The role of enteric inhibitory motoneurons in peristalsis in the isolated guinea-pig small intestine

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1. Peristalsis is a co-ordinated motor behaviour in which an anally propagated contraction of the circular muscle propels intraluminal contents. The role of excitatory motoneurons in peristalsis is well established; however the role of enteric inhibitory motoneurons is unknown.

2. A combination of a nitric oxide synthase inhibitor and apamin, which blocks relaxation of the circular muscle of guinea-pig small intestine mediated by enteric inhibitory motoneurons, was used to investigate the role of inhibitory motoneurons in peristalsis in isolated segments of guinea-pig small intestine.

3. N^{ω}-nitro-L-arginine methyl ester (L-NAME, 400 μM) and N^{ω}-nitro-L-arginine (L-NOArg, 100 μM) significantly reduced the threshold volume required to trigger emptying of the intestine. This effect was reversed by L-arginine (4 mM) and L-arginine alone increased the threshold volume for initiation of peristalsis. Sodium nitroprusside (0·1–10 μM), which generates nitric oxide, also increased the threshold volume. L-NAME, L-NOArg, L-arginine and sodium nitroprusside did not alter the maximal intraluminal pressure generated during emptying. Contraction of the longitudinal muscle during the initial phase of fluid infusion was significantly increased by L-NAME and L-NOArg and reduced by sodium nitroprusside (1 nM to 10 μM).

4. Apamin (0·5 μM) did not significantly alter the threshold volume necessary to initiate peristalsis or contraction of the longitudinal muscle. However, the maximal pressure generated when the intestine was emptying was significantly increased. Furthermore, short segments of circular muscle contracted apparently randomly, before peristaltic emptying was triggered.

5. A combination of L-NAME and apamin completely disrupted peristalsis. Contractions of the circular muscle did not always start at the oral end. Stationary contractions as well as contractions propagating orally and anally were observed.

6. It is concluded that enteric inhibitory motoneurons are crucial for peristalsis to occur. They are important in setting the threshold at which peristaltic emptying is triggered, via nitric oxide. They are essential for the propagation of the circular muscle contraction, via an apamin-sensitive mechanism of transmission. Contraction of the longitudinal muscle during peristalsis is partly inhibited by a nitric oxide-mediated mechanism.

Peristalsis is a co-ordinated pattern of motor behaviour which occurs in the gastrointestinal tract and allows the contents to be propelled in an anal direction. This behaviour is controlled by neurons in the enteric nervous system, and in isolated segments of intestine it occurs in a reproducible manner for several hours (Trendelenburg, 1917; Kosterlitz & Lees, 1964; Costa & Furness, 1982). Two phases of peristalsis have been described in response to the slow infusion of liquid which stretches the intestinal wall: the preparatory and emptying phases (Trendelenburg, 1917; Kosterlitz & Lees, 1964). The role of excitatory and inhibitory motoneurons in the preparatory phase of peristalsis has been investigated. Radial stretch elicits reflex contraction of the longitudinal muscle (Kosterlitz & Robinson, 1959) and inhibitory motoneurons to the circular muscle are involved in an accommodation mechanism which relaxes the circular muscle (Waterman, Costa & Tonini, 1994). At a threshold level of distension the emptying phase occurs, consisting of a strong contraction of the circular muscle at the oral end which propagates anally. Whereas the role of excitatory motoneurons in the emptying phase is well established (Kosterlitz & Lees,

1964; Costa & Furness, 1982), the role of inhibitory motoneurons in this phase has not been established because until recently drugs which block inhibitory neuromuscular transmission have not been available.

Enteric inhibitory motoneurons do not use noradrenaline or acetylcholine and are often referred to as non-adrenergic and non-cholinergic (NANC). They are present throughout the gastrointestinal tract in all species studied (for reviews see Costa & Furness, 1982; Taylor & Bywater, 1988; Hoyle & Burnstock, 1989; Sanders & Ward, 1992). In the guinea-pig, there are at least two mechanisms of NANC inhibitory transmission (Niel, Bywater & Taylor, 1983; Costa, Furness & Humphreys, 1986). Both mechanisms mediate inhibitory transmission to the circular muscle of the small intestine and are distinguished by their sensitivity to the bee venom extract, apamin. Apamin blocks small conductance calcium-dependent potassium channels in the intestinal smooth muscle (Banks *et al.* 1979; Maas, Den Hertog, Ras & Akker, 1980). Apamin-sensitive inhibitory transmission is responsible for the fast inhibitory junction potential (Niel *et al.* 1983; Bywater & Taylor, 1986). ATP, or a related purine, has been proposed to be the transmitter of enteric inhibitory motoneurons (see Hoyle & Burnstock, 1989) and evidence suggests that ATP mediates the apamin-sensitive mode of transmission to the circular muscle of the guinea-pig small intestine (Costa *et al.* 1986; Crist, He & Goyal, 1992). Apamin-resistant inhibitory transmission produces a slow inhibitory junction potential in circular smooth muscle cells (Niel *et al.* 1983; Bywater & Taylor, 1986). Recently, the presence of nitric oxide synthase has been demonstrated in the enteric inhibitory motoneurons to the circular muscle (Costa *et al.* 1992) and nitric oxide has been shown to mediate apamin-resistant transmission from these neurons. Analogues of L-arginine which inhibit the synthetic enzyme for nitric oxide block slow inhibitory junction potentials (Lyster, Bywater, Taylor & Watson, 1992) and apamin-resistant relaxation of the circular muscle layer (Humphreys, Costa & Brookes, 1991). A combination of apamin and an arginine analogue blocks the relaxation mediated by enteric inhibitory motoneurons (Humphreys *et al.* 1991).

In the present study, we have used this combination of drugs to investigate the role of inhibitory motoneurons in peristalsis in the guinea-pig small intestine. The results demonstrate that enteric inhibitory motoneurons to the circular muscle play a critical role in the triggering and propagation of the wave of contraction in peristalsis.

METHODS

Guinea-pigs (strain: IMVS coloured) of either sex and weighing between 250 and 350 g were stunned and bled via the carotid arteries. Segments of small intestine just distal to the ligament of Treitz were removed, flushed of luminal contents and placed in Krebs solution at room temperature, gassed with 95% O_2–5% CO_2, until required. The composition of the Krebs solution was (mM): NaCl, 118; KCl, 4·7; NaH_2PO_4, 1·0; $NaHCO_3$, 25; $MgSO_4$, 1·2; D-glucose, 11·0; and $CaCl_2$, 2·5; pH 7·4.

Peristalsis was studied using the method of Tonini, Frigo, Lecchini, D'Angelo & Crema (1981) with modifications as described previously (Waterman, Costa & Tonini, 1992). Segments of small intestine, approximately 7–8 cm in length, were mounted horizontally in a 115 ml organ bath. The organ bath contained Krebs solution maintained at 36–37 °C and gassed with 95% O_2–5% CO_2. The Krebs solution was replaced at least every 30 min. Peristalsis was elicited by infusing Krebs solution into the oral end of the intestinal lumen at a rate of 0·63 ml min^{-1}.

Longitudinal muscle contraction was recorded with a Harvard Bioscience isotonic transducer with tension set at 7·5 mN. Intraluminal pressure at the anal end of the length of intestine was measured using a Statham pressure transducer. Signals were recorded using a MacLab data acquisition system with a bridge amplifier (Analog Digital Instruments, Sydney, Australia) and analysed using MacLab Chart v. 3.2 software on a Macintosh IIsi computer. The volume expelled at the end of each wave of contraction was measured with a measuring cylinder. The residual volume remaining in the intestine after a peristaltic wave could be estimated by siphoning fluid from the intestine with a fixed length of tubing at the end of each experiment. The intraluminal pressure following removal of the residual volume was defined as the baseline pressure and the length of intestine under these conditions was considered to be the resting length. Each peristaltic wave caused the expulsion of fluid from the anal end of the intestine via a one-way valve. Fluid leaving the end of the tubing activated a balanced switch that switched off the infusion pump for 10 s to allow the intestine time to finish emptying before more fluid entered.

Parameters measured

The emptying phase of peristalsis is elicited when the intestine is radially distended to a threshold level (Yanagiya, Ohkubo & Shimada, 1958; Ginzel, 1959) and consists of a wave of circular muscle contraction propagating in an anal direction. The threshold volume required to trigger emptying was measured as the volume infused into the intestine plus the residual volume remaining in the intestine at the end of the peristaltic wave (Waterman *et al.* 1992). The intraluminal pressure at threshold and the maximal ejection pressure when the intestine was emptying were also measured. Longitudinal muscle contraction was measured as the average contraction throughout the period of fluid infusion, expressed as millimetres of contraction per millilitre infused.

Video imaging

Intestinal activity was recorded using a Panasonic colour WV-CL504 camera with zoom lens connected to a Panasonic AG-7355 video cassette recorder. Images were recorded at a rate of 25 s^{-1}. The time, day, date and frame number were superimposed on the video images using a Pro Video TD100 time–day–date generator (Pro-Video Systems Pty Ltd, Adelaide, Australia). After the experiment, the video recording was played back into a Macintosh Quadra 900 equipped with a SuperMac VideoSpigot Pro video board. Images were grabbed at 0·4 s intervals and stored on the computer using SuperMac Screenplay software. NIH Image v. 1.45 software was used to convert the images to silhouettes. Each image of the intestine was divided into ten rings of equal length and the average diameter of these rings was measured. The range of diameters was divided into five equal bin widths and each ring of the intestine shaded according to its diameter.

Drugs used

The following drugs were used: N^{ω}-nitro-L-arginine methyl ester (L-NAME), N^{ω}-nitro-L-arginine (L-NOArg), sodium nitroprusside, apamin and tetrodotoxin (Sigma, St Louis, MO, USA); D-NOArg (Bachem, Bubendorf, Switzerland); and L-arginine (BDH, Poole, Dorset, UK). [β-Ala^8]NKA(4–10) was a gift from Dr C. A. Maggi (Menarini Farmeceutica, Florence, Italy).

All drugs except apamin were dissolved in distilled water. Apamin was dissolved in 0·1% trifluoroacetic acid (TFA). TFA at a final concentration of 0·005% did not have any significant effect on peristalsis. L-NAME and L- and D-NOArg were prepared on the day of the experiment. Sodium nitroprusside, L-arginine and apamin were prepared as stock solutions and stored at −4 °C until required. All drugs were administered in volumes less than 1% of the organ bath volume.

Experimental design and analysis of results

Data are presented as the means ± S.E.M. of experiments using n guinea-pigs. Control activity was recorded for at least 10 min before the addition of a drug. The drug was allowed to equilibrate for 10–20 min before peristalsis was again elicited. Comparisons between parameters in the presence and absence of a drug were made using paired t tests. In experiments involving addition of L-NAME and L-arginine, comparisons with control were made using paired t tests with Bonferroni's correction (Ingelfinger, Mosteller, Thibodeau & Ware, 1983). Effects of five different concentrations of sodium nitroprusside were tested using repeated measures ANOVA followed by a Newman–Keuls test. $F_{(x,y)}$ refers to the F ratio where x represents the degrees of freedom of drug concentrations and y the degrees of freedom for error. A probability of less than 0·05 was regarded as significant.

RESULTS

Response of intestine to fluid distension

Infusion of fluid into the intestine initially caused radial distension and contraction of the longitudinal muscle. Once a threshold level of distension was reached, peristaltic emptying was triggered. This consisted of a wave of circular muscle contraction propagating from the oral to the anal end of the intestine, as described previously (Kosterlitz & Lees, 1964). The effect on peristaltic emptying of drugs which either block or mimic inhibitory neuronal transmission was investigated.

Effects of L-NAME and L-NOArg

L-NAME (400 μM) and L-NOArg (100 μM) were used to investigate whether nitric oxide plays a role in peristalsis. Nitric oxide is synthesized by the enzyme nitric oxide synthase from the precursor L-arginine (Knowles, Palacios, Palmer & Moncada, 1989). N^{ω}-nitro-L-arginine methyl ester (L-NAME) and N^{ω}-nitro-L-arginine (L-NOArg) are arginine analogues which competitively inhibit nitric oxide synthase (Hobbs & Gibson, 1990; Rees, Palmer, Schulz, Hodson & Moncada, 1990). L-NAME was used at a concentration of 400 μM which has been shown previously to inhibit completely nitric oxide synthesis (Rees *et al.* 1990). L-NOArg was used at a concentration of 100 μM, which produces 90% inhibition of nerve-mediated relaxation of the rat anococcygeus muscle (Hobbs & Gibson, 1990).

Peristaltic emptying was not blocked by L-NAME or L-NOArg; a wave of circular muscle contraction still propagated from oral to anal. However, the volume of fluid required to produce a threshold level of radial distension (i.e. the threshold volume) was reduced by both drugs. L-NAME reduced the threshold volume by 33·0 ± 4·8% ($n = 4$; $t = 4·70$; $P = 0·04$) and this effect was reversed by the subsequent addition of 4 mM L-arginine (Fig. 1). L-NOArg reduced the threshold volume by 35·0 ± 4·1% ($n = 6$; $t = 8·489$; $P = 0·0004$). Reducing the threshold volume resulted in an increase in the frequency of emptying of the intestine (Fig. 2). L-Arginine alone increased the threshold volume by 30·7 ± 14·4% from 0·39 ± 0·02 to 0·51 ± 0·03 ml ($n = 4$; $t = 4·82$; $P = 0·02$). D-NOArg (100 μM), the inactive stereoisomer of L-NOArg, did not have any significant effect on threshold volume (0·51 ± 0·09 ml *versus* 0·46 ± 0·07 ml in controls; $n = 4$; $t = 2·50$; $P > 0·05$). Thus, a nitric oxide synthetic pathway is involved in setting the threshold distension required to trigger emptying of the intestine.

The pressure in the intestine at threshold in control preparations varied considerably between experiments (95% confidence limits: 77–120 Pa; $n = 16$). This parameter was not altered by L-NAME (120 ± 23% of controls; $n = 4$; $t = 0·765$; $P > 0·05$), L-NOArg (112 ± 17% of controls; $n = 6$; $t = 0·512$; $P > 0·05$), L-arginine (85 ± 12% of controls; $n = 4$; $t = 1·446$; $P > 0·05$) or D-NOArg (99 ± 3% of controls; $n = 4$; $t = 0·418$; $P > 0·05$).

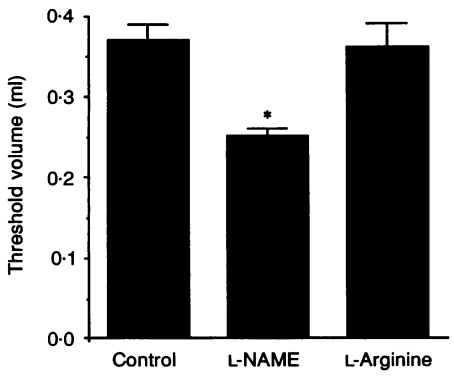

Figure 1. The effect of blocking nitric oxide synthesis on the threshold volume required to trigger peristaltic emptying
The threshold volume was significantly reduced by inhibiting nitric oxide synthesis with 400 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME). This effect was reversed by adding 4 mM L-arginine. Bars indicate means and S.E.M. for 4 experiments. Data were analysed using paired t tests with Bonferroni's correction. * $P < 0·05$.

The maximal ejection pressure generated during the peristaltic contraction was not significantly altered by the addition of L-NAME (1·20 ± 0·17 *versus* 1·50 ± 0·36 kPa in controls; $n = 4$; $t = 1·06$; $P > 0·05$) or L-NOArg (1·24 ± 0·18 *versus* 1·24 ± 0·15 kPa in controls; $n = 6$; $t = 0·023$; $P > 0·05$). Furthermore, this parameter was not significantly altered by L-arginine (1·46 ± 0·44 *versus* 1·08 ± 0·19 kPa in controls; $n = 4$; $t = 2·04$; $P > 0·05$). D-NOArg increased maximal ejection pressure by 8·8% (1·41 ± 0·26 *versus* 1·31 ± 0·24 kPa in controls; $n = 4$; $t = 8·489$; $P = 0·0034$), suggesting that this drug may have some non-specific effects.

The average contraction of the longitudinal muscle during the period of fluid infusion was significantly increased by L-NOArg, from 4·5 ± 1·7 mm ml^{-1} in controls to 15·0 ± 3·8 mm ml^{-1} ($n = 6$; $t = 3·375$; $P < 0·02$). D-NOArg did not have any significant effect (3·5 ± 1·7 mm ml^{-1} *versus* 2·8 ± 1·0 mm ml^{-1} in controls; $n = 4$; $t = 0·653$; $P > 0·05$). These results suggest that nitric oxide or a similar chemical normally inhibits longitudinal muscle contraction during peristalsis.

In empty segments of intestine, L-NOArg increased intraluminal pressure by 18·3 ± 5·4 Pa ($n = 6$; $t = 3·40$; $P = 0·02$). There was no significant effect of D-NOArg on baseline pressure ($\Delta P = 7·4 \pm 4·7$ Pa; $n = 4$; $t = 1·574$; $P > 0·05$). This indicates that nitric oxide is released in the absence of distension and produces relaxation of the circular muscle layer, as has been reported elsewhere (Ozaki, Blondfield, Hori, Publicover, Kato & Sanders, 1991; Waterman *et al.* 1994). In two out of five quiescent preparations, phasic contractions of the longitudinal muscle layer were induced at a frequency of 3–5 min^{-1}. Contractions of the longitudinal muscle layer occurred at this frequency in nineteen out of thirty-seven control preparations from twenty-two animals. Thus endogenous nitric oxide inhibits spontaneous, phasic contractions of the longitudinal muscle as well as distension-evoked contractions.

Effect of sodium nitroprusside

Sodium nitroprusside (SNP) spontaneously releases nitric oxide and has been used previously to mimic the effects of the endogenous inhibitory transmitter (Moncada, Palmer & Higgs, 1991). Concentrations from 1 nM to 10 μM sodium nitroprusside were tested by adding cumulatively to the preparation.

The threshold volume required to trigger the emptying phase of peristalsis was significantly increased by 0·1 μM and higher concentrations of SNP (see Fig. 3). The maximal ejection pressure generated during emptying was not significantly altered by SNP at any of the concentrations tested ($F_{(5,15)} = 1·622$; $P = 0·2143$). All concentrations of SNP tested significantly reduced longitudinal muscle contraction (Fig. 4; $F_{(5,15)} = 21·119$; $P < 0·0001$) in a concentration-dependent manner.

Effect of apamin

Apamin was used at a concentration of 0·5 μM to block a component of inhibitory transmission to the circular muscle. This concentration has previously been shown to block the effect of the endogenous inhibitory transmitter and of ATP and its analogues in some regions of the gastrointestinal tract (Vladimirova & Shuba, 1978; Banks *et al.* 1979; Costa *et al.* 1986) but has no effect on responses of guinea-pig gastrointestinal smooth muscle to exogenous

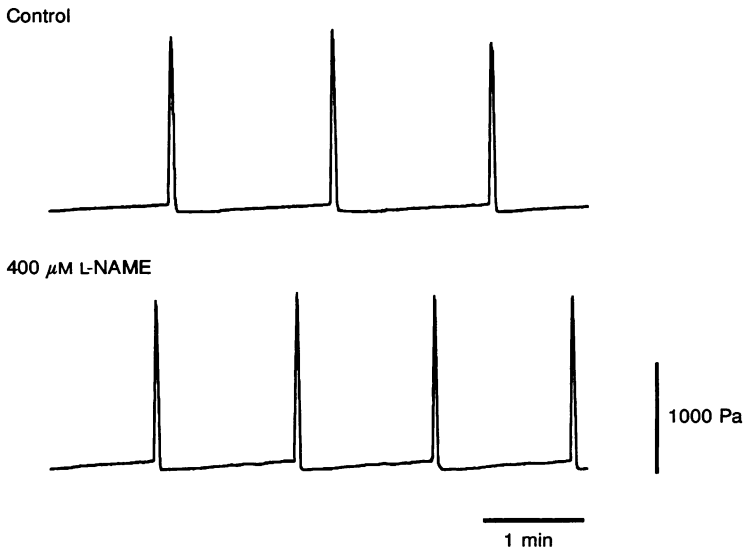

Figure 2. Effect of inhibition of nitric oxide synthesis on peristalsis
Peristalsis was stimulated by infusing Krebs solution into the intestinal lumen at a constant rate. The recordings of intraluminal pressure show that the frequency of emptying of the intestine increased when 400 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) was added to the organ bath, due to a decrease in the threshold distension required to trigger emptying.

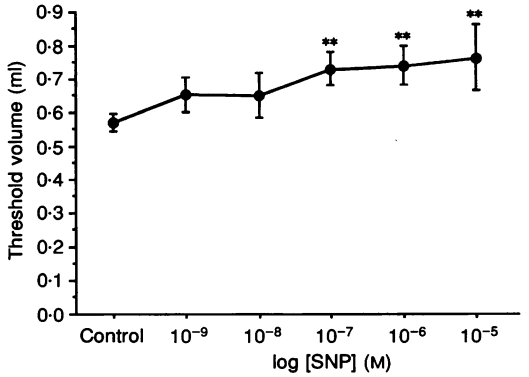

Figure 3. The effect of sodium nitroprusside on the threshold volume required to trigger emptying of the intestine
Threshold volume was significantly increased by concentrations of sodium nitroprusside greater than or equal to 0·1 μM. The graph shows the means and S.E.M. of results from 6 experiments. Data were analysed using repeated measures ANOVA followed by a Newman–Keuls test. ** $P < 0{\cdot}01$ compared to control.

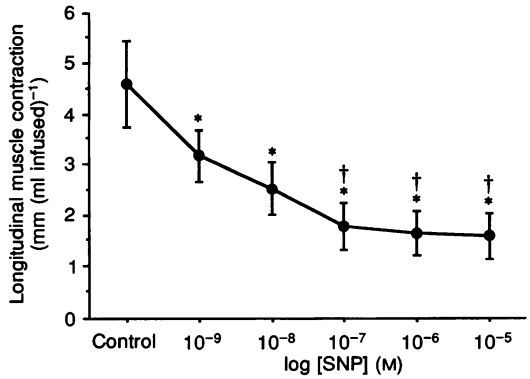

Figure 4. The effect of sodium nitroprusside on maximal longitudinal muscle contraction
Sodium nitroprusside reduced the amplitude of longitudinal muscle contraction in a concentration-dependent manner. The graph shows the means and S.E.M. of results from 6 experiments. Data were analysed using repeated measures ANOVA followed by a Newman–Keuls test. * $P < 0{\cdot}01$ compared to control; † $P < 0{\cdot}01$ compared to 1 mM SNP.

vasoactive intestinal peptide, isoprenaline or papaverine (Costa *et al.* 1986).

Apamin increased the maximal ejection pressure generated by the intestine when emptying from $1{\cdot}86 \pm 0{\cdot}35$ to $2{\cdot}12 \pm 0{\cdot}33$ kPa ($n = 7$; $t = 3{\cdot}16$; $P = 0{\cdot}0196$) but did not affect the threshold volume that triggers this phase ($0{\cdot}53 \pm 0{\cdot}08$ ml *versus* $0{\cdot}51 \pm 0{\cdot}07$ ml in controls; $n = 7$; $t = 0{\cdot}349$; $P > 0{\cdot}05$) (Fig. 5), the pressure at threshold ($95 \pm 11\%$ of controls; $n = 7$; $t = 1{\cdot}210$; $P > 0{\cdot}05$) or longitudinal muscle contraction ($2{\cdot}27 \pm 0{\cdot}67$ mm ml^{-1}, *versus* $2{\cdot}16 \pm 0{\cdot}5$ in controls; $n = 7$; $t = 0{\cdot}537$; $P > 0{\cdot}05$). Apamin greatly altered the pattern of contraction during peristalsis (Fig. 6). The circular muscle appeared to contract almost simultaneously along the length of the intestine and a longer segment of intestine was contracted at any given time. Furthermore, the extent of the maximal contraction was increased, since the minimum diameter was reduced. In the presence of apamin, the almost simultaneous contraction of the circular muscle was still able to empty the intestine. Following emptying, the intestine remained highly contracted. In five out of seven experiments, single or multiple contractions of short segments of circular muscle appeared at different locations prior to the circular muscle contraction along the whole segment. These results suggest that apamin-sensitive inhibitory neuromuscular transmission occurs during peristalsis.

In resting, empty segments of intestine, apamin did not significantly alter intraluminal pressure ($n = 4$; $t = 0{\cdot}829$; $P > 0{\cdot}05$) or the length of the segment ($n = 4$; $t = 0{\cdot}633$; $P > 0{\cdot}05$).

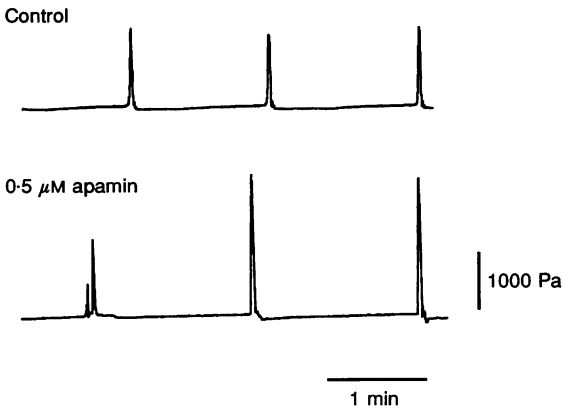

Figure 5. The effect of apamin on peristalsis
Apamin (0·5 μM) increased the maximal ejection pressure generated when the intestine was emptying, but did not alter the frequency of emptying. Apamin also caused contractions of short segments of circular muscle during the initial filling phase. The first, smaller peak in pressure was due to a stationary contraction of the circular muscle at the anal end of the segment.

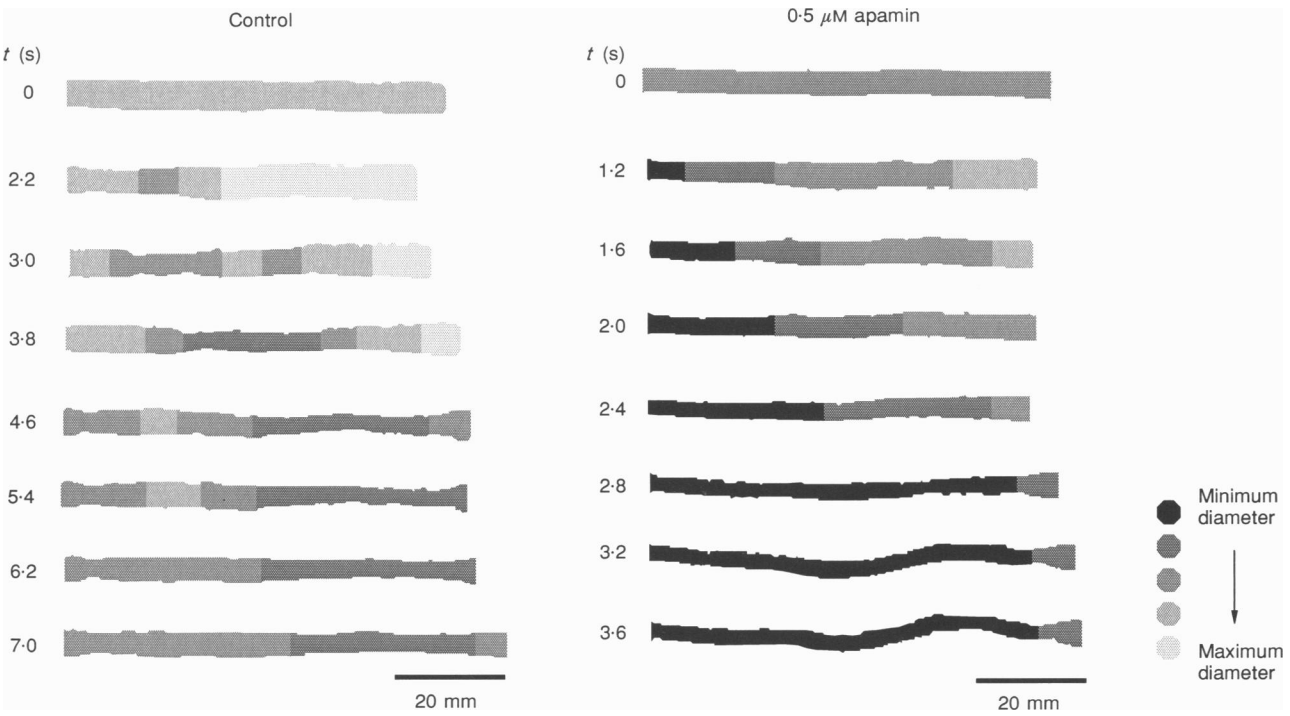

Figure 6. Silhouettes of the intestine showing the effect of apamin on peristalsis
The silhouettes have been shaded according to the diameter of the intestine, with darker shades indicating a smaller diameter and therefore greater shortening of the circular muscle. Time zero shows the intestine just prior to initiation of the circular muscle contraction. Apamin (0·5 μM) increased the shortening of the circular muscle and the length of intestine which was contracted at any given time. The circular muscle contracted almost simultaneously along the length of intestine after the addition of apamin. Note that the duration of the emptying phase after apamin was much shorter than in the controls.

The effects of apamin on peristalsis could be explained by a direct depolarizing effect of the drug on the circular muscle. To determine whether this was the case, the compliance of the intestine in response to fluid distension at a rate of 0·31 ml min^{-1} was measured in the presence of 0·6 μM tetrodotoxin, to block neural activity, and apamin plus tetrodotoxin. Apamin did not have any significant effect on compliance at either low distension volumes (volume less than 80% of the volume required to trigger emptying in control preparations; $n = 4$; $t = 1{\cdot}321$; $P > 0{\cdot}05$) or high volumes (volume equal to 133% of threshold volume; $n = 4$; $t = 0{\cdot}571$; $P > 0{\cdot}05$). Under identical conditions the NK_2 receptor agonist, [β-Ala^8]-NKA(4–10), which directly depolarizes the circular muscle (M. Costa, unpublished results) caused a significant reduction in compliance at low ($n = 5$; $t = 4{\cdot}34$; $P < 0{\cdot}02$) but not high levels of distension ($n = 5$; $t = 2{\cdot}462$; $P = 0{\cdot}07$). These results suggest that the effect of apamin on peristalsis is not due to direct depolarization of the circular muscle.

Combined effect of L-NAME and apamin

Peristalsis was severely disrupted in the presence of both L-NAME and apamin ($n = 5$; see Fig. 7). With both drugs in the bath, contractions appeared to occur randomly in response to infusion of even small volumes of fluid and consequently a threshold volume could not be established. Contractions no longer propagated in an oral-to-anal direction along the length of the intestine and did not occur consistently just at the oral end but started part way along the intestine. Contractions were observed to be stationary or to propagate orally or anally. As a result of this disruption, a co-ordinated peristaltic contraction could no longer be recognized from the pressure traces and only small, irregular changes in pressure were observed (Fig. 8).

DISCUSSION

This study has demonstrated that inhibitory motoneurons to the circular muscle are activated during peristalsis and that blocking transmission from these neurons disrupts peristalsis. This suggests that inhibitory motoneurons are essential for the initiation of a localized contraction of the circular muscle and its co-ordinated anal propagation during peristalsis.

Role of inhibitory motoneurons in peristalsis

It can be postulated that the stimulus for peristalsis is detected by stretch (volume) receptors or tension (pressure) receptors. Increasing transmural pressure across the

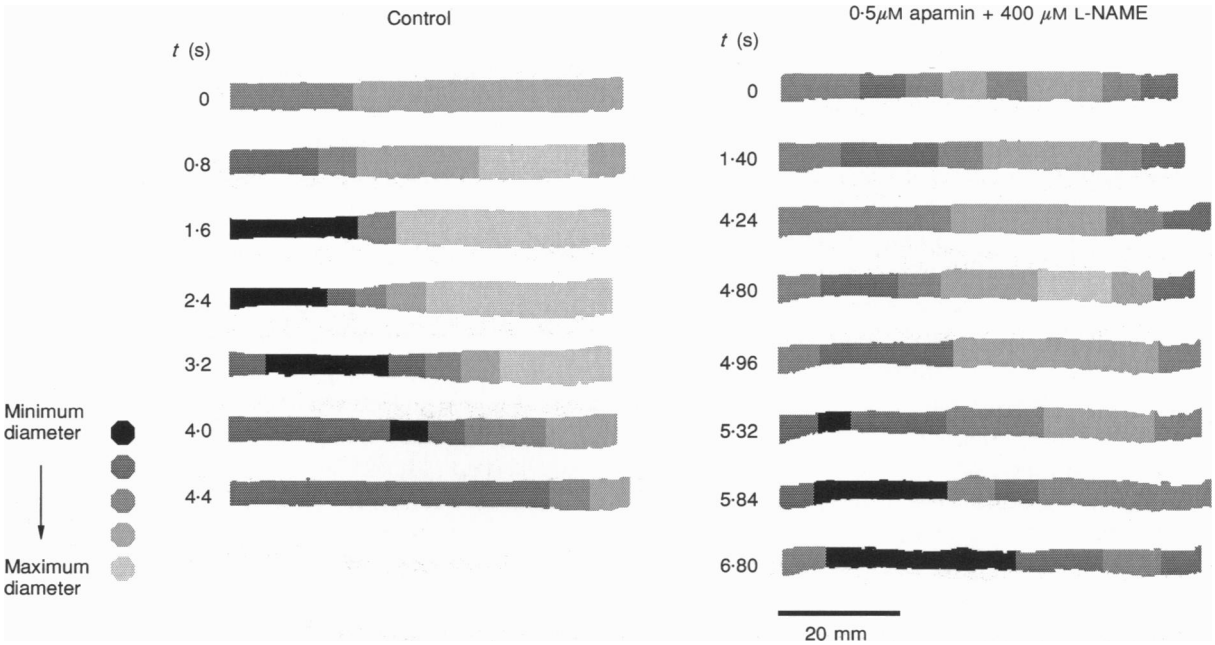

Figure 7. Silhouettes of the intestine showing the combined effect of apamin and inhibition of nitric oxide synthesis on peristalsis
The silhouettes have been shaded according to the diameter of the intestine, with darker shades indicating a smaller diameter and therefore greater shortening of the circular muscle. Time zero in the control shows the intestine just prior to initiation of the circular muscle contraction and in the second panel shows the intestine when it contains an equivalent volume of fluid. Following the addition of 0·5 μM apamin and 400 μM L-NAME to block inhibitory transmission to the circular muscle, contractions occurred randomly along the intestine. The figure shows contractions at the anal end and one-quarter of the way along the intestine in the first 4 s. A co-ordinated contraction propagating from the oral to the anal end was not observed. Furthermore, the intestine did not become as distended as in controls, despite containing a similar volume of fluid.

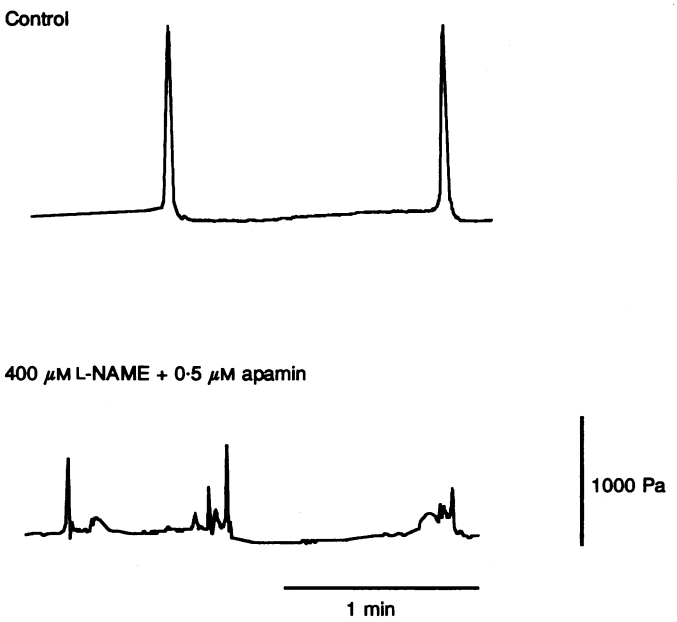

Figure 8. Combined effect of apamin and inhibition of nitric oxide synthesis on peristalsis
Complete inhibition of inhibitory transmission to the circular muscle severely disrupted peristalsis. Irregular, unco-ordinated contractions appeared as small changes in the pressure trace. Clearly defined phases of filling and emptying did not occur.

intestinal wall whilst maintaining a constant diameter with an external sleeve was shown not to be sufficient to trigger the emptying phase of peristalsis (Yanagiya *et al.* 1958; Ginzel, 1959). Therefore change in stretch (volume) is the major stimulus for peristalsis. This hypothesis is confirmed by the present experiments in which increases in the frequency of peristalsis produced by L-NAME and L-NOArg were accompanied by a decrease in volume distension but not by a change in the pressure at threshold.

Localized distension in isolated segments of intestine elicits both orally directed excitatory and anally directed inhibitory reflexes to the circular muscle (Hirst, 1979; Costa & Furness, 1982; Smith, Bornstein & Furness, 1990). The proposal that these excitatory and inhibitory reflexes are involved in peristalsis has been discussed by several investigators (Bayliss & Starling, 1899; Cannon, 1912; Crema, 1970; Hirst, 1979; Costa & Furness, 1982). There is good evidence that excitatory reflexes are essential for peristalsis, since blocking transmission from the final excitatory motoneurons in the reflex prevents peristalsis (Trendelenburg, 1917). However, this is the first time that transmission from the final motoneurons of the descending inhibitory reflex has been blocked during peristalsis, demonstrating their involvement.

In peristalsis, distension occurs during the initial filling phase, prior to the contraction of the circular muscle at the oral end which marks the beginning of peristaltic emptying. Throughout the emptying phase, distension continues to occur in the segment of intestine anal to the propagating contraction, due to the propelled volume of fluid. It is likely that in both cases, distension activates descending inhibitory reflexes and therefore inhibitory motoneurons. Clearly the emptying phase of peristalsis also involves the activation of excitatory motoneurons. It is to be expected that the excitatory and inhibitory inputs to the muscle must interact with one another. Blockade of transmission from enteric inhibitory motoneurons would thus be expected to increase the overall excitability of the preparation, bringing the circular muscle closer to its 'threshold' for contraction. This would cause a decrease in the threshold volume for initiation of peristalsis and an increase in the rate of propagation of the contraction during the emptying phase.

The results of the present study are consistent with the proposal that peristalsis is the result of coactivation of excitatory and inhibitory reflexes in a distended segment of intestine (Hukuhara & Fukuda, 1963; Costa & Furness, 1982). Initially when the intestine is slowly distended, there is a simultaneous activation of ascending excitatory and descending inhibitory reflexes along the length of the intestine, with the inhibitory reflex predominating (Waterman *et al.* 1994). Throughout the preparatory phase, enteric inhibitory motoneurons maintain the circular muscle in a relaxed state, as demonstrated by the increase in circular muscle tone produced by drugs which block inhibitory neuromuscular transmission (Waterman *et al.* 1994). At a particular intraluminal volume (the threshold volume), a contraction occurs at the oral end, indicating a shift from net inhibition to net excitation. As a result of this contraction of the oral end, enteric neurons sensitive to distension are deactivated; thus the descending inhibitory reflex is no longer activated from the contracted region. In this way the inhibition immediately anal to the contraction is reduced and the ascending excitatory reflex thus prevails, leading to contraction and the apparent anal propagation of the contraction. As this process continues, the contraction propagates anally. We therefore propose that the sequential removal of descending inhibition is essential for the anal propagation of the circular muscle contraction. Blockade of transmission from enteric inhibitory motoneurons would prevent the sequential removal of descending inhibitory reflexes and the contraction would occur almost synchronously in every ring of circular muscle. The occurrence of multiple contractions following blockade of inhibitory neuromuscular transmission (present study) is consistent with this proposal. A computer simulation of the coactivation of enteric reflexes confirms the interpretation of the results presented in this paper (Mayo, Brookes & Costa, 1992).

Relative roles of the two mechanisms of inhibitory transmission in peristalsis

There are inhibitory motoneurons with short and long projections, but they all utilize an apamin-sensitive mechanism (Bornstein, Costa, Furness & Lang, 1986) and both short and long inhibitory motoneurons contain nitric oxide synthase immunoreactivity (Costa *et al.* 1992). The differential effect of nitric oxide synthase inhibitors and apamin on the different parameters of peristalsis suggests that the two mechanisms may play different roles in peristalsis. It remains to be established whether a single functional class of inhibitory motoneurons uses the two mechanisms alternately or two functionally distinct classes of inhibitory motoneurons each use one mechanism preferentially.

Nitric oxide appears to be involved in setting the threshold for emptying, since the threshold volume was reduced by L-NAME and L-NOArg. This effect is specific since the actions of L-NAME were reversed by L-arginine, and D-NOArg did not have any significant effect. Furthermore, L-arginine alone and sodium nitroprusside had the opposite effect to the nitric oxide synthase inhibitors. This interpretation is consistent with the role of nitric oxide from inhibitory motoneurons in increasing the compliance of the intestinal wall during the preparatory phase (Waterman *et al.* 1994). Similar conclusions have been reached by R. Ciccocioppo, L. Onori, E. Messori, S. M. Candura, T. Coccini & M. Tonini (unpublished observations) in experiments on rabbit colon. In a recent study, Sugisawa, Komori, Takewaki & Ohashi (1991) found that sodium nitroprusside stimulated peristalsis by reducing the threshold for triggering the emptying phase

of peristalsis and that L-NOArg did not have any significant effect. The lack of a role for endogenous nitric oxide and the opposite effect of sodium nitroprusside reported by these investigators may be due to the different method of studying peristalsis. It is well known that peristalsis 'fatigues' rapidly when the Trendelenburg preparation is used (as in Sugisawa *et al.* 1991). In contrast, the method used in the present study allows peristalsis to be studied continuously for several hours without fatigue.

The apamin-sensitive mechanism does not appear to be involved in setting the threshold for peristalsis since apamin did not affect the threshold volume. However, this mechanism is involved in the timing of the circular muscle contraction underlying propagation, since with apamin in the bath the contraction occurred almost simultaneously along the segment. The increase in maximal ejection pressure by apamin is likely to be due to this altered temporal pattern of contraction.

The differential effects of the drugs on the different components of peristalsis may be a reflection of the different time courses of action of the inhibitory transmitters on the smooth muscle. Nitric oxide-mediated transmission is likely to involve direct activation of soluble guanylate cyclase and production of cGMP, which in turn alters contractile mechanisms (Vincent & Hope, 1992). In the circular muscle of the guinea-pig small intestine, this form of transmission produces a relaxation (Humphreys *et al.* 1991) and is relatively slow (rise time of slow inhibitory junction potential, IJP, ~1·4 s; Bywater & Taylor, 1986). On the other hand, apamin-sensitive transmission produces fast inhibitory junction potentials (rise time of fast IJP, ~400 ms; Bywater & Taylor, 1986; Crist *et al.* 1992) and is responsible for a component of the inhibitory transmission mediating relaxation of the circular muscle of the guinea-pig small intestine (Costa *et al.* 1986). Vasoactive intestinal peptide (VIP) has also been shown to be present in all the inhibitory motoneurons to the circular muscle (Costa *et al.* 1992). We have not investigated the effect of agents that interfere with VIP. However, our results indicate that the apamin-sensitive and nitric oxide-mediated mechanisms are essential for the emptying phase of peristalsis.

Inhibition of the longitudinal muscle during peristalsis

Fluid distension of the intestine elicits contraction of the longitudinal muscle which is mediated by acetylcholine release from excitatory longitudinal muscle motoneurons (Kosterlitz & Robinson, 1959). The present study indicates that this contraction is reduced in amplitude by nitric oxide, via an apamin-insensitive mechanism. Nitric oxide-mediated inhibition of the longitudinal muscle in strips of guinea-pig small intestine has previously been reported (Osthaus & Galligan, 1991). The site of nitric oxide synthesis, however, is not clear. Nitric oxide synthase is not present in longitudinal muscle motoneurons (Costa *et al.* 1992). However, it is present in a subpopulation of descending interneurons in the myenteric plexus of the guinea-pig small intestine (Costa *et al.* 1992). It is therefore possible that nitric oxide synthesized in these neurons may inhibit longitudinal muscle contraction by inhibiting excitatory longitudinal muscle motoneurons or by diffusing to the longitudinal muscle, causing inhibition directly.

The exact circuitry of the enteric neurons involved in the process of peristalsis has not been identified; however the results of this and other work (see Introduction) clearly indicate that excitatory and inhibitory motoneurons to the circular muscle and excitatory motoneurons to the longitudinal muscle are part of the circuitry.

In conclusion, we have demonstrated that transmission from enteric inhibitory motoneurons plays a crucial role in setting the threshold for initiation of the emptying phase of peristalsis and in allowing co-ordinated contractions to propagate in an oral-to-anal direction along the intestine.

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