinea-pig myenteric plexus. *J. Physiol.*, **198**, 92–94P.

AMBACHE, N. & FREEMAN, M.A. (1968b). Atropine-resistant longitudinal muscle spasms due to excitation of non-cholinergic neurones in Auerbach's plexus. *J. Physiol.*, **199**, 705–727.

AMBACHE, N., VERNEY, J. & ZAR, M.A. (1970). Evidence for the release of two atropine-resistant spasmogens from Auerbach's plexus. *J. Physiol.*, **207**, 761–782.

BARTLET, A.L. (1974). Action of putative transmitters in the chicken vagus nerve/oesophagus and Remak nerve/rectum preparations. *Br. J. Pharmac.*, **51**, 549–558.

BARTLET, A.L. & HASSAN, T. (1971). Contraction of chicken rectum to nerve stimulation after blockade of sympathetic and parasympathetic transmission. *Q. J. exp. Physiol.*, **56**, 178–183.

BENNETT, M.R. (1966). Transmission from intramural excitatory nerves to the smooth muscle cells of the guinea-pig taenia coli. *J. Physiol.*, **185**, 132–147.

BENNETT, T. (1969). Nerve-mediated excitation and inhibition of the smooth muscle cells of the avian gizzard. *J. Physiol.*, **204**, 669–686.

BURN, J.H. & RAND, M.J. (1960). The relation of circulating noradrenaline to the effect of sympathetic stimulation. *J. Physiol.*, **150**, 295–305.

FRANCO, R., COSTA, M. & FURNESS, J.B. (1979). Evidence for the release of endogenous substance P from intestinal nerves. *Naunyn-Schmiedebergs Arch. Pharmac.*, **306**, 195–201.

HASSAN, T. (1969). A hyoscine-resistant contraction of isolated chicken oesophagus in response to stimulation of parasympathetic nerves. *Br. J. Pharmac.*, **36**, 268–275.

OHASHI, H. (1971). An electrophysiological study of transmission from intramural excitatory nerves to the smooth muscle cells of the chicken oesophagus. *Jap. J. Pharmac.*, **21**, 586–596.

OHASHI, H., NAITO, K., TAKEWAKI, T. & OKADA, T. (1977). Non-cholinergic excitatory junction potentials in smooth muscle of chicken rectum. *Jap. J. Pharmac.*, **27**, 379–387.

OHASHI, H. & OHGA, A. (1967). Transmission of excitation from the parasympathetic nerve to the smooth muscle. *Nature*, **216**, 291–292.

PATON, W.D.M. (1954). Transmission and block in autonomic ganglia. *Pharmac. Rev.* **6**, 59–67.

TAKEWAKI, T. & OHASHI, H. (1977). Non-cholinergic excitatory transmission to intestinal smooth musle cells. *Nature*, **268**, 749–750.

TAKEWAKI, T., OHASHI, H. & OKADA, T. (1977). Non-cholinergic and non-adrenergic mechanism in the contraction and relaxation of the chicken rectum. *Jap. J. Pharmac.*, **27**, 105–115.

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