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Analysis of contributions of acetylcholine and tachykinins to neuro-neuronal transmission in motility reflexes in the guinea-pig ileum

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1 The roles of acetylcholine (ACh) and tachykinins in neuro-neuronal transmission during ascending excitatory and descending inhibitory reflexes were studied by recording intracellular reflex responses of the circular muscle to physiological stimuli. Experiments were carried out in opened segments of guinea-pig ileum in an organ bath that was partitioned so that three regions could be independently exposed to drugs.

2 Ascending excitatory reflexes evoked by either distension from the serosal side or compression of the mucosa were depressed by 55% and 85%, respectively, in the presence of hexamethonium (200 μM) and by 30% and 45%, respectively, by a desensitizing concentration of the selective NK_3 receptor agonist, senktide (1 μM), in the chamber in which reflexes were initiated. Together, hexamethonium and senktide abolished reponses to compression. A residual response to distension persisted. This was abolished by hyoscine (1 μM).

3 Hexamethonium (200 μM) abolished ascending reflexes when applied to the region between the stimulus and the recording sites, or to the recording chamber.

4 Descending reflex responses were reduced by 35% by synaptic blockade in the stimulus chamber with physiological saline containing 0.1 mM Ca^{2+} plus 10 mM Mg^{2+}. Senktide (1 μM) in the stimulus chamber reduced distension reflexes to the same extent as synaptic blockade, whereas hexamethonium (200 μM) and hyoscine (1 μM) depressed responses by less than 20%. Responses to compression were reduced by 40% by senktide alone, while senktide and hexamethonium together reduced responses by 60%, an effect similar to synaptic blockade. Under these conditions, hyoscine in the stimulus chamber restored reflexes evoked by distension, but did not alter those evoked by mucosal compression.

5 Total synaptic blockade in the intermediate chamber, between stimulus and recording sites, reduced descending reflex responses by more than 90%. In contrast, hexamethonium (200 μM) had no effect and hyoscine (1 μM) reduced only the responses to distension (by 30%). Senktide (1 μM) depressed responses to both stimuli by approximately 80%.

6 Application of hexamethonium (200 μM) to the recording chamber depressed descending reflex responses to distension applied in the near stimulation chamber by 15%, but had no effect on responses to compression in the near chamber or to either stimulus applied in the far chamber.

7 Descending reflexes evoked by near chamber stimuli were unaffected by hyoscine (1 μM) or senktide (1 μM) applied to the recording chamber; hyoscine enhanced reflexes evoked by compression in the far chamber by 50%.

8 For the ascending excitatory reflex pathway, it is concluded that transmission from sensory neurones is mediated by ACh acting via both nicotinic and muscarinic receptors, and by tachykinins acting at NK_3 receptors. Transmission from ascending interneurones appears to be predominantly via nicotinic receptors. The descending inhibitory pathways are more complex, and while transmission from sensory neurones involves nicotinic, muscarinic and NK_3 receptor-dependent components, transmission from descending interneurones to inhibitory motor neurones is neither cholinergic nor due to tachykinins acting via NK_3 receptors.

Keywords: Enteric nervous system; peristalsis; motility; enteric reflexes; tachykinins; NK_3 receptors; ileum; substance P; neurokinin

Introduction

The passage of food along the gastrointestinal tract is facilitated by two reflexes, the ascending excitatory reflex and the descending inhibitory reflex, which modulate contractions of intestinal circular muscle (Bayliss & Starling, 1899; Hukuhara *et al.*, 1958; Costa & Furness, 1976). These polarized reflexes can be activated by distension of the gut wall or by mechanical or chemical stimulation of the mucosa (Bayliss & Starling, 1899; Hukuhara *et al.*, 1958; Smith & Furness, 1988; Smith *et al.*, 1990; 1991; Grider & Jin, 1994). In the guinea-pig small intestine, reflexes evoked by mechanical stimuli persist in preparations in which the extrinsic nerves have been severed and allowed to degenerate (Furness *et al.*, 1995), indicating that the sensory neurones, interneurones and motor neurones responsible for these reflexes are intrinsic to the enteric nervous system. Indeed, most of the neuro-neuronal connections are probably contained within the myenteric plexus (Bornstein, 1994).

While the basic elements of motility reflex circuits have been characterized electrophysiologically and anatomically (Costa *et al.*, 1992; Bornstein, 1994; Furness *et al.*, 1994), the pharmacology of transmission within these pathways remains uncertain. Nicotinic receptor antagonists abolish propulsion of

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intestinal content in intact preparations, indicating that acetylcholine (ACh) acting at nicotinic synapses is essential for peristalsis (Crema *et al.*, 1970; Costa & Furness, 1976; Yagasaki *et al.*, 1979; Tonini *et al.*, 1981). However, detailed analyses of the components of the reflexes underlying peristalsis suggest that ACh is not the only transmitter at ganglionic synapses. For example, descending inhibitory reflexes evoked by distension are unaffected by hexamethonium when the distance between the stimulus and recording site is less than 20 mm, and only slightly depressed at distances up to 35 mm (Costa *et al.*, 1986; Smith & Furness, 1988; Smith *et al.*, 1990). Moreover, studies in which a divided organ bath was used to allow selective application of drugs to distinct sites along reflex pathways have revealed that noncholinergic transmission may be important for the initiation and conduction of both ascending and descending reflexes (Tonini & Costa, 1990; Yuan *et al.*, 1994; Grider, 1994).

A plethora of putative neurotransmitters have been identified in nerve terminals in myenteric ganglia (Furness *et al.*, 1988; Ekblad *et al.*, 1991) and many of these have been demonstrated to mimic synaptic responses in myenteric neurones (Wood & Mayer, 1978; Palmer *et al.*, 1987; Wood, 1987; Galligan & Bertrand, 1994). Amongst the possible transmitters, tachykinins are found in sensory neurones intrinsic to both myenteric and submucous ganglia (Steele *et al.*, 1991; Kirchgessner *et al.*, 1992) and there is evidence that tachykinins at least partly mediate slow excitatory synaptic potentials (e.p.s.ps) in myenteric neurones (Katayama & North, 1978; Morita *et al.*, 1980; Johnson *et al.*, 1981). These effects are probably mediated via NK_3 receptors, as these have been identified on myenteric neurones, and agonists at this receptor evoke release of ACh and substance P (SP) from the myenteric plexus and can produce neurogenic contractions and relaxations of intestinal circular muscle (Laufer *et al.*, 1985; Guard & Watson, 1987; Guard *et al.*, 1990; Yau *et al.*, 1992; Maggi *et al.*, 1993). However, physiological roles for tachykinins at enteric neuro-neuronal synapses have not yet been identified.

The present study sought to investigate the relative roles of tachykinins and ACh in neuro-neuronal transmission during both ascending excitatory and descending inhibitory motility reflexes evoked by distension or by mechanical deformation of the mucosal villi. A divided organ bath preparation was used, so that drugs could be applied selectively to affect transmission at different sites along the reflex pathways (Yuan *et al.*, 1994; 1995).

Methods

Tissue preparation

Guinea-pigs of either sex and weighing between 200 and 350 g were stunned with a blow to the head and killed by severing the carotid arteries and spinal cord. A 5–10 cm segment of ileum was taken approximately 10–25 cm from the ileocaecal junction and flushed clean with physiological saline containing (in mM): NaCl 118, $NaHCO_3$ 25, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaH_2PO_4 1.0 and glucose 11, bubbled with 95% O_2, 5% CO_2. The segment was then opened along the mesenteric border and pinned flat, mucosa uppermost, in an organ bath.

In order to analyse transmission between the different neural components of peristaltic reflex circuits, plastic partitions, 1 mm thick, were placed across the segment to divide the bath into three chambers (Figure 1; Yuan *et al.*, 1994). These were sealed with silicone grease and their integrity tested by the application of phenol red dye (0.01%) into each chamber separately. No leakage of dye was observed.

In the chamber at one end of the segment, the tissue was rolled over to expose the serosa and allow microelectrode impalements of the circular muscle to be made; this chamber was designated the recording chamber (Figure 1). Reflexes were evoked by stimulating the intestine in either the adjacent (near stimulation) chamber or the chamber at the other end of the preparation (far stimulation chamber). For studies of the descending reflex, the segment was set up with the anal end in the recording chamber, while in ascending reflex experiments, the orientation of the preparation was reserved.

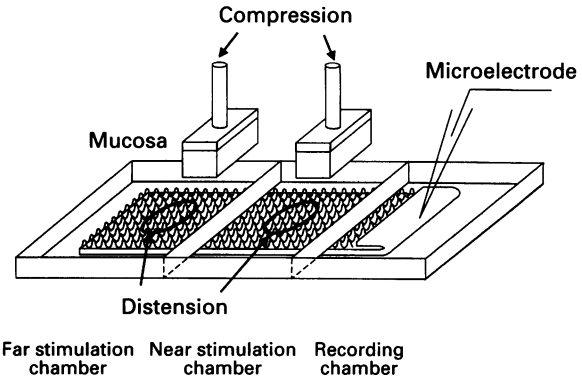

Figure 1 Diagram of the divided organ bath used for recording motility reflexes in segments of isolated ileum. Distension or mucosal compression stimuli were applied in the near or far stimulation chambers, and the reflex responses of the circular muscle were monitored in the recording chamber. Each chamber was independently perfused and partitions (15 mm apart) prevented fluid mixing between chambers.

Each chamber was separately perfused with physiological saline containing nicardipine (3 μM), which blocks L-type calcium channels in smooth muscle, and reduces contractions without affecting the junction potential responses of the circular muscle or preventing initiation of reflexes (Smith & Furness, 1988). This allows the electrical responses of the muscle to be recorded by an intracellular microelectrode in the absence of muscle movement that may dislodge the electrode. The perfusion solutions were bubbled with 95% O_2, 5% CO_2 maintained at 37°C and continuously perfused into each chamber by a gravity feed system at a rate of 0.7 ml min^{-1}.

Intracellular recording

After an equilibration period of 1 h, a glass microeletrode (filled with 2 M KCl, resistance 25–60 MΩ) was advanced from the serosal side of the tissue through the longitudinal muscle and myenteric plexus to impale a circular muscle cell. The potential difference across the cell membrane was amplified by a high impedance electrometer (WPI KS-700), displayed on an oscilloscope (Gould 240) and recorded with a computerised polygraph system (Axotape 1.2.01; Axon Instruments Inc.). Impalements were considered adequate if the resting membrane potential of the cell was more negative than −40 mV and stable for at least 30 s. In many preparations, slow wave oscillations of the membrane potential were observed.

Experimental protocol

Stimuli were applied to the segment in the near or far stimulation chambers, either by distending the gut wall, or by distorting the mucosa with a compressor block. Rubber balloons, held in metal supports (diameter 5 mm) were embedded in the base of the bath in both stimulation chambers. The distances from the centres of the balloons in the near and far stimulation chambers to the recording sites were approximately 12 and 27 mm respectively. Distension was applied by inflating the balloons with an injection of 0.15 ml distilled water to stretch the overlying segment of intestine. This distension volume evokes submaximal reflex responses (Furness *et al.*, 1995). The time courses of the distension stimuli were monitored by sliding potentiometers attached to the balloon inflation syringes.

The compressor consisted of a rectangular base made from Sylgard polymer (bottom surface 5 × 11 mm and flat) and a

sponge rubber supporting block attached to a vertical lever arm. Compression was applied over the centre of the distension balloons by advancing the block manually with a calibrated rackwork bosshead. Depth of compression of the mucosal surface was fixed at 1 mm with a locking screw. The entire device was mounted on a mobile stage to enable the compression block to be positioned over the bath and easily moved between stimulus sites.

Four stimuli were used: near chamber distension, near chamber compression, far chamber distension and far chamber compression. Each stimulus was applied to the segment for approximately 6 s, separated by intervals of at least 2 min. Previous studies have shown that such a regime does not produce a rundown in reflex responses to repeated stimuli (Yuan *et al.*, 1991).

The change in membrane potential of the circular muscle cells evoked by each stimulus was recorded, and the peak amplitude, latency, time to peak and half-peak duration of the responses were calculated. As the time of onset of a compression stimulus could not be accurately determined, latencies were calculated for responses to distension only. Initially, control responses were established to each stimulus. Drugs were then added to the solution perfusing the appropriate chamber and allowed to equilibrate with the tissue for 15 min, after which the stimuli were repeated and the responses recorded. The drug solution was then drained from the chamber and perfusion with control solution was recommenced. Recovery responses were recorded after a further 15 min.

The use of a divided organ bath allowed investigation of transmission at different sites along the reflex pathways. Transmission in the region of stimulus initiation was examined by testing the effects of drugs added to the near stimulation chamber on reflex responses evoked by stimuli applied in this chamber. Transmission between the stimulus and recording regions was examined by measuring reflex responses to stimuli applied in the far stimulation chamber in the presence of drugs added to the intermediate (near stimulation) chamber. Similarly, transmission near the recording site was examined by testing the effects of drugs added to the recording chamber.

Contractile studies

To test the effectiveness of drugs used in the reflex experiments, 1 cm segments of guinea-pig ileum were mounted longitudinally in organ baths (volume 2.5 ml) and bathed with physiological saline gassed with 95% O_2, 5% CO_2 at 37°C. Each segment was connected by a silk tie to an isotonic force transducer (resting tension 1 g) and contractile responses were monitored by a computerised polygraph (DATAQ). Non-cumulative concentration-effect curves were established by adding increasing concentrations of agonists to the bath for 2.5 min, at intervals of 30 min, either alone, or in the presence of antagonists, added 15 min prior to the agonists.

Analysis of results

For reflex experiments, the mean and standard error (s.e.mean) of the response parameters over a number of animals were calculated for each stimulus and treatment condition. In the text, n refers to the number of animals used. Means were compared between control and drug treatments by Student's paired t statistic. Comparisons of effects between drug treatments were made using t tests for unpaired data, or two-way analysis of variance, as appropriate. In contraction experiments, responses to the agonists were expressed as percentages of the response evoked by carbachol (10 mM). Concentration-effect curves in the presence and absence of senktide were compared by two-way analysis of variance. In all statistical comparisons performed, differences were considered significant if $P<0.05$.

Drugs

The following drugs were used: hyoscine hydrobromide, hexamethonium maleate, nicardipine, dimethylphenylpiperazinium iodide (DMPP; Sigma, U.S.A.); suramin (Bayer, Australia); dihydro-β-erythroidine (Research Biochemicals Incorporated, U.S.A.); carbachol (BDH Chemicals Inc. U.S.A.); and senktide (Auspep, Australia). Spantide was synthesized by Dr Roger Murphy of the Department of Pharmacology, University of Melbourne. All drugs were dissolved in physiological saline prior to each experiment except for senktide which was dissolved in 0.1 M acetic acid, and nicardipine which was dissolved in 100% ethanol and stored at 4°C.

Results

Control responses

Circular muscle cells had resting membrane potentials of 50 ± 1 mV ($n = 128$). Compression and distension stimuli consistently evoked compound excitatory junction potentials (e.j.ps) in the circular muscle oral, and compound inhibitory junction potentials (i.j.ps) anal to the stimulus. The shapes of these responses varied between preparations, but were both commonly biphasic, comprising an initial fast component followed by a slower response. In both pathways, reflex responses were reproducible over the time period required to investigate drug effects.

Measurement of intervals between distension stimuli and responses in the circular muscle enabled the estimation of apparent conduction velocity in each pathway. The initiation latency for ascending reflexes (220 ± 15 ms) was significantly greater than the initiation latency for descending reflexes (90 ± 6 ms) evoked by distension ($P<0.001$), but the conduction velocities (0.10 and 0.12 ms^{-1}, respectively) did not differ between pathways.

Ascending pathway

Effect of drugs applied at the site of stimulus Transmission between neurones in the region of reflex initiation was examined by testing the effects of drugs added to the near stimulation chamber on reflex responses evoked by stimuli applied in this chamber (Figure 2). Hexamethonium (200 μM), a nicotinic receptor antagonist, reduced the amplitude of ascending reflex e.j.ps evoked by distension and compression by approximately 55% and 85%, respectively ($P<0.01$ for distension; $P<0.001$ for compression, compared with controls). Responses returned to control levels 15 min after washout of the drug. When senktide (1 μM) was added to the near stimulation chamber to desensitize NK_3 receptors, the amplitudes of ascending e.j.ps evoked from within this chamber were also significantly decreased, by 30% and 45% respectively ($P<0.05$ for distension; $P<0.01$ for compression). The latencies and temporal properties of e.j.ps were not altered by hexamethonium or senktide.

Simultaneous application of hexamethonium and senktide abolished e.j.ps evoked by mucosal compression. A small response to distension persisted; this was removed in 7 out of 8 preparations by the further addition of the muscarinic receptor antagonist, hyoscine (1 μM), to the near chamber perfusate. The presence of synapses in the near stimulation chamber was confirmed by application of physiological saline containing 0.1 mM Ca^{2+} plus 10 mM Mg^{2+} (see methods) to block synaptic transmission. When this modified saline was added to the near stimulation chamber, reflex responses to both distension and compression in this chamber were abolished.

Effect of drugs applied at sites along the ascending pathway Ascending excitatory reflexes evoked by stimulation in the far chamber and conducted through the near stimulation

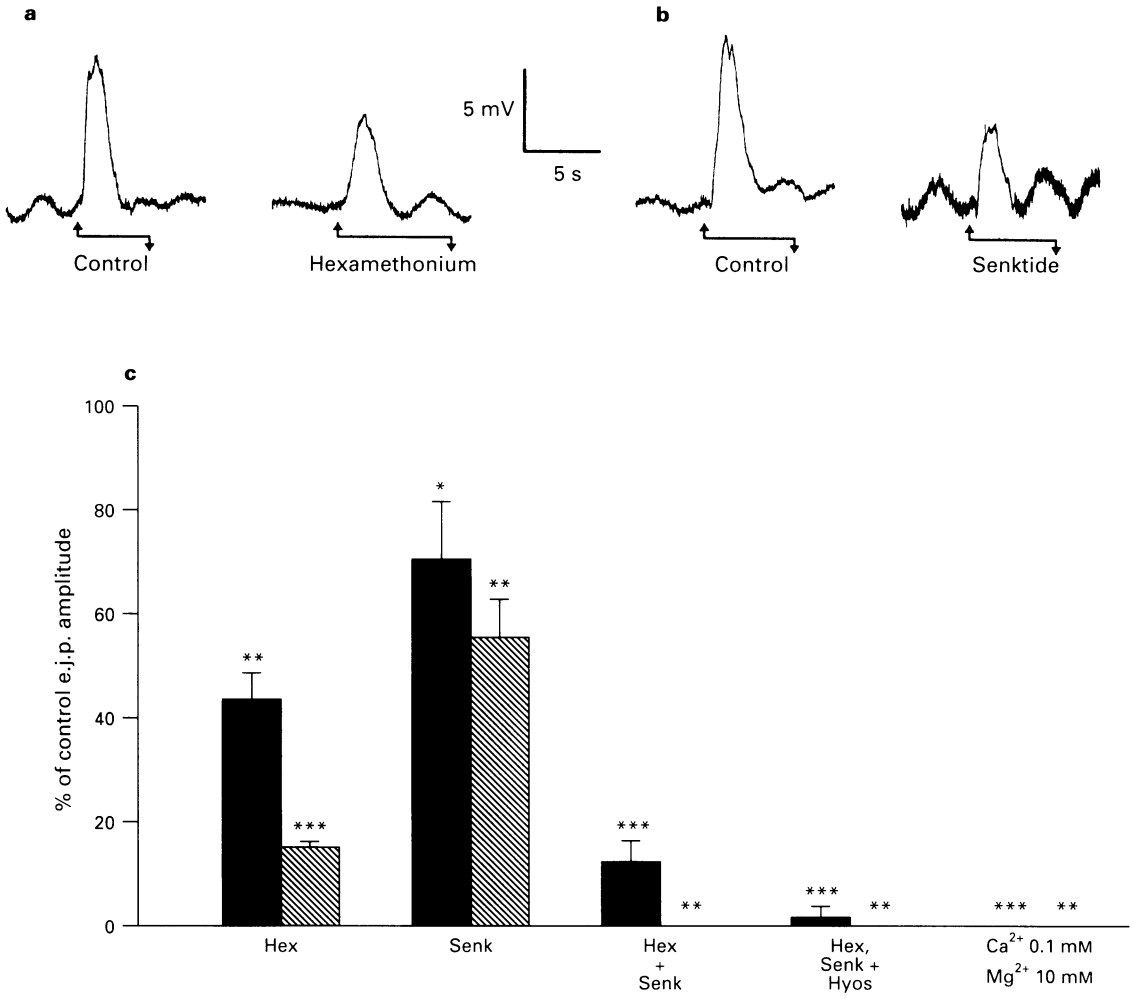

Figure 2 Effect of drugs applied in the near stimulation chamber on ascending responses evoked from within this chamber. (a) Hexamethonium (200 μM) reduced the amplitude of e.j.ps evoked by distension. Arrows indicate the period of distension stimulus. (b) Desensitization of NK_3 receptors with senktide (1 μM) also depressed responses. (c) Amplitudes of e.j.ps evoked by distension (solid columns) or mucosal compression (hatched columns) in the presence of drugs expressed as a percentage of the amplitude of control respones (mean $\pm$ s.e.mean, $n = 4-8$). *Significantly different from control ($P < 0.05$); **significantly different from control ($P < 0.01$); ***significantly different from control ($P < 0.001$); Hex = hexamethonium (200 μM); Hyos = hyoscine (1 μM); Senk = senktide (1 μM). Total synaptic blockade was achieved by perfusion of the stimulation chamber with physiological saline containing 0.1 mM Ca^{2+} and 10 mM Mg^{2+}.

chamber were completely abolished when hexamethonium (200 μM) was added to the intermediate (near stimulation) chamber (Figure 3). Synaptic blockade in the intermediate chamber with saline containing 0.1 mM Ca^{2+} plus 10 mM Mg^{2+} also abolished ascending reflexes. In contrast, senktide applied at this site had no effect on e.j.ps evoked from the far stimulation chamber. Nicotinic receptor blockade with hexamethonium in the recording chamber abolished ascending reflexes initiated from either stimulation chamber ($n = 4$).

Descending pathway

Effect of drugs applied at the site of stimulus In contrast to the ascending pathway, synaptic blockade in the near stimulation chamber with saline containing 0.1 mM Ca^{2+} plus 10 mM Mg^{2+} only partly depressed descending reflexes evoked from within this chamber. I.j.ps activated by distension and compression were reduced to 65% and 25% of control responses, respectively (Figure 4).

The nicotinic receptor antagonists, hexamethonium (200 μM) or dihydro-β-erythroidine (10 μM), added to the near stimulation chamber, had little effect on the amplitudes of i.j.ps evoked by stimuli applied in this chamber. Hexamethonium reduced responses to distension by less than 20% ($P < 0.05$) and did not attenuate responses to mucosal compression, while the only effect of dihydro-β-erythroidine was to reduce the duration of responses to near chamber compression by 20% ($P < 0.05$). Hyoscine (1 μM) reduced the amplitudes of i.j.ps evoked by distension by 10%, but was ineffective on responses to compression. Application of senktide (1 μM) to the near stimulation chamber reduced the amplitude of i.j.ps evoked by distension or compression in this chamber by approximately 40% in each case ($P < 0.05$). The effect of senktide on responses to distension was similar to the reduction observed when all synaptic transmission in this chamber was blocked. In addition, the mean latency of i.j.ps evoked by distension was significantly increased by 10% in the presence of senktide ($P < 0.01$). Spantide (10 μM), an NK_1 and NK_2 receptor antagonist had no effect on reflex amplitudes, when applied to the stimulus region.

Simultaneous addition of hexamethonium (200 μM) and senktide (1 μM) to the stimulation chamber reduced descending reflex responses evoked by compression to an extent similar to the effect of synaptic blockade. When hyoscine (1 μM) was also added to the near stimluation chamber perfusate, the amplitudes of responses evoked by near chamber compression were again much smaller than control values ($P < 0.001$), but the magnitude of these reductions was no different from the effects of combined application of hexamethonium and senktide. In contrast, responses to near chamber distension returned to control levels when hyoscine was added in the presence of hexamethonium and senktide (Figure 4).

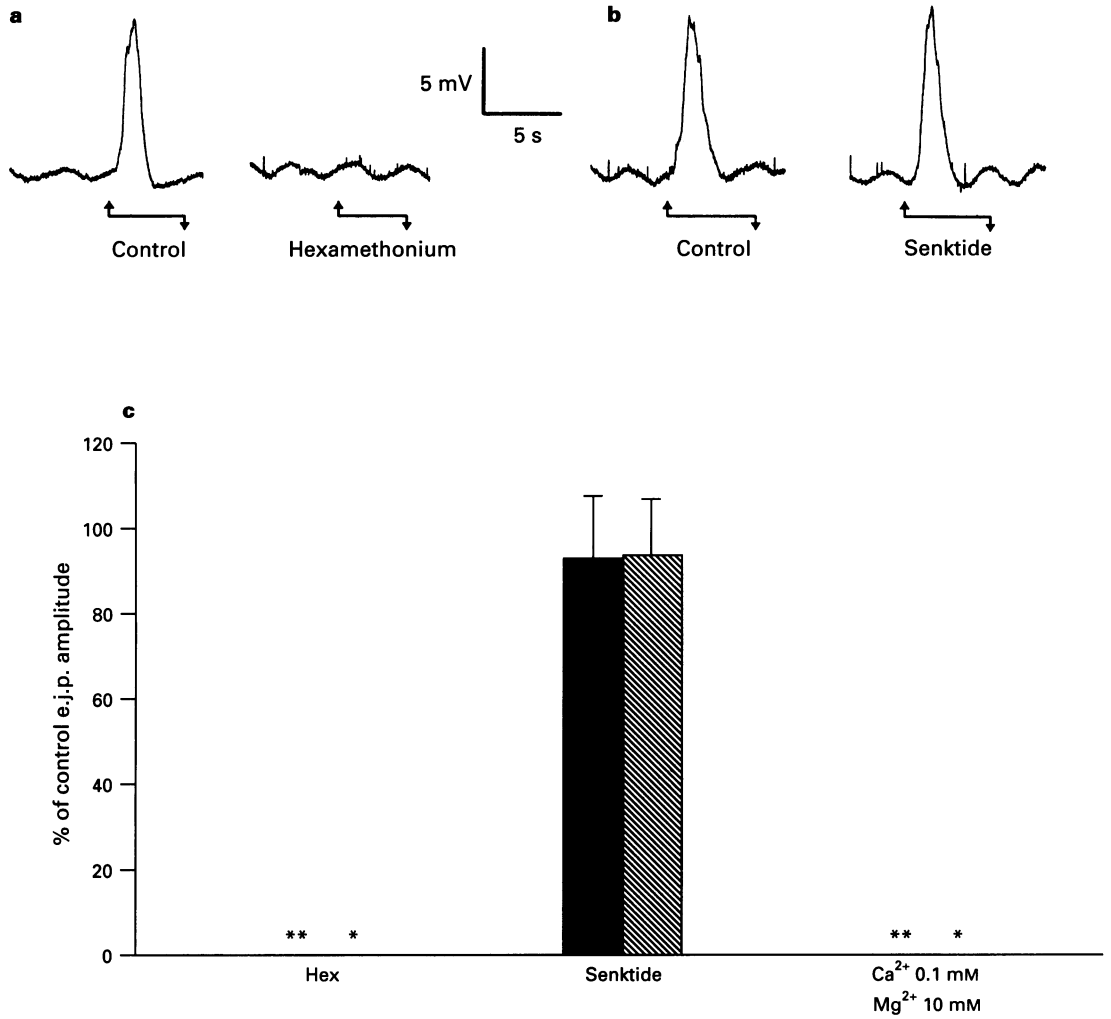

Figure 3 Effect of drugs applied in the near (intermediate) chamber on ascending reflex responses evoked from the far stimulation chamber. (a) Hexamethonium (200 μM) abolished e.j.ps evoked by distension. Arrows indicate the period of distension stimulus. (b) Reflexes were unaffected by senktide (1 μM). (c) Amplitudes of e.j.ps evoked by distension (solid columns) or mucosal compression (hatched columns) in the presence of drugs expressed as a percentage of the amplitude of control responses (mean $\pm$ s.e.mean, $n = 4-6$). *Significantly different from control ($P<0.05$); **significantly different from control ($P<0.01$); Hex = hexamethonium (200 μM).

Effect of drugs applied to the region between stimulus and recording sites on descending reflexes I.j.ps evoked by distension or compression in the far stimulation chamber were almost abolished by perfusion of the intermediate (near stimulation) chamber with physiological saline containing 0.1 mM Ca^{2+} plus 10 mM Mg^{2+} (Figure 5). However, the amplitude of these reflexes was unaffected by either nicotinic receptor antagonist (hexamethonium or dihydro-β-erythroidine) in the intermediate chamber. Hyoscine in this chamber reduced the amplitudes of responses to distension by 30% ($P<0.05$), but had no effect on reflexes evoked by compression. Application of senktide to the intermediate chamber depressed reflexes elicited by far chamber stimulation to 25% (distension) and 30% (compression) of control values ($P<0.001$). However, spantide (10 μM) had no effect on any of the response parameters. When applied together to this intermediate region, hexamethonium and senktide, with or without hyoscine, had no greater effect than that of senktide alone.

Effect of drugs applied to the recording chamber on descending reflexes Application of hexamethonium to the recording chamber slightly reduced the mean amplitude only of descending reflexes evoked by distension in the near stimulation chamber (Figure 6). Responses evoked from the far stimulation chamber were unaffected. Indeed, when the drug was added to both near stimulation and recording chambers simultaneously, reflex responses to near chamber stimuli were only slightly reduced, to approximately 75% of control values ($P<0.01$ for distension; $P<0.05$ for compression). Hexamethonium had no effect on response latency or duration.

Hyoscine in the recording chamber appeared to enhance descending reflexes slightly but only the mean amplitude of the responses evoked by far chamber compression was significantly greater than control ($P<0.01$). Also, the mean times to peak of the responses to far chamber stimuli were reduced by 20% for distension and 15% for compression ($P<0.05$). Latency and duration of reflex responses were unaffected by hyoscine. Application of senktide to the recording chamber did not alter reflex responses evoked from either stimulation chamber.

Effect of an ATP receptor antagonist High concentrations of the P_2 receptor antagonist, suramin (Hoyle *et al.*, 1990), have been shown to block hexamethonium-resistant fast e.p.s.ps recorded in some myenteric neurones (Galligan & Bertrand, 1994). However, when applied to the near stimulation chamber, suramin (100 μM) had no effect on descending reflex responses to either far or near chamber stimulation ($n = 6-7$).

Effectiveness of drug treatments

In the present study, desensitization of NK_3 receptors was demonstrated by measuring the contractile effect of senktide on isolated segments of ileum in the absence and presence of a

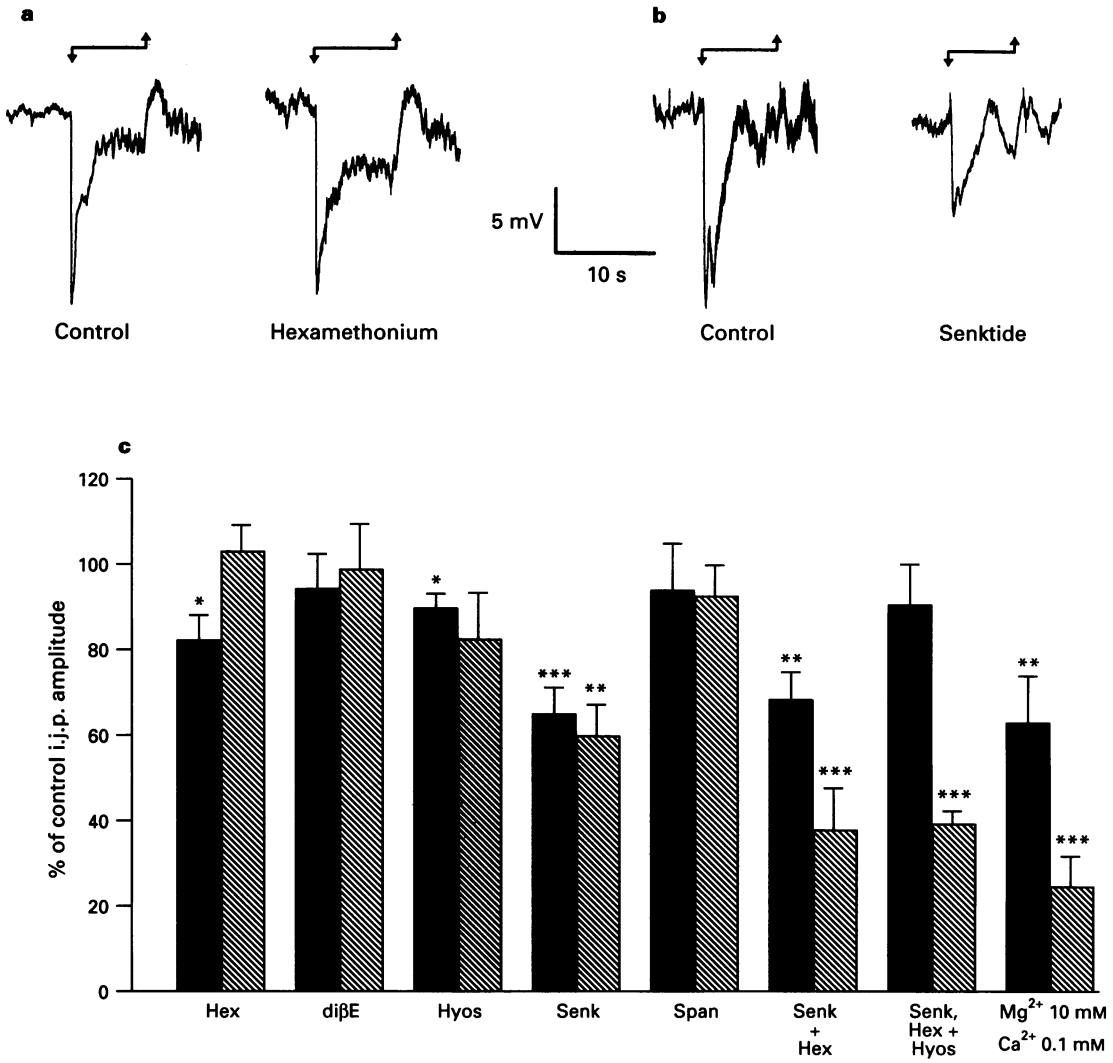

Figure 4 Effect of drugs applied in the near stimulation chamber on descending reflex responses evoked from within this chamber. (a) Hexamethonium (200 μM) only slightly depressed i.j.ps evoked by distension stimuli. Arrows indicate the period of distension stimulus. (b) Desensitization of NK_3 receptors with senktide (1 μM) substantially depressed reflex responses. (c) Amplitudes of i.j.ps evoked by distension (solid columns) or mucosal compression (hatched columns) in the presence of drugs expressed as a percentage of the amplitude of control responses (mean $\pm$ s.e.mean, $n=6-8$). *Significantly different from control ($P<0.05$); **significantly different from control ($P<0.01$); ***significantly different from control ($P<0.001$); Hex = hexamethonium (200 μM); diβE = dihydro-β-erythroidine (10 μM); Hyos = hyoscine (1 μM); Senk = senktide (1 μM); Span = spantide (10 μM).

desensitizing concentration of the drug. Senktide evoked a concentration-related contraction of longitudinal segments of guinea-pig ileum. Responses to senktide (10 nM – 1 μM) were completely abolished by prior exposure to 1 μM senktide ($P<0.0001$). No effects of senktide on contractile responses to cholinoceptor agonists, carbachol (10 nM – 10 μM) or DMPP (0.1 – 100 μM) were observed (Figure 7). The concentration of dihyrdo-β-erythroidine used in reflex experiments was shown to block nicotinic receptors effectively by completely abolishing contractile responses evoked by DMPP (0.1 – 100 μM).

Discussion

This study suggests that ACh acting at nicotinic receptors and tachykinins acting via NK_3 receptors each make substantial contributions to transmission between enteric neurones during ascending excitatory and descending inhibitory motility reflexes in the guinea-pig small intestine. Muscarinic receptors appear to have a relatively minor role. The data also suggest that other neurotransmitters or receptors, as yet unidentified, play a significant role in the transmission of descending reflexes. The deducted sites of actions of the transmitters investigated are illustrated in Figure 8.

Transmission from sensory neruones in ascending reflex pathways

The present results indicate that sensory neurones mediating ascending reflexes may employ both ACh and tachykinins as transmitters. Nicotinic receptor blockade reduces, but does not abolish, ascending excitatory reflexes at the site of reflex initiation in the divided organ bath, as previously reported by Tonini & Costa (1990). The reduction was probably due to an action at nicotinic synapses at the outputs of sensory neurones, which have only very short oral projections (Bornstein *et al.*, 1991b; Kirchgessner *et al.*, 1992). This is supported by the observation that blockade of all synaptic transmission in the near stimulation chamber abolished excitatory reflexes evoked from this chamber. It therefore appears that sensory neurones release ACh to excite nicotinic receptors on ascending interneurones. The residual response in the presence of nicotinic receptor blockade appears to be largely due to tachykinins acting at NK_3 receptors on ascending interneurones, as it is substantially depressed by desensitization with senktide. A further, relatively minor, component of transmission from sensory neurones to interneurones appears to be mediated by ACh acting on muscarinic receptors (Tonini & Costa, 1990). These conclusions are consistent with immunohistochemical

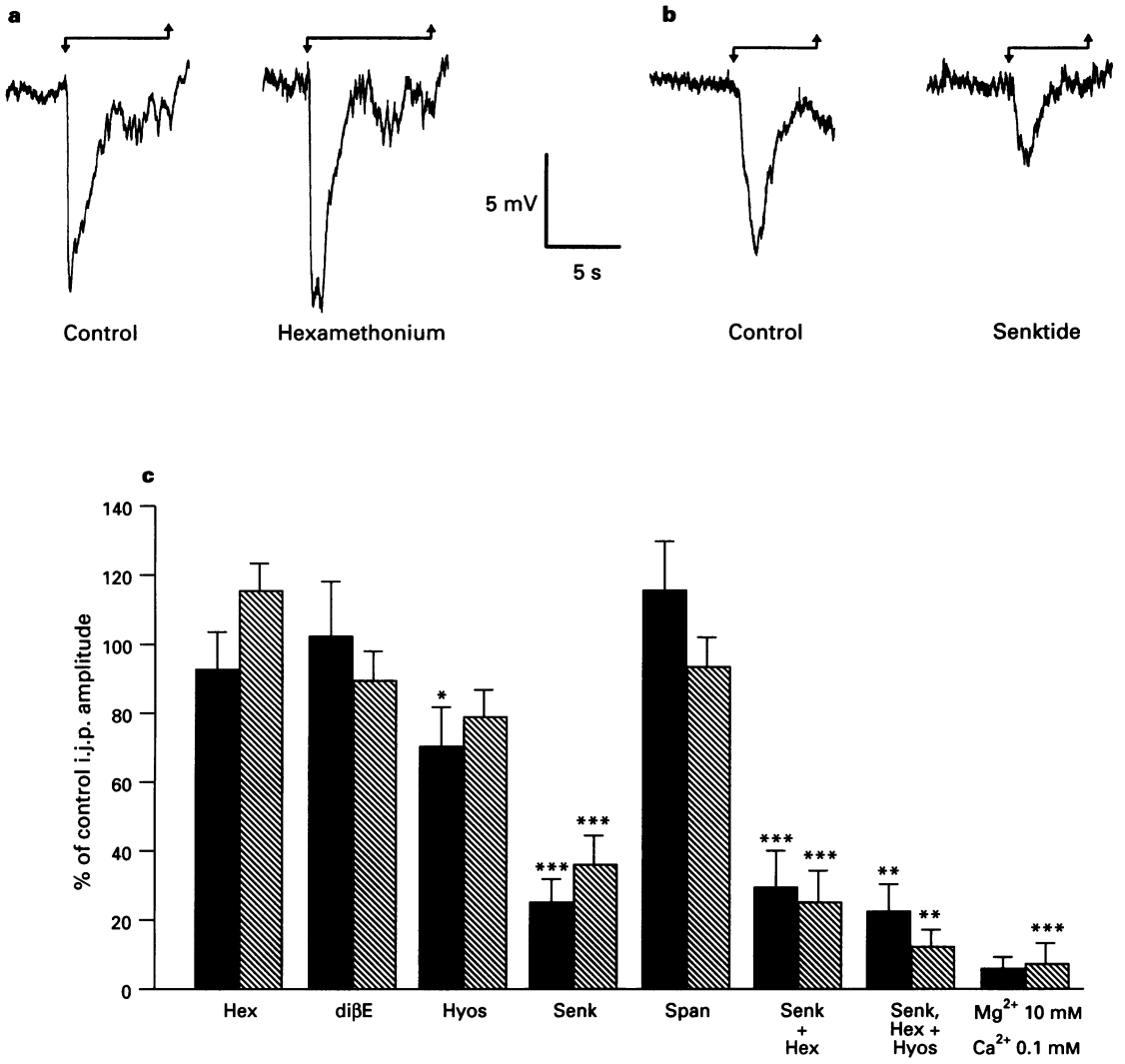

Figure 5 Effect of drugs applied in the near stimulation chamber on descending reflex responses evoked from the far stimulation chamber. (a) Nicotinic receptor blockade with hexamethonium (200 μM) had no effect on i.j.ps evoked by distension stimuli. Arrows indicate the period of distension stimulus. (b) Desensitization of NK_3 receptors with senktide (1 μM) greatly depressed reflex responses. (c) Amplitude of i.j.ps evoked by distension (solid columns) or mucosal compression (hatched columns) in the presence of drugs expressed as a percentage of the amplitude of control responses (mean $\pm$ s.e.mean, $n = 5-8$). *Significantly different from control ($P<0.05$); **significantly different from control ($P<0.01$); ***significantly different from control ($P<0.001$); Hex = hexamethonium (200 μM); diβE = dihydro-β-erythroidine (10 μM); Hyos = hyoscine (1 μM); Senk = senktide (1 μM); Span = spantide (10 μM).

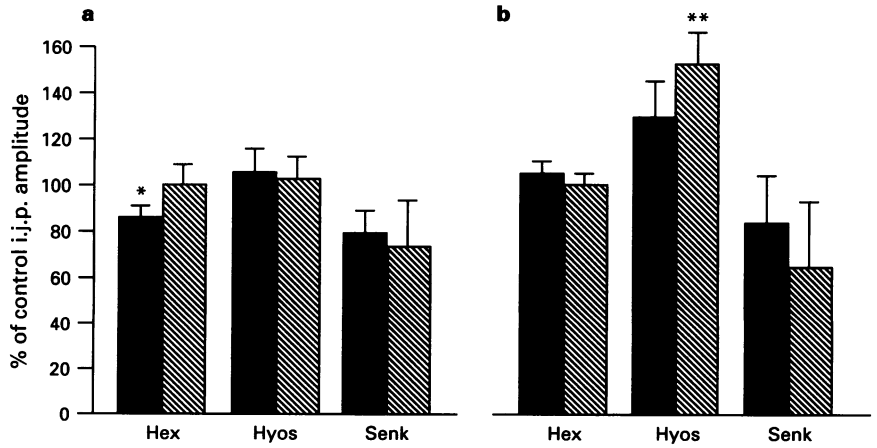

Figure 6 Effect of drugs added to the recording chamber on descending reflex responses evoked by stimuli applied in (a) the far stimulation chamber or (b) the near stimulation chamber. Each graph shows the amplitude of i.j.ps evoked by distension (solid columns) or mucusal compression (hatched columns) in the presence of drugs added to the recording chamber expressed as a percentage of the amplitude of control responses (mean $\pm$ s.e.mean, $n = 6$). *Significantly different from control ($P<0.05$); **significantly different from control ($P<0.01$); Hex = hexamethonium (200 μM); Hyos = hyoscine (1 μM); Senk = senktide (1 μM).

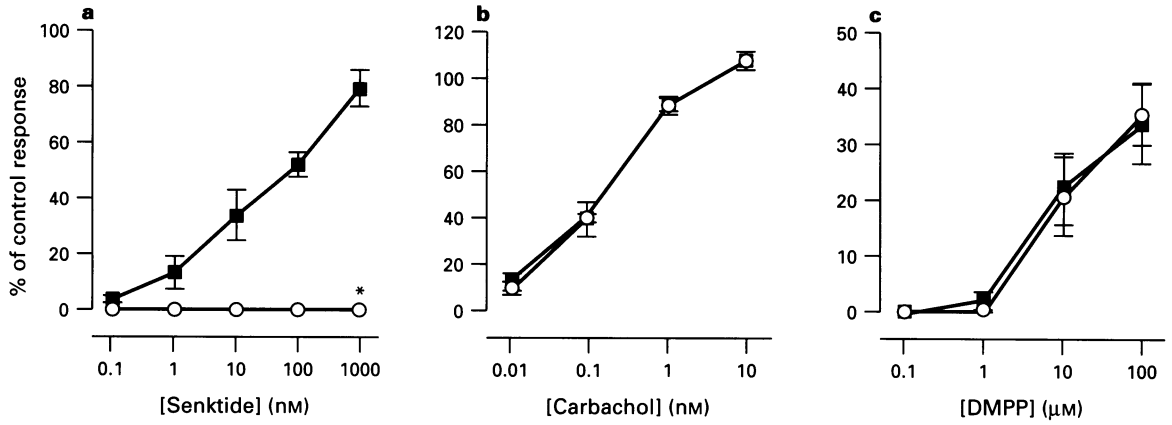

Figure 7 Effect of NK_3 receptor desensitization with senktide on contractions of isolated segments of ileum evoked by senktide and cholinoceptor agonists. (a) Concentration-effect curves for senktide alone (■) and in the presence of senktide (1 μM, O; $n=4$). (b) Carbachol alone (■) and in the presence of senktide (1 μM, O; $n=4$). (c) DMPP alone (■) and in the presence of senktide (1 μM, O; $n=4$). Results are expressed as mean ± s.e.mean. *Significantly different from control curve ($P<0.01$).

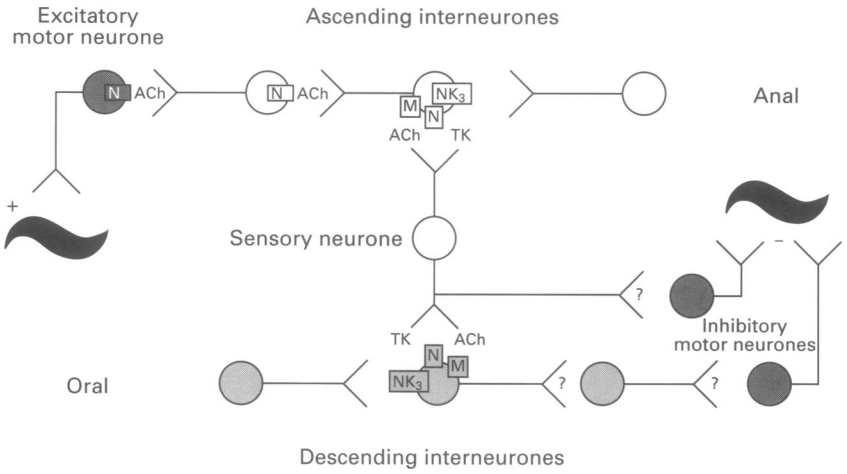

Figure 8 Diagram summarising the roles of acetylcholine (ACh) acting via nicotinic (N) and muscarinic (M) receptors, and tachykinins (TK) acting via NK_3 receptors at neuro-neuronal synapses in ascending and descending reflex pathways to the circular muscle in the guinea-pig ileum.

evidence which indicate that many, if not all, intrinsic sensory neurones of the small intestine are immunoreactive for both choline acetyltransferase (ChAT) and tachykinins (Song *et al.*, 1991; Steele *et al.*, 1991; Kirchgessner *et al.*, 1992).

Transmission from ascending interneurones

Transmission from interneurones at sites further along the ascending pathways appears to be exclusively mediated by nicotinic receptors. As both sensory neurones and excitatory motor neurones have predominantly short oral projections, reflexes evoked from the far chamber would be conducted to the recording site by interneurones which synapse with other interneurones in the intermediate chamber. While desensitization of NK_3 receptors in the near (intermediate) chamber had no effect on reflexes evoked from the far stimulation chamber, nicotinic receptor blockade in the region between stimulus and recording sites completely abolished reflex responses. Furthermore, hexamethonium in the recording chamber abolished ascending reflexes evoked by stimuli applied in either stimulation chamber. These results indicate that transmission between ascending interneurones and from ascending interneurones to excitatory motor neurones is via ACh acting on nicotinic receptors. These conclusions are consistent with both electrophysiological results indicating that the only responses that can be evoked in myenteric neruones by activation of ascending reflexes are bursts of fast e.p.s.ps (Bornstein *et al.*, 1991a; Smith *et al.*, 1992) and immunohistochemical data demonstrating that all neurones which project orally to other ganglia are cholinergic (Brookes *et al.*, 1991; Steele *et al.*, 1991). Although ascending interneurones are also immunoreactive for tachykinins, the present results suggest that these peptides do not function as primary transmitters between these neurones.

Transmission from sensory neurones in descending reflex pathways

The descending nerve pathways which mediate inhibition of the circular muscle are more complex than ascending excitatory pathways. Reflexes evoked by stimulation in the near chamber were only partly depressed when all synaptic transmission in this chamber was blocked by saline containing 0.1 mM Ca^{2+} plus 10 mM Mg^{2+}. This result, which is similar to that obtained by Costa *et al.* (1995) in a study using measurements of circular muscle contractility, indicates that descending reflexes are partly mediated by pathways containing sensory neurones with short anally-directed processes and partly by pathways in which sensory neurones project for longer distances anally. The substantially smaller effect of synaptic blockade on reflexes stimulated by distension compared with mucosal compression suggests a greater involvement of sensory neurones with long anal projections in mediating reflexes evoked by distension stimuli.

Transmission from the short anal projections of sensory neurones was investigated by comparing the effects of drugs

applied to the near stimulation chamber with the effect of synaptic blockade on reflexes evoked from this chamber. Responses to both distension and compression were virtually unaffected by the presence of nicotinic receptor antagonists in this chamber. In contrast, desensitization of NK_3 receptors in the near chamber significantly attenuated reflexes evoked from this chamber, reducing responses to distension to the same level as total synaptic blockade. This suggests that tachykinins mediate the major component of transmission from short anally-projecting sensory neurones. This action appears to be exclusively via NK_3 receptors, in view of the very high selectivity of senktide and also because the NK_1 and NK_2 receptor antagonist, spantide, had no effect on the descending reflex when applied in the stimulation chamber. However, due to the poor selectivity of spantide, a functional role of NK_1 and NK_2 receptors cannot be excluded. Indeed, NK_1 receptors have recently been localized to descending interneurones and inhibitory motor neurones in the guinea-pig ileum (Portbury *et al.*, 1996), and there is evidence that such receptors can be activated by NK_1 receptor agonists (Burcher & Stamatakos, 1994). Experiments are currently in progress in our laboratory using recently developed selective NK_1 and NK_2 receptor antagonists.

Hyoscine slightly depressed distension reflexes when applied to the stimulation chamber, implying a role, possibly modulatory, for muscarinic receptors at synapses at the output of short processes of sensory neurones. As activation of tachykinin or muscarinic receptors evokes only slow excitatory synaptic responses in myenteric neurones (Katayama & North, 1978; North & Tokimasa, 1982; Morita *et al.*, 1980; Johnson *et al.*, 1981), it appears that transmission at first order synapses in the descending pathway may be mediated almost entirely by slow events. This is consistent with a recent study which indicated that myenteric sensory neurones communicate with other neurones via slow e.p.s.ps (Kunze *et al.*, 1993).

While hyoscine did not modulate the combined effect of nicotinic receptor blockade and NK_3 receptor desensitization on reflexes evoked by near chamber compression, its presence in this chamber restored reflexes evoked by distension stimuli. This may have been due to blockade of muscarinic presynaptic inhibitory receptors on sensory neurones (North & Tokimasa, 1982). This selective effect of hyoscine represents the first pharmacological distinction between reflexes evoked by distension and those activated by mucosal deformation, and supports earlier physiological studies which concluded that these reflexes are mediated by distinct populations of sensory neurones (Smith *et al.*, 1991; 1992).

The residual responses to near chamber stimuli in the presence of 0.1 mM Ca^{2+} plus 10 mM Mg^{2+} saline were mediated by sensory neurones with relatively long anal processes that projected into the recording chamber before synapsing. However, hexamethonium, hyoscine or senktide in the recording chamber did not significantly attenuate reflex responses evoked from the near stimulus chamber. This suggests that transmission from the long anally-directed terminals of sensory neurones is not mediated by ACh or by tachykinins acting via NK_3 receptors.

Transmission between descending interneurones

To investigate transmission between interneurones in descending pathways, reflexes were evoked from the far stimulation chamber and drugs applied to the intervening chamber. These reflexes were almost abolished when the physiological saline in the intermediate chamber contained 0.1 mM Ca^{2+} plus 10 mM Mg^{2+}, confirming the presence of synapses in this region. While many of these synapses were probably between descending interneurones, far chamber stimuli would also be expected to activate sensory neurones which synapsed in the intervening chamber. Although NK_3 receptor desensitization in the near chamber profoundly depressed, but did not abolish, conduction of the descending reflex through this chamber, it is not possible to deduce whether this effect is on transmission from sensory neurones or interneurones. However, the failure of nicotinic receptor antagonists in the intervening chamber to alter reflexes evoked from the far chamber indicates that synapses between descending interneurones do not operate via release of ACh onto nicotinic receptors.

Transmission from descending interneurones to inhibitory motorneurones

As most inhibitory motor neurones project anally for less than 2 mm (Bornstein *et al.*, 1986; Brookes *et al.*, 1991), when descending pathways were activated by stimuli in the far chamber, the majority of synapses that were activated in the recording chamber would be between interneurones and motor neurones. Because neither NK_3 receptor desensitization nor nicotinic receptor blockade in the recording chamber affected reflexes evoked from the far chamber, it appears that transmission from descending interneurones to motor neurones is not mediated by ACh or by tachykinins acting at NK_3 receptors.

Identity of transmitters released from descending interneurones

Previous electrophysiological data shows that activation of descending reflexes evokes fast (Hirst *et al.*, 1975; Smith *et al.*, 1992), but rarely slow (Bornstein *et al.*, 1991a), e.p.s.ps in myenteric neurones. Together with the results of the present study, this indicates that another fast transmitter, other than ACh, is involved in the conduction of descending reflexes. This might be considered surprising, because at least three classes of cholinergic descending interneurones have been identified in immunohistochemical studies (Costa *et al.*, 1992). However, a population of noncholingeric interneurones has also been identified and these make large numbers of synaptic contacts with other non-cholinergic descending interneurones and with inhibitory motor neurones (Pompolo & Furness, 1995; Young *et al.*, 1995).

Recent evidence suggests that ATP may play a role in transmission, as some fast e.p.s.ps in the myenteric plexus are sensitive to the P_2 receptor antagonist, suramin, rather than to hexamethonium (Galligan & Bertrand, 1994). However, in the present study, suramin in the near stimulation chamber had no effect on descending reflexes evoked from either stimulation chamber. Investigation of the effect of suramin in the recording chamber was not performed, as any effect on transmission to motor neurones could not be discriminated from an action at inhibitory neuromuscular junctions (Hoyle *et al.*, 1990; McConalogue *et al.*, 1995). Thus, the possibility that ATP mediates transmission from descending interneurones to inhibitory motor neurones cannot be excluded.

In summary, the results of the present study indicate that transmission from sensory neurones in the ascending excitatory reflex pathway is mediated by ACh acting via both nicotinic and muscarinic receptors, and by tachykinins acting at NK_3 receptors, while transmission from ascending interneurones appears to be predominantly via nicotinic receptors. The descending inhibitory pathways are more complex, and while transmission from sensory neurones involves nicotinic, muscarinic and NK_3 receptor-dependent components, transmission from descending interneurones to inhibitory motor neurones is neither cholinergic nor due to tachykinins acting via NK_3 receptors.

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