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Presynaptic, muscarinic inhibition of non-adrenergic, non-cholinergic neuromuscular transmission in the chicken rectum

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1 Cholinergic inhibition of the non-adrenergic, non-cholinergic (NANC) transmission was investigated in the chicken isolated rectum with Remak's nerve attached.

2 Stimulation of Remak's nerve (RT stimulation) at frequencies higher than 5 Hz elicited a late, slow contraction of the rectum in addition to an initial, fast NANC contraction. The late, slow contraction was blocked by atropine (0.25 $\mu g\,ml^{-1}$), potentiated by physostigmine (50 $ng\,ml^{-1}$) and accompanied by an overflow of acetylcholine into the vascular perfusate, indicating the existence of cholinergic innervation to the rectum via Remak's nerve.

3 RT stimulation (10 pulses at 0.5–1.0 Hz) elicited NANC-mediated excitatory junction potentials (e.j.ps). The e.j.p. amplitude declined at the second stimulus and then increased to reach a plateau. Atropine, by abolishing this decrease in amplitude, increased the mean amplitude of the e.j.ps during trains of stimuli but atropine did not affect the amplitude of the first e.j.p. Physostigmine reduced the mean e.j.p. amplitude, and this action was readily antagonized by atropine.

4 A single intramural nerve stimulation delivered 2 s or less before RT stimulation with trains of stimuli, suppressed the amplitude of the first e.j.p. of the train. This effect was abolished by atropine.

5 Atropine in concentrations high enough to affect the e.j.p. amplitude had no effect on the resting membrane potential, the threshold for generating an action potential, or membrane resistance of the smooth muscle.

6 It is concluded that RT stimulation at low frequencies causes the release of acetylcholine simultaneously with the NANC transmitter. The released acetylcholine acts mainly on prejunctional muscarinic receptors and mediates an inhibitory effect on the release of the NANC transmitter.

Introduction

The excitatory innervation to the smooth muscle of the chicken rectum is mainly non-adrenergic, non-cholinergic (NANC) (Takewaki *et al.*, 1977). Antimuscarinic drugs, e.g., atropine and hyoscine, potentiate the excitatory junction potential (e.j.p.) elicited in response to electrical stimulation of the NANC neurone (Ohashi *et al.*, 1977; Takewaki & Ohashi, 1977). To account for these findings, cholinergic neurones are thought to be implicated in the inhibition of NANC neurotransmission in the chicken rectum. A similar role for cholinergic neurones in the regulation of transmitter release from peripheral adrenergic nerves has been proposed in other tissues, including heart, artery, vein, spleen and retractor penis muscle (Langer, 1977; Klinge & Sjöstrand, 1977; Muscholl, 1979; Starke, 1981; Kuriyama & Suzuki, 1981). Recently, muscarinic inhibition of acetylcholine release from the myenteric plexus has been demonstrated in the guinea-pig ileum (Kilbinger & Wagner, 1979).

The existence of a cholinergic innervation to the chicken rectum has not been demonstrated nor changes observed in the membrane potential during exposure to acetylcholine (Bartlet & Hassan, 1971; Bartlet, 1974; Takewaki *et al.*, 1977). Activation of muscarinic receptors may increase the ionic permeability of the smooth muscle membrane leading to a depolarization and a reduction in membrane resistance, as in most gastrointestinal smooth muscles (Bolton, 1979). If so, acetylcholine released from cholinergic nerve fibres may reduce the e.j.p. through an action on the smooth muscle cells (postsynaptic site) as well as by an action on the presynaptic excitatory nerve terminals which innervate them.

The aims of the present study were to investigate the presence of a cholinergic innervation to the chicken rectum and to determine the effects of atropine on NANC neurotransmission in an attempt to verify the basis of cholinergic inhibition of the transmission.

This study revealed the existence of a cholinergic muscarinic inhibitory innervation of the chicken rectum which was presynaptic and not due to activation of muscarinic receptors on the postsynaptic site. A preliminary account of some of these results has been published previously (Komori *et al.*, 1980b; Komori & Ohashi, 1981).

Methods

Measurement of contractile responses

White Leghorn chickens of either sex, more than 50 days old, were killed by a blow on the neck and bled. The whole rectum was removed together with Remak's nerve and the blood supply. A one cm section of the anal end of Remak's nerve trunk was detached carefully from adhering tissue. A few nerve branches supplying the rectum were cut just after they left Remak's nerve trunk and were dissected free from the mesentery. The rectum was set up in a 50 ml organ bath filled with Krebs-Henseleit solution (composition (mM): NaCl 118.9, KCl 4.6, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgCl_2$ 2.4 and glucose 11.1), kept at 30°C and bubbled with a 95% O_2 plus 5% CO_2. The anal end of Remak's nerve trunk was placed in a bipolar suction electrode and the nerve branches into another bipolar suction electrode for stimulation of the nerves with trains of 10 rectangular pulses of 0.2 ms duration at various frequencies at supramaximal intensity (Nihon Khoden, MSE-3). Longitudinal changes in tension of the rectum were measured isometrically by a force-displacement transducer (Nihon Khoden, SB-1T) and recorded.

In some experiments, the isolated rectum was also perfused with Krebs-Henseleit solution via the caudal mesenteric artery at a rate of 3.0 ml min^{-1}. The venous effluent was collected and infused into a small organ bath (0.3 ml) which contained a longitudinal strip of guinea-pig ileum to detect and assay acetylcholine. Both the bathing and perfusing media contained physostigmine (2 $\mu g\,ml^{-1}$). The venous effluent was collected for 60 s, 20 s after the beginning of nerve stimulation of Remak's nerve (2 or 10 Hz for 30 s). This experimental procedure was repeated every 30 min. All collected samples were kept at −20°C until assayed.

Measurements of e.j.ps and electrotonic potentials

The isolated rectum was sectioned longitudinally along the side opposite the edge which contained the axon terminals and pinned out in an organ bath (10 ml) through which Krebs-Henseleit solution flowed continuously at a rate of 2–4 ml min^{-1}. Besides stimulation of the external nerves, field stimulation of the intramural nerves with rectangular pulses of 0.2 ms duration was achieved using a pair of Ag-AgCl electrodes (1.0 mm in diameter) (Komori & Ohashi, 1982). Excitatory NANC responses to stimulation of the external nerves, but not those to field stimulation have been shown to be blocked by hexamethonium (Takewaki & Ohashi, 1977; Komori & Ohashi, 1982).

The methods of intracellular recording of membrane potential were similar to those previously described (Takewaki & Ohashi, 1977). Glass microelectrodes (40 to 80 MΩ resistance) were filled with 3 M KCl. The microelectrode was inserted into cells from the serosal surface of the tissue. To record responses to field stimulation, the microelectrode was positioned at a distance of 1.0 mm from the point of stimulation. To evoke and record electrotonic potentials, strips, about 20 mm in length and 1.5 mm in width, were dissected from the longitudinal layer of the rectum and mounted in an organ bath as described by Abe & Tomita (1968).

In most experiments, isoprenaline (0.25 $\mu g\,ml^{-1}$) was added to the bathing solution to suppress spontaneous electrical and mechanical activity. This concentration of isoprenaline hyperpolarized the membrane by about 10 mV, but did not alter membrane resistance (Komori *et al.*, 1980a). Fresh drug was frequently added because of its instability in Krebs-Henseleit solution.

Measurement of compound action potentials in Remak's nerve branches

One nerve branch (about 0.1 mm in diameter and 10 mm in length) originating in the ganglia in the trunk of Remak's nerve was cut just before entering the rectal wall, isolated from the mesentery, and introduced into a monopolar suction electrode to record action potentials evoked by stimulating the trunk of Remak's nerve. Action potentials were amplified by an RC-coupled amplifier with a time constant of 300 ms, displayed on an oscilloscope and photographed. Action potentials represent firing in the postganglionic axons of the NANC nerves (Kanazawa *et al.*, 1980).

Assay of acetylcholine

Samples of the venous effluent were assayed against standard solutions of acetylcholine on the longitudinal muscle strip (5 cm long) obtained from the proximal to the terminal 10 cm of the guinea-pig ileum (Paton & Zar, 1968), sensitized with physostigmine,

5 ng ml^{-1}. To inhibit the release of endogenous acetylcholine from the assay muscle strip, morphine, 10 μg ml^{-1}, was added to the bathing medium. The assay bath contained 3 ml Krebs solution (composition (mM): NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose, 1.1), maintained at 30°C and bubbled with a 95% O_2 plus 5% CO_2. The active substance in the effluent was qualitatively confirmed to be acetylcholine, since the contraction of the assay muscle induced by the effluent was prevented by atropine, and was lost after boiling the effluent for 5 min in alkali.

Drugs

Drugs used were carbamylcholine chloride (Merck), acetylcholine chloride (Tokyokasei), morphine hydrochloride (Tanabe), physostigmine salicylate (Merck), atropine sulphate (Merck), (−)-isoprenaline sulphate (Merck), hyoscine-N-butylbromide (Tanabe) and timepidium bromide (Tanabe). The concentrations in the text and figures are expressed as the final salt concentrations in the bathing and perfusing solutions, unless otherwise stated.

Statistical significance of the results were evaluated using Student's *t* test for paired and unpaired data.

Results

Cholinergic nerve-mediated contractions and stimulation-evoked release of acetylcholine

Electrical stimulation of the trunk of Remak's nerve (RT stimulation) produces a non-adrenergic, non-cholinergic (NANC) contraction of the isolated chicken rectum (Bartlet & Hassan, 1971; Bartlet, 1974; Takewaki *et al.*, 1977). In the present experiments the NANC contraction reached a maximal tension within 7 s after the beginning of nerve stimulation and then decayed completely within 20 s. Above 5 Hz, the initial contraction was followed frequently by a late contraction which was slower in time course than the initial NANC contraction. Atropine (0.25 μg ml^{-1}) markedly reduced the late, slow contraction, but enhanced the NANC fast contraction (Figure 1). Electrical stimulation of the branches of Remak's nerve to the rectum (RB stimulation) also produced a contractile response. This response was small but substantially similar to that elicited by RT stimulation; the lowest frequency required to produce the mechanical response (1 Hz), was higher than that required using RT stimulation. Figure 2 shows mechanical responses to RB stimulation at three different frequencies before and after cumulative application of physostigmine (up to

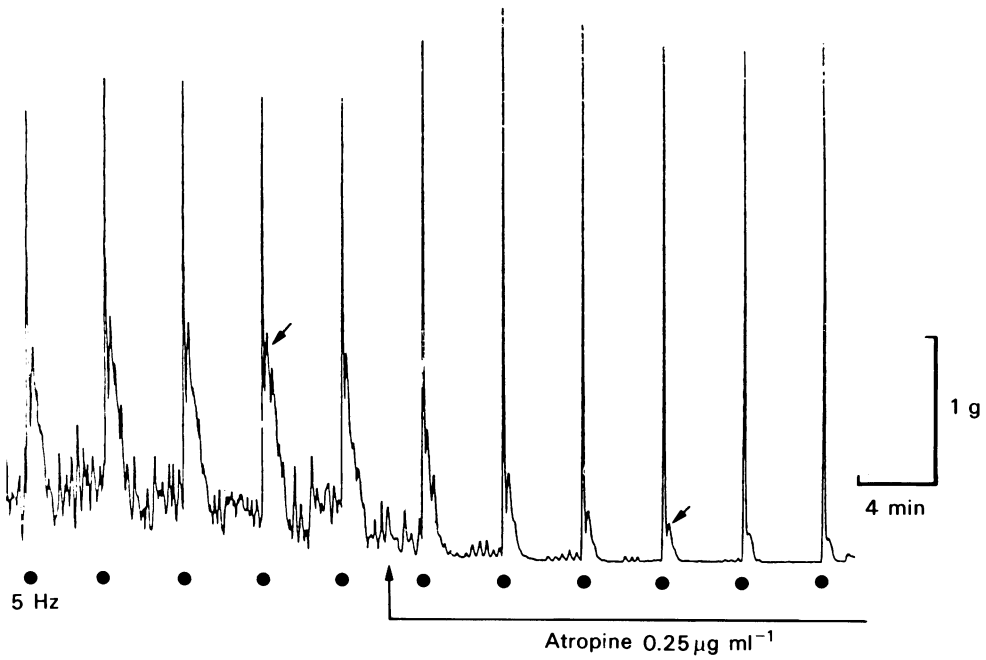

Figure 1 Effect of atropine (0.25 μg ml^{-1}, arrow ↑) on the contractile responses of the rectum to stimulation of Remak's nerve 10 pulses at 5 Hz, 0.2 ms (●). Atropine enhanced the fast but suppressed the slow component (↙) of the response.

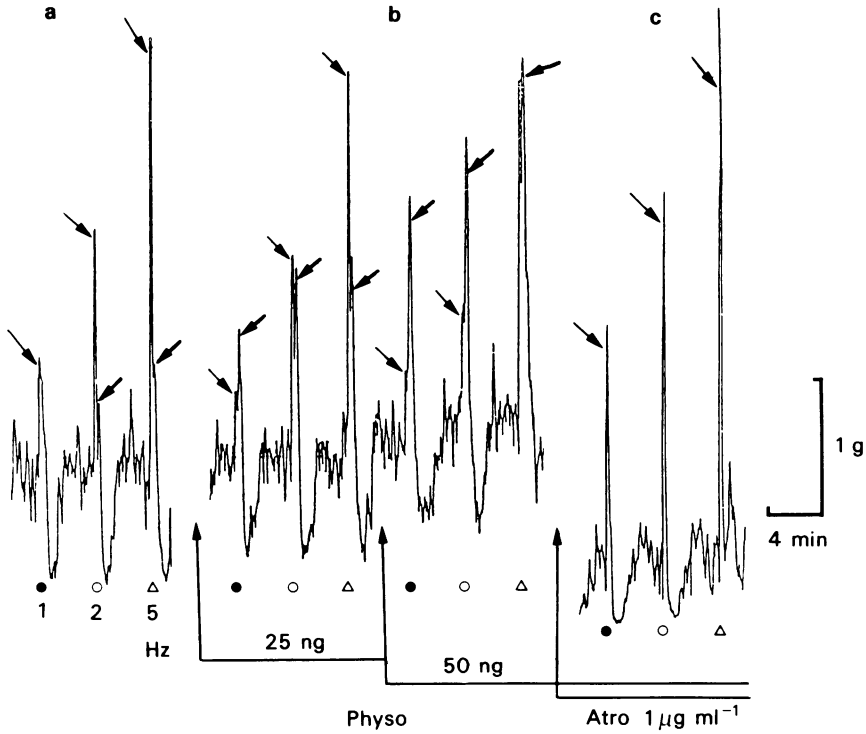

Figure 2 Effects of cumulatively added physostigmine (Physo 25 to 50 ng ml^{-1}) at first and second arrows, on the biphasic contractile responses of the chicken rectum to stimulation of Remak's nerve branches and of atropine (Atro 1 μg ml^{-1}) at third arrow on these effects. The nerve branches were stimulated with trains of 10 square-wave pulses of 0.2 ms duration at 1 (●), 2 (○) and 5 Hz (△). (a) Control responses; (b) 15 min after exposure to physostigmine, 25 ng ml^{-1}; (c) 10 min after additional exposure to atropine. Physostigmine suppressed the initial, fast component (thin arrow, ↘) but enhanced the late, component (thick arrow, ↙) of the response. Atropine reversed the inhibitory effect of physostigmine on the initial, fast component and also potentiated it, but abolished the late, component. The magnitude of the relaxation produced by the nerve stimulation varied with changes in tone, but was not primarily affected by these drugs.

50 ng ml^{-1}). At 2 and 5 Hz, a biphasic response was elicited, comprising an initial NANC contraction and a later and smaller contraction. The cholinesterase inhibitor decreased the initial NANC component, but increased the late component, of the biphasic contractile response. On addition of atropine (1 μg ml^{-1}) to the bathing medium containing physostigmine, the late component disappeared and the initial NANC component was enhanced. These results indicate that the late contraction is mediated

Table 1 The output of acetylcholine from the isolated, perfused chicken rectum

Stimulation		*Acetylcholine* (ng g^{-1} wet wt. tissue min^{-1})
Remak's nerve trunk	Control	0.6 ± 0.2 (n = 8)
	2 Hz	1.9 ± 0.7 (n = 4)
	10 Hz	5.9 ± 3.9 (n = 4)
Remak's nerve branch	Control	0.7 ± 0.1 (n = 8)
	2 Hz	2.2 ± 0.7 (n = 4)
	10 Hz	3.2 ± 0.5 (n = 4)

Values are mean ± s.e.mean output of acetylcholine per stimulation period (30 s at 2 Hz or 10 Hz).

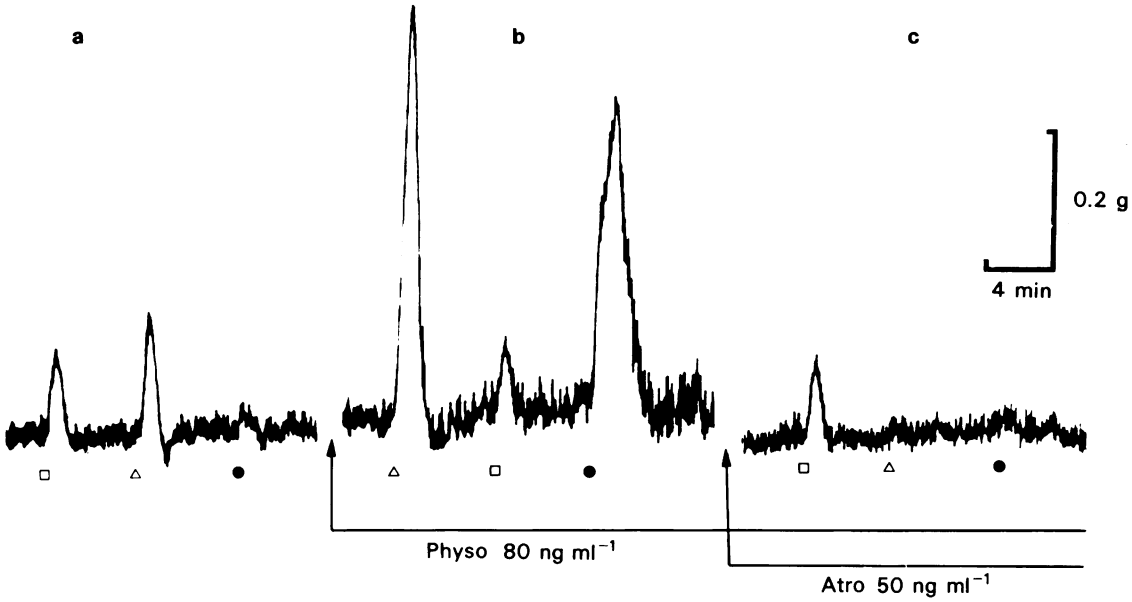

Figure 3 Effects of physostigmine (Physo 80 ng ml^{-1}) alone and with atropine (Atro 50 ng ml^{-1}) on the responses of the longitudinal smooth muscle of the guinea-pig ileum to the venous effluent from the isolated, perfused chicken rectum during stimulation of Remak's nerve, substance P (3 ng ml^{-1}) and acetylcholine (5 ng ml^{-1}). Physostigmine and atropine were added to the perfusing solution, whereas substance P and acetylcholine were added to the effluent. (a) Control responses to substance P (□), acetylcholine (△), and Remak's nerve stimulation (0.2 ms, 30 Hz for 30 s; ●); (b) 10 min after changing to a solution containing physostigmine; (c) 15 min after changing to a solution containing physostigmine and atropine. This indicates the existence of a cholinergic innervation via Remak's nerve to the chicken rectum.

by acetylcholine through activation of muscarinic receptors on the smooth muscle. Relaxation of the rectum occasionally occurred following contractile responses to RT or RB stimulation, the extent of which depended on the muscle tone.

In the isolated, perfused chicken rectum, the acetylcholine concentration in the venous effluent was increased following both RT and RB stimulation (see Table 1 and Figure 3). Figure 3 shows contractile responses of the longitudinal smooth muscle of the guinea-pig ileum to substance P, acetylcholine and venous effluent during RT stimulation. Substance P was used to detect any deterioration of the assay preparation. Contractile responses to acetylcholine and the effluent were both enhanced by physostigmine and abolished by atropine, but there was little change in the responses to substance P. This indicates the presence of a cholinergic innervation to the chicken rectum.

Effects of atropine and physostigmine on excitatory junction potentials (e.j.ps)

E.j.ps in response to both RT and field stimulation of the intramural nerves in the chicken isolated rectum are believed to be mediated by neurotransmitter released from NANC nerve terminals, while transmission is depressed by a muscarinic, cholinergic mechanism (Takewaki & Ohashi, 1977). To confirm this cholinergic function, the effects of atropine and physostigmine on e.j.ps in response to repetitive nerve stimulation were studied.

When either the trunk of Remak's nerve or the intramural nerves were stimulated with trains of submaximal stimuli at low frequencies (0.5 to 1.0 Hz), a series of e.j.ps was recorded from the same cell provided a good impalement was maintained. In response to RT stimulation, the e.j.p. amplitude declined following the second stimulus and then gradually increased to attain a steady level which was greater than the amplitude of the first e.j.p. (Komori & Ohashi, 1981; 1982). Prolonged recordings of successive e.j.ps could be made only when the depolarization remained sub-maximum. This was frequently achieved by decreasing the stimulus intensity.

To quantify the effect of atropine, the extrinsic nerve trunk or the intrinsic nerves were stimulated with trains of ten stimuli of a given intensity at 0.5 Hz and the resulting e.j.ps were recorded from 6 to 14 cells selected at random before and after application of atropine (0.25 μg ml^{-1}). The e.j.p. amplitude

Table 2 Effects of drugs on e.j.p. amplitude

Preparation	*Stimulation*	*No. of cells*	*E.j.p. amplitude* (mV) *Control*	*After drug*	*Drug*	*Mean ratio control/treated*
1	Intramural nerve	6	5.6 ± 0.3	8.5 ± 0.3*	Atropine	1.5
2	Intramural nerve	7	6.9 ± 0.2	9.8 ± 0.5*	Atropine	1.4
3	Intramural nerve	7	6.2 ± 0.3	10.1 ± 0.4*	Atropine	1.6
4	Remak's nerve branch	14	2.9 ± 0.1	3.8 ± 0.1*	Atropine	1.3
5	Remak's nerve trunk	7	6.6 ± 0.3	8.9 ± 0.3*	Atropine	1.4
6	Remak's nerve trunk	9	5.0 ± 0.1	8.4 ± 0.2*	Atropine	1.7
7	Remak's nerve trunk	13	5.7 ± 0.2	7.4 ± 0.2*	Buscopan	1.3
8	Remak's nerve trunk	11	8.6 ± 0.3	12.9 ± 0.4*	SA-504	1.5
9‡	Remak's nerve trunk	10	5.1 ± 0.1	2.6 ± 0.1*	Physostigmine	0.5
				8.3 ± 0.2*	Physostigmine + atropine	1.6

The first ten e.j.ps following nerve stimulation at 0.5 Hz were recorded from each of 6 or 14 cells before and after drug application and their amplitudes were averaged for each preparation. Values are mean ± s.e.mean.
*Values significantly different from control values ($P < 0.001$). Antimuscarinics were used at a concentration of 0.25 $\mu g\,ml^{-1}$.
‡Physostigmine and atropine were used at a concentration of 50 $ng\,ml^{-1}$ and 1 $\mu g\,ml^{-1}$, respectively. Buscopan = hyoscine-N-butylbromide. SA-504 = timepidium bromide.

before and after atropine was averaged for each preparation. Data pooled from 6 preparations are given in Table 2. The e.j.p. amplitude was found to be significantly increased by atropine in all 6 preparations ($P < 0.001$). The experiments also showed that atropine abolished the decrease in size of the e.j.ps during trains of stimuli. Similar results were obtained with two other antimuscarinic drugs, hyoscine-N-butylbromide and timepidium bromide. Therefore, the atropine-induced potentiation of the e.j.p. is attributable to its antimuscarinic action, i.e. to blockade of the muscarinic action of acetylcholine released from cholinergic nerve terminals.

The effect of physostigmine on the e.j.p. was examined in the same way as for the antimuscarinic drugs. Physostigmine decreased the e.j.p. amplitude significantly ($P < 0.001$) (Table 2). The effect of physostigmine was readily reversed by atropine.

Thus, a cholinergic function mediated by muscarinic receptors is present and depresses transmission from the NANC nerve to the smooth muscle presynaptically or postsynaptically.

Is the cholinergic induced inhibition presynaptic in origin?

(i) *Effects of atropine on the time course of the e.j.p.* Stimulation of the trunk of Remak's nerve involves ganglionic transmission (Kanazawa *et al.*, 1980). Inhibitory muscarinic receptors are present in sympathetic ganglia (Eccles & Libet, 1961; Libet, 1967; Kosterlitz *et al.*, 1968; Libet & Tosaka, 1969). To test whether muscarinic receptors in the ganglion are involved in the cholinergic induced inhibition, the effect of atropine on compound action potentials recorded from post-ganglionic axons of the NANC nerves evoked by RT stimulation was examined in six different preparations. Compound action potentials were elicited by trains of ten stimuli using similar values of pulse intensity and frequency to those required to elicit e.j.ps and atropine was added to the perfusion medium of the caudal mesenteric artery to give easy access of the drug to the ganglion cells (Kanazawa *et al.*, 1980). Atropine neither changed the pattern of discharge nor the shape of the com-

pound action potentials, as observed by Kikuchi *et al.* (1982). This ruled out the possibility that atropine affected the e.j.p. by interacting with muscarinic receptors at the ganglionic synapse.

The latency, time to peak and half-decay time of the fully formed e.j.p. were measured before and after application of atropine and compared with controls. Atropine caused no changes in these temporal parameters, except that the duration of the rising phase of the e.j.p. in response to RT stimulation was slightly increased. In addition, the exponential decay of the e.j.p. remained unchanged in the presence of atropine. The mean values of the time constant of 274 ± 11.3 ms (mean $\pm$ s.e.mean, $n = 7$) for the e.j.p. in response to RT stimulation, and 273.5 ± 6.5 ms ($n = 7$) for the e.j.p. in response to intramural nerve stimulation are not significantly different from their respective control values.

(ii) *Effects of atropine on the effects of carbamylcholine on membrane potential and membrane resistance* Acetylcholine depolarizes the membrane of intestinal smooth muscles by increasing membrane permeability to ions (Bolton, 1979). To see whether this was true of the smooth muscle of the chicken rectum, a more stable cholinester, carbamylcholine, was used.

On addition of carbamylcholine to the bathing medium, the membrane potential was decreased, and the depolarizing effect was reversible. Figure 4a shows the log dose-response curves. The degree of depolarization increased in a dose-dependent manner. During depolarization of the membrane, spike potentials with varied amplitudes (from less than 5 mV to 60 mV) discharged at frequencies of 1.0 to 4.0 s^{-1}. Depolarization of more than 30 mV led to blockade of spike discharges. The effect of carbamylcholine at any concentration tested was completely antagonized by atropine. In Figure 4b, electrotonic potentials in the presence and absence of carbamylcholine, 0.5 $\mu g\,ml^{-1}$, are shown. It can be seen that carbamylcholine reduced the time course and size of the electrotonic potential, in addition to the depolarization of the membrane, and these effects are readily reversed by atropine. When the concentration of carbamylcholine was increased to 1 $\mu g\,ml^{-1}$, no electrotonic potential could be recorded because of an intense increase in membrane conductance. Qualitatively similar results were obtained with acetylcholine in concentrations of 10 and 1 $\mu g\,ml^{-1}$.

If activation of muscarinic receptors postsynaptically on the smooth muscle had been involved in the cholinergic induced decrease in the NANC response,

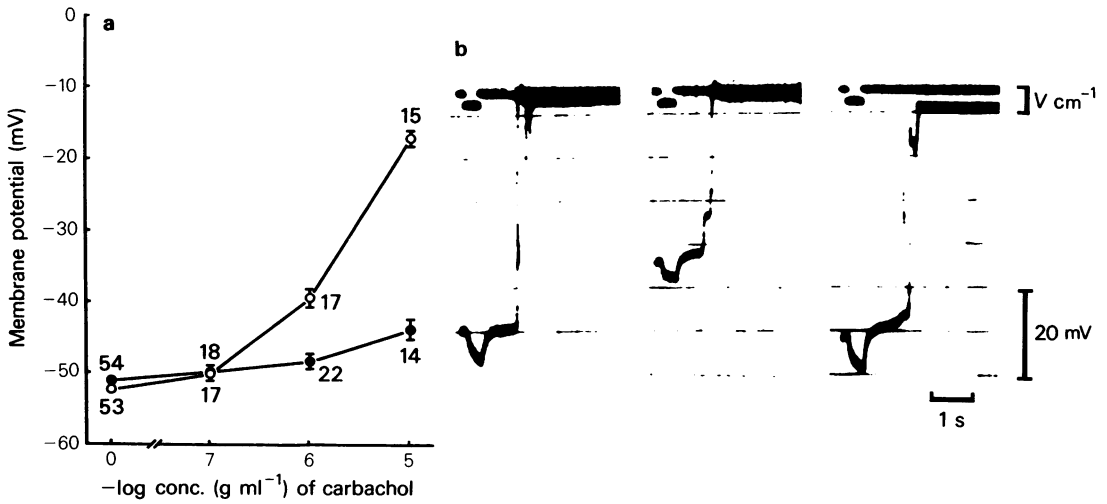

Figure 4 Effects of carbachol on the membrane potential and membrane resistance of the smooth muscle of the chicken rectum. (a) The dose-response (depolarization) relationship of carbachol before (○) and in the presence (●) of atropine, 0.25 $\mu g\,ml^{-1}$. Abscissa scale: log concentration of carbachol ($g\,ml^{-1}$). Ordinate scale: change in membrane potential (mV). Each point represents mean $\pm$ s.e.mean. The number of results is indicated by the figures near each point. (b) Electrotonic potentials recorded from the longitudinal smooth muscle of the chicken rectum in response to hyperpolarizing current pulses of 800 ms duration. Left, control; middle, 3 min after carbachol, 0.5 $\mu g\,ml^{-1}$; right, 5 min after atropine, 0.25 $\mu g\,ml^{-1}$. Upper trace: applied field strength ($V\,cm^{-1}$); lower trace; change in membrane potential. Activation of muscarinic receptors on the smooth muscle decreases both membrane potential and membrane resistance.

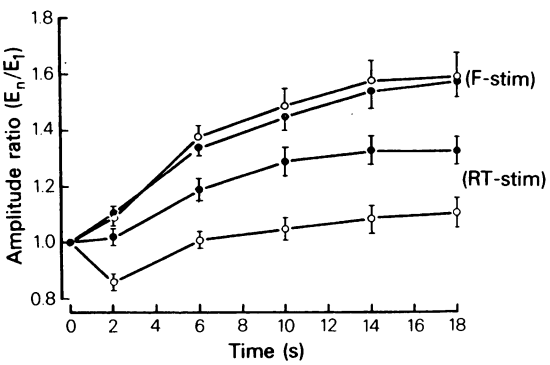

Figure 5 Effect of atropine on the size of e.j.ps in the chicken rectum. The amplitude (ordinate), compared to the first e.j.p. (E1), of the second (E2), fourth (E4), sixth (E6), eighth (E8) and tenth (E10) e.j.p. in a train elicited by repetitive stimulation (0.5 Hz) of Remak's nerve (RT-stim) and of intramural nerves (F-stim) plotted against the time after commencing stimulation (abscissa). (○), Control; (●), in the presence of atropine. 0.25 $\mu g\,ml^{-1}$. Each point represents a mean value of 5–9 measurements ± s.e.mean (vertical bar). See text.

changes in membrane potential and membrane resistance should have preceded and should have been parallel to the atropine-induced potentiation of the e.j.p.

(iii) *Effects of atropine on membrane potential and membrane resistance* The mean resting membrane potential in 41 cells of 6 preparations was -54.7 ± 0.7 mV (mean ± s.e.mean) in normal medium and -54.8 ± 0.7 mV after 1 $\mu g\,ml^{-1}$ of atropine, a concentration which was high enough to potentiate the e.j.p. Atropine-induced potentiation of e.j.ps during repetitive nerve stimulation at 0.5 Hz occurred with no change in the basal membrane potential. Electrotonic potentials and the slope of the curve relating the steady-state amplitude of electrotonic potentials to applied voltage gradients between the stimulating plates remained unchanged in the presence of atropine, indicating no apparent change in membrane resistance.

The threshold for generating an action potential was determined by passing depolarizing current in the absence and presence of atropine. The data obtained from 11 cells in two preparations gave a mean threshold of -42.3 ± 0.8 mV (mean ± s.e.mean) in the presence of atropine, which did not differ significantly from the mean value of -41.8 ± 0.9 mV in normal medium. The threshold was also measured by the level of depolarization of the e.j.p. required to initiate an action potential. Atropine did not change this threshold.

Thus, there was no evidence to suggest that the cholinergic inhibition of the NANC response is caused by activation of muscarinic receptors located postsynaptically on the smooth muscle.

Some characteristics of the cholinergic inhibitory function

(i) *Effects of atropine on facilitation of the e.j.p.* Figure 5 shows the amplitude of successive e.j.ps elicited by ten stimuli at 0.5 Hz compared to the first e.j.p. and plotted against time after the beginning of stimula-

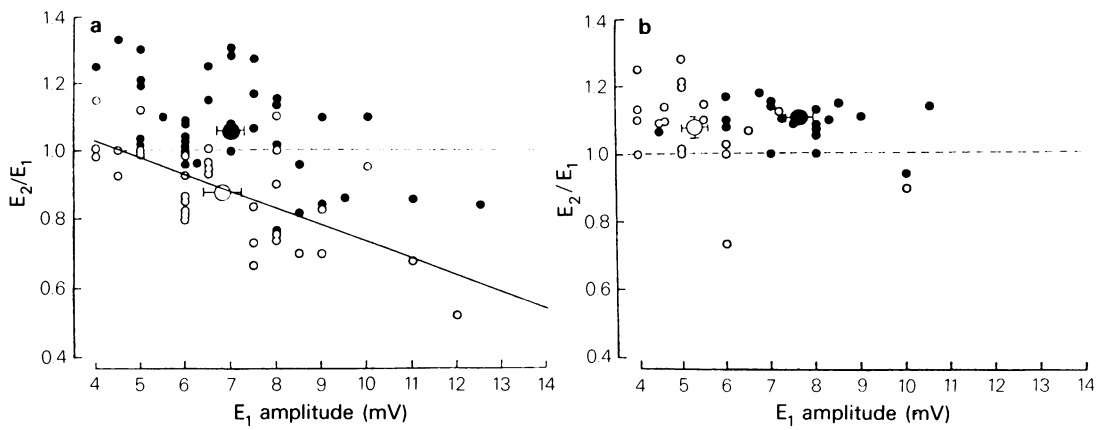

Figure 6 Amplitude ratio of the second e.j.p. (E_2) to the first e.j.p. (E_1) plotted against the amplitude of E_1 in the presence (●) and absence (○) of atropine, 0.25 $\mu g\,ml^{-1}$. (a) Remak's nerve stimulation; (b) intramural nerve stimulation. Larger closed and open circles represent mean values ± s.e.means (horizontal and vertical bars) in the presence and absence of atropine (± s.e.mean within the circles is omitted). The difference between the means is statistically significant only in the vertical direction in (a) and only in the horizontal direction in (b). The calculated regression line for open circles in (a) is given as $E_2/E_1 = 1.23 - 0.05\,E_1$ ($r = -0.67$, $n = 33$, $P < 0.001$). See text.

tion (in stimulus order). With intramural nerve stimulation the curves before and after atropine are close together, whereas with RT stimulation, the curve after atropine deviated from that before. However, atropine potentiated the amplitude of the first e.j.p. in response to intramural nerve stimulation, but not appreciably that to RT stimulation. This may account for the deviation between the curves before and after atropine in the case of RT stimulation. To elucidate this possibility, the effect of atropine on the first and second e.j.ps was examined by plotting the amplitude ratio of the second e.j.p. to the first e.j.p. (E_2/E_1) against the first e.j.p. amplitude (E_1) in the absence and presence of atropine (Figure 6). It was found that there is a weak negative correlation between the values of E_2/E_1 and E_1 ($r = -0.67$, $n = 33$, the regression line, $E_2/E_1 = 1.23 - 0.05\,E_1$, $P < 0.001$) in the case of RT stimulation. Atropine produced a statistically significant shift of the ratio of E_2/E_1 upwards from 0.88 ± 0.02 (mean $\pm$ s.e.mean, $n = 33$) to 1.06 ± 0.02 ($n = 37$) ($P < 0.001$) in keeping with the correlation, but it did not significantly affect the first e.j.p. amplitude (6.8 ± 0.4 mV in normal medium and 7.0 ± 0.3 mV in the presence of atropine). On the contrary, there is no correlation between E_2/E_1 and E_1 in the case of intramural nerve stimulation, and atropine increased significantly the first e.j.p. amplitude from 5.3 ± 0.3 mV (mean $\pm$ s.e.mean, $n = 29$) to 7.6 ± 0.3 mV ($n = 20$) ($P < 0.001$), but it did not change the ratio of E_2/E_1 (1.07 ± 0.03 in normal medium and 1.10 ± 0.01 in the presence of atropine). Taking this difference between intramural nerve stimulation and RT stimulation into account, if the amplitude of the third and following e.j.ps were compared to the second e.j.p. in Figure 5, the curve after atropine coincided nearly with that before atropine in both cases of nerve stimulation. It is, therefore, suggested that atropine had no effect on the facilitation of e.j.ps. Figure 7 shows the results of an experiment where RT stimulation with paired stimuli at 1 s intervals was preceded by a single intramural nerve stimulus. The first e.j.p. in response to RT stimulation was consistently reduced and invariably became atropine-sensitive. The most plausible explanation for this is that intramural nerve stimulation caused activation of the cholinergic inhibitory process, leading to a decrease in the size of the first e.j.p.

These findings corroborate the view that there is little or no involvement of the cholinergic induced inhibition in the response to the first stimulus of each train for RT stimulation and that the depression is characterized by slow onset after RT stimulation.

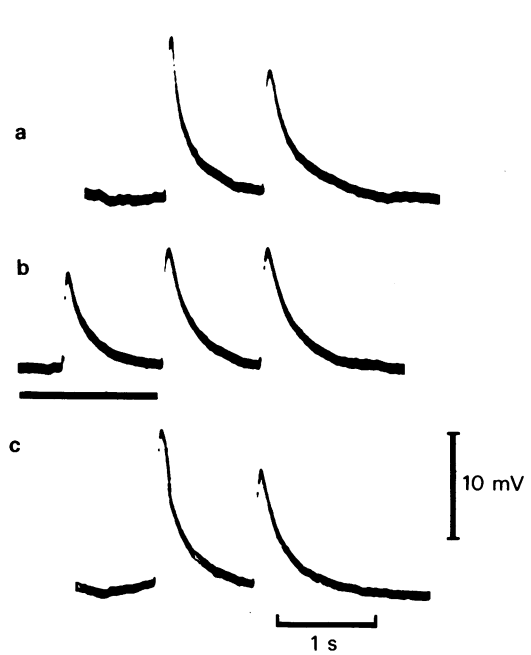

Figure 7 Effect of preceding intramural nerve stimulation on the amplitude of the first e.j.p. elicited by Remak's nerve stimulation with paired pulses of 1 s interval. E.j.ps evoked by paired square-wave pulses of 0.2 ms duration applied to Remak's nerve, (a) alone; (b) 1000 ms after a single intramural nerve stimulation with square-wave pulse of 0.2 ms duration (thick horizontal bar); (c) again without preceding intramural nerve stimulation. Records in (a–c) were made in the same cell at about 2 min intervals. This suggests that intramural nerve stimulation activates a cholinergic inhibitory process, leading a diminution of the first e.j.p.

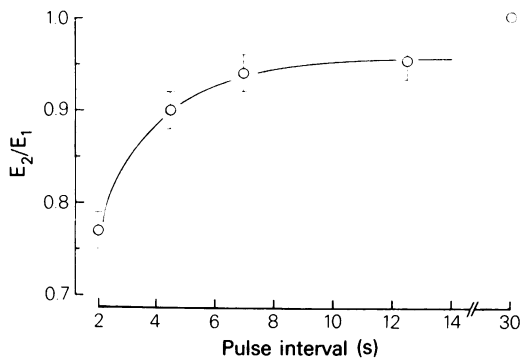

Figure 8 Time course of recovery from the cholinergic depression of e.j.p. E.j.ps were elicited by Remak's nerve stimulation with paired square-wave pulses of 0.2 ms duration at varied intervals and recorded from the same cell. Amplitude ratios of the second e.j.p. (E_2) to the first e.j.p. (E_1) were plotted against stimulus interval. The line was drawn to fit the points by eye. The graph shows that recovery from the depression proceeds along an almost exponential time course with a time constant of 4 s.

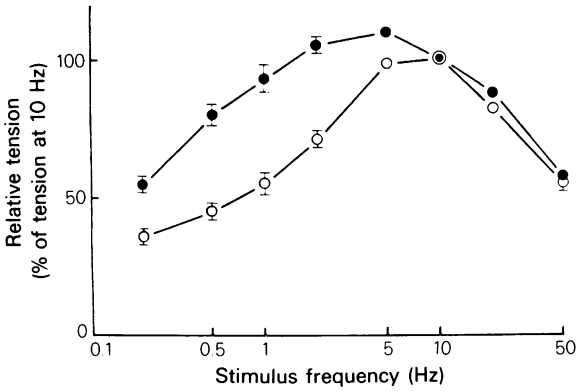

Figure 9 Effect of atropine, 0.25 $\mu g\,ml^{-1}$ on the relationship between the contractile responses of the rectum and the frequency of stimulation of Remak's nerve. Abscissa scale: log stimulus frequency; ordinate scale: % change in the magnitude of the contractile responses (the magnitude of the contractile response to stimulation at 10 Hz was taken as 100%). Trains of 10 square-wave pulses of 0.2 ms duration were used for the nerve stimulation. Plotted results were obtained from 6 experiments each in the presence (●) and absence (○) of atropine. Vertical bars show s.e.mean. This suggests that the muscarinic inhibitory process functions more efficiently at low stimulus frequencies.

(ii) *Recovery from the cholinergic induced inhibition of e.j.ps* The kinetics of the recovery of e.j.ps from inhibition are shown in Figure 8 in which the ratios of E_2/E_1 are plotted against the stimulus interval of paired stimuli. The inhibition was found to decrease initially with an almost exponential time course with a time constant of 4 s, but took about 30 s to be reversed completely.

(iii) *Dependence on stimulus frequency* Figure 9 shows contractile responses of the rectum to a train of ten pulses of RT stimulation at frequencies of 0.2, 0.5, 1, 2, 5, 10, 20 and 50 Hz, corresponding to train lengths from 0.2 to 50 s. Atropine (0.25 $\mu g\,ml^{-1}$) enhanced the responses at frequencies up to 5 Hz, but not at 10, 20, or 50 Hz. The drug also increased the magnitude of contractile responses to paired pulses at 50 and 500 ms intervals, and the extent of the increase was significantly greater for the response to paired pulses of 500 ms interval ($P < 0.01$).

These findings suggest that the muscarinic inhibitory process functions more efficiently at low stimulus frequencies.

Discussion

The present results are in keeping with the suggestion (Ohashi *et al.*, 1977; Takewaki & Ohashi, 1977) that the functional output of the non-adrenergic, non-cholinergic (NANC) excitatory neurones in the chicken rectum is regulated by cholinergic neurones. Stimulation of the cholinergic nerves may be responsible for the muscarinic inhibition of the NANC excitatory junction potentials (e.j.ps). Thus stimulation of the trunk of Remak's nerve (RT stimulation) or its branches caused on atropine-sensitive, physostigmine-sensitive contraction of the chicken rectum and an increase in the outflow of acetylcholine in the venous effluent from the isolated, perfused chicken rectum.

The cholinergic inhibition is not mediated at the ganglionic synapse; atropine had no effect on compound action potentials evoked by RT stimulation (preganglionic nerve stimulation) in the postganglionic axons of the NANC nerves (Kikuchi *et al.*, 1982). Nor is it a physiological antagonism by acetylcholine acting on the smooth muscle membrane, since exogenously applied acetylcholine or carbamylcholine produced a shift in the membrane potential in the same direction as the e.j.p. (depolarization). Atropine did not increase the sensitivity of the smooth muscle membrane to the NANC nerve transmitter since the drug failed to change the amplitude of the first e.j.p. in response to trains of RT stimulation. It appears, then that either acetylcholine acts on muscarinic receptors on the smooth muscle to increase membrane conductance (reduce membrane resistance), which for a given junctional current, would lead to a smaller potential change at the membrane, or that acetylcholine acts on muscarinic receptors on the NANC nerve terminals to inhibit output of the unknown transmitter.

Acetylcholine and carbamylcholine induced atropine sensitive depolarizations and increased membrane conductance. However, atropine increased the e.j.p. amplitude in concentrations which did not produce changes in the membrane potential, the steady-state size and time course of electrotonic potentials, and slope of the V–I curve. This serves to rule out the possibility that acetylcholine released from cholinergic nerve terminals acts on muscarinic receptors on the smooth muscle. Additional support is the failure of atropine to alter the exponential decay of the e.j.p. which is determined by the time constant of the muscle membrane (Komori & Ohashi, 1982). The cholinergic inhibition is unlikely to be due to a decrease in the number of junctions involved, since atropine potentiated the e.j.p. evoked at a given stimulus intensity and did not produce any change in the compound action potential recorded from the postganglionic NANC axons. It appears, then, that the cholinergic inhibition results from activation of muscarinic receptors on the NANC nerve terminals which modulate the release of the unknown transmitter. A muscarinic inhibition of transmitter release has

also been demonstrated in both peripheral adrenergic (see Introduction) and cholinergic neurones (Kilbinger & Wagner, 1979). Cholinergic inhibition of NANC transmission in the present investigation was prominent at low stimulus frequencies, and in this respect, was analogous to that in both adrenergic and cholinergic nerves.

So far, the direct action of acetylcholine on the NANC nerve terminals has been considered. However, acetylcholine may stimulate muscarinic receptors on neighbouring non-neuronal structures and release a substance which could modulate the release of the unknown excitatory transmitter, as suggested in the negative feedback process involved in the release of noradrenaline from adrenergic nerve fibres (Hedqvist, 1976). The finding that the first e.j.p. following repetitive stimulation of the trunk of Remak's nerve was rarely affected by atropine, but was reduced by prior intramural nerve stimulation and was atropine-sensitive, suggests that the cholinergic inhibition is mediated by acetylcholine released by nerve stimulation rather than spontaneously.

The slow onset of the cholinergic inhibition after stimulation of the trunk of Remak's nerve cannot be explained simply by the delay of 100 ms or more (Purves, 1974; Bolton, 1976) of activation of muscarinic receptors by acetylcholine, since involvement of cholinergic inhibition was shown in the first e.j.p. following intramural nerve stimulation. This might be due to a delay in conduction of the nerve impulse somewhere along the cholinergic pathway.

The present results suggest that the muscarinic receptors on the NANC nerve terminals are sensitive to concentrations of acetylcholine below the threshold for contraction or depolarization of the smooth muscle membrane. An alternative possibility is that acetylcholine release occurs in the vicinity of NANC nerve terminals so that the acetylcholine cannot readily diffuse to muscarinic receptors on the smooth muscle.

The NANC contractile response produced by stimulation of the trunk of Remak's nerve was enhanced by atropine only when the nerve was stimulated at low frequencies. However, the acetylcholine output was found to be larger with 10 Hz than with 2 Hz. A possible interpretation for these observations is that if the cholinergic inhibitory function operates during nerve stimulation at high frequencies it is masked by the facilitation of e.j.ps which is unchanged in the presence of atropine and becomes stronger as the pulse interval is shortened.

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