Br. J. Pharmacol. (1990), **101**, 1011–1015

Differences in control of descending inhibition in the proximal and distal regions of rat colon

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1 Descending inhibition in the proximal and distal portions of rat colon was studied separately, *in vitro*.

2 In the proximal colon, localized distension with a small balloon caused three types of response (contraction; relaxation; relaxation, then contraction) of the circular muscle on the anal side of the distended region.

3 Distension caused descending relaxation of circular muscle in all segments of the proximal colon, although for this prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was necessary in some segments to increase muscle tone.

4 Atropine and guanethidine did not inhibit this descending relaxation, but tetrodotoxin did.

5 Hexamethonium inhibited the descending relaxation in 14 of 17 preparations of proximal colon tested, but not in the others.

6 In the distal colon, distension consistently caused an increase in the tone of the circular muscles. Descending relaxation was observed only after development of higher tone. Atropine and guanethidine did not inhibit the relaxation, but tetrodotoxin did.

7 Hexamethonium did not inhibit the descending relaxation in most of the preparations of distal colon examined.

8 AF64A, an inhibitor of choline uptake, inhibited the response mediated by cholinergic neurones *in vitro* to electrical transmural stimulation of the longitudinal muscle of proximal colon.

9 Treatment of colonic preparations with AF64A *in vitro* resulted in inhibition of descending relaxation in those of proximal, but not those of distal, colon.

10 The participation of intrinsic cholinergic neurones in the descending neuronal pathway is strongly suggested by the results in the proximal colon, but less so in the distal colon.

11 The tone and spontaneous contractile activity of colonic circular muscles are discussed in relation to their neuronal control.

Introduction

Stimuli applied to the intestinal wall produce contraction on the oral side (ascending contraction) and relaxation on the anal side (descending relaxation) of the stimulated region, resulting in peristaltic movements ('The law of the intestine', Bayliss & Starling, 1899). Descending relaxation has been observed in cat colon (Crema, 1970), dog intestine (Bayliss & Starling, 1899), rabbit colon (Julé, 1980) and guinea-pig colon (Costa & Furness, 1976). Myenteric neurones are known to contain neural pathways responsible for peristalsis of the intestine (reviewed by Kosterlitz & Lees, 1964; North, 1982). These neuronal pathways have been studied by inflation of a small balloon in the intestine (Frigo & Lecchini, 1970) or by mechanical distension (Costa & Furness, 1976) to produce a localized stimulus. Hirst & McKirdy (1974) demonstrated the presence of a descending neural pathway in the guinea-pig small intestine excited by distension of the intestinal wall or transmural electrical stimulation. They also suggested the involvement of cholinergic interneurones in the pathway, because the electrical response was inhibited by (+)-tubocurarine. The neural pathway of the descending reflex contains afferent sensory neurones, interneurones and inhibitory motor neurones. The inhibitory neurones have been suggested to be non-adrenergic, non-cholinergic (NANC; Costa & Furness, 1976). Grider & Makhlouf (1986) showed that the NANC inhibitory neurones in rat colon receive an input from cholinergic interneurones and release vasoactive intestinal peptide. Other studies have demonstrated cholinergic myenteric neurones whose depolarizing effect is reversibly blocked by hexamethonium (Sato *et al.*, 1973; Nishi & North, 1973; North *et al.*, 1980; Tokimasa *et al.*, 1981).

In the present work, we studied the responses of the circular muscles of the proximal and distal portions of rat colon to inflation of a balloon in the lumen. We also examined the participation of cholinergic interneurones in the descending inhibitory pathway by use of ethylcholine mustard aziridinium ion (AF64A), which has been shown to cause selective injury of cholinergic neurones in the peripheral nervous system (Mantione *et al.*, 1983a; McArdle & Hanin, 1986; Hoyle *et al.*, 1986).

Methods

Male Wistar rats (250–350 g) were used. They were stunned by a blow on the head and bled via the carotid artery. Segments of the proximal and distal colon were removed and placed in Tyrode solution consisting of (in $g l^{-1}$) NaCl 8, KCl 0.2, $CaCl_2$ 0.2, $MgCl_2$ 0.1, NaH_2PO_4 0.05, $NaHCO_3$ 1.0 and glucose 1.0. The faeces in the excised segments were gently flushed out with Tyrode solution. Segments of 4 to 6 cm of the proximal colon and distal colon were used. In all experiments the preparation was kept in 30 ml of Tyrode solution at 37°C aerated with 95% O_2 and 5% CO_2. The preparation was equilibrated for at least 30 min before the experiment was started. Drugs were added to the organ bath in volumes of less than 1% of the bathing solution.

Recording of responses of colonic circular muscle to the stimulus of distension

Colonic segments were held horizontally with the side adherent to the mesentery at the bottom in a specially designed organ bath (Figure 1). The middle of the segment was connected by a stainless-steel hook at the joint of the mesentery to an anchor fixed to the bottom of the bath. A rubber balloon, connected to a syringe by thin polyethylene tubing, was introduced into the lumen and positioned at the middle of the segment. The balloon was inflated with 0.1 to 0.2 ml of

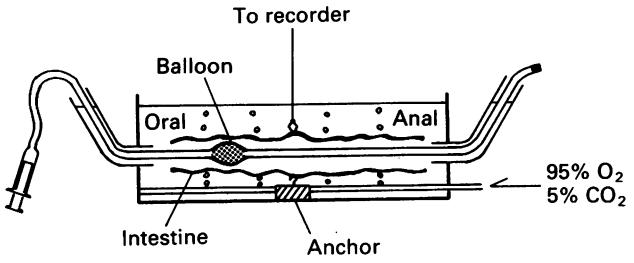

Figure 1 Diagram of the system used for recording the responses of isolated colonic segments to balloon inflation.

warm water from the syringe to produce slightly greater local distension than that produced by a faecal bolus. The duration of distension was 30 or 60 s. The mechanical response of the circular muscle about 1.0 cm anal to the balloon was recorded, by connecting a frog heart clip to a small area of the wall opposite to the anchor and then connecting the clip via a thread to an isotonic transducer (TD-112A, Nihonkohden, Tokyo, Japan). Both ends of the segment were free. This arrangement allowed preferential recording of the response of the circular muscle, since the balloon was deflated immediately after the development of the responses to avoid an effect on the spontaneous activity. The circular muscle was subjected to a resting load of 0.5 g.

Recording of responses of longitudinal muscle to electrical transmural stimulation

Colonic segments were suspended in an organ bath filled with aerated Tyrode solution, maintained at 37°C. The oral end of the segment was attached to a transducer and the anal end mounted on an anodal electrode placed at the bottom of the bath. Responses of the longitudinal muscle to transmural stimulation, with trains of 50 pulses of 0.1 ms width and supramaximal voltage (usually 30 V) at a frequency of 10 Hz, were recorded isotonically and successively with a 10 min interval between tests and described in the preceding paragraph.

Preparation of AF64A

AF64A was prepared from ethylcholine mustard (AF64) picrate as described by Fisher *et al.* (1982). Briefly, AF64 picrate was dissolved in saline and picrate was removed with anion exchange resin. The solution was then adjusted to pH 7.4 by addition of solid $NaHCO_3$, stood for 1 h at room temperature and then used as AF64A. AF64A was prepared just before each experiment. The conversion of AF64 to AF64A measured by the iodine-thiosulphate method (Sandberg *et al.*, 1984) was 91.5%, so the concentration of AF64A was corrected on the basis of this value.

Treatment of colon segments with AF64A

Colonic segments *in vitro* were treated with AF64A prepared as described above by adding the agent into the bath at a final concentration of 62.5 μM.

Drugs

Ethylcholine mustard picrate (AF64) was a gift from Mitsubishi Kasei Kogyo Co., Yokohama, Japan. Tetrodotoxin was a gift from Sankyo Co., Osaka, Japan, and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) a gift from Ono Pharmaceutical Co., Osaka, Japan. $PGF_{2\alpha}$ was dissolved in ethanol and added to the organ bath at a final concentration of 10 ng ml^{-1} in 0.1% ethanol. This concentration of ethanol did not affect the responses. All other chemicals were of analytical grade and dissolved in distilled water.

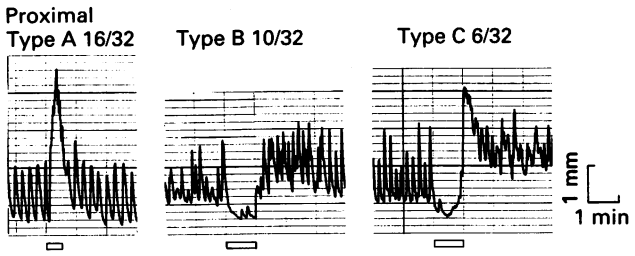

Figure 2 Responses of proximal segments of rat colon to balloon inflation. The segments were radially distended with a small balloon and the responses of the circular muscle 1 cm anal to the balloon were recorded. Small rectangles indicate 30 s distension and large ones 60 s distension. Ratios indicate the number of preparations that showed the indicated response to the total numbers of preparations studied. Details of experimental conditions are described in the Methods.

Results

Descending responses of the proximal colon to local distension

At rest, circular muscle of the proximal colon exhibited spontaneous rhythmic contractions. On stimulation, three types of response were observed on the anal side of an inflated balloon (Figure 2). Of 32 preparations, 16 showed only contraction (designated as type A), 10 showed only relaxation (type B) and 6 showed relaxation followed by contraction (type C). The type B response occasionally changed to a type C response with spontaneous decrease in the resting tone of the preparation during the experiment. The type of response, especially development of relaxation, appeared to be closely related to the resting tone of the preparation, because the type B response was elicited in preparations that initially exhibited a type A response to local distension when their resting tone was increased by treatment with $PGF_{2\alpha}$ (Figure 3).

Hexamethonium inhibited the descending relaxation in 14 of 17 preparations of the proximal colon, but did not affect relaxation in the other 3 preparations (Figure 4). Guanethidine (4 μM) did not have any significant effect on the descending relaxation, but tetrodotoxin (1 μM) completely blocked the relaxation of all preparations tested. Tetrodotoxin slightly stimulated the spontaneous contractile activity of circular muscle, as has been found previously (Manzini *et al.*, 1986). Atropine (1 μM) did not diminish the inhibitory phase of the response. These results indicate that NANC neurones mediate the descending relaxation of the proximal colon.

Descending responses of distal colon to local distension

Preparations of the distal colon exhibited either no spontaneous contraction of circular muscle or slight contraction at low frequency, even after a long period of equilibration in the

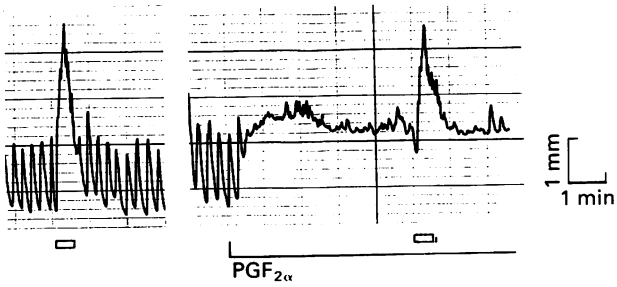

Figure 3 Effect of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on proximal colonic segments that exhibited the type A response. $PGF_{2\alpha}$ (10 ng ml^{-1}) was added to the organ bath. For further details see legend of Figure 2 and Methods.

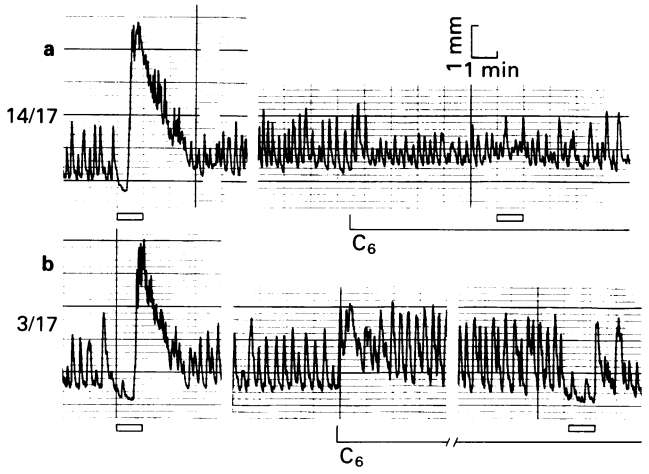

Figure 4 Effect of hexamethonium (C_6, 500 μM) on descending response of a proximal colon to balloon inflation. The descending response was either abolished (a) or not affected (b). For further details see legend of Figure 2 and Methods.

organ bath. Almost all preparations showed only contraction of circular muscle anal to the distended region in the initial 3 or 4 tests with local distension. However, the resting tone of the circular muscle gradually increased during successive distensions and never returned to the pre-stimulus level, even after repeated changes of the bathing solution. When a higher resting tone was acquired in this way, the preparation began to exhibit descending relaxation followed by contraction instead of contraction only (Figure 5). Neither atropine nor guanethidine affected the inhibitory response. Tetrodotoxin increased the resting tone and abolished the response to local distension, as in the proximal portion of the colon. In 17 experiments in which the effect of hexamethonium treatment was investigated, descending relaxation was abolished in only one case, partially inhibited in 6 and unaffected in the other 10. This agent increased the tone in all preparations (Figure 6).

Effect of AF64A treatment on the cholinergic response of rat proximal colon

Electrical transmural stimulation induced a small phasic contraction followed by a transient rapid relaxation and a large rebound contraction. These responses were consistently observed after each stimulus during a period of 2 h. The initial small phasic contraction seemed to be cholinergic because atropine abolished it without affecting the following two phases of the response (Figure 7). Treatment of the colonic segment with AF64A *in vitro* resulted in selective inhibition of the small phasic contraction in all 4 preparations tested within 2 h, with no other changes (Figure 8).

Effect of AF64A on descending relaxation

The descending relaxation of the proximal colon in response to local distension was inhibited by pretreatment with AF64A

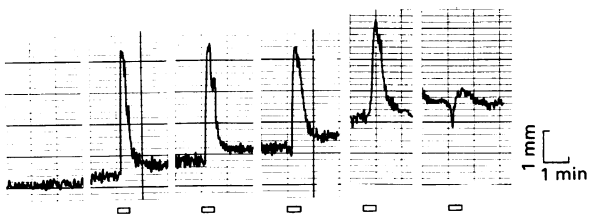

Figure 5 Change in response of the distal colon to balloon distension with time. The preparation was distended repeatedly with a small balloon. For further details see legend of Figure 2 and Methods.

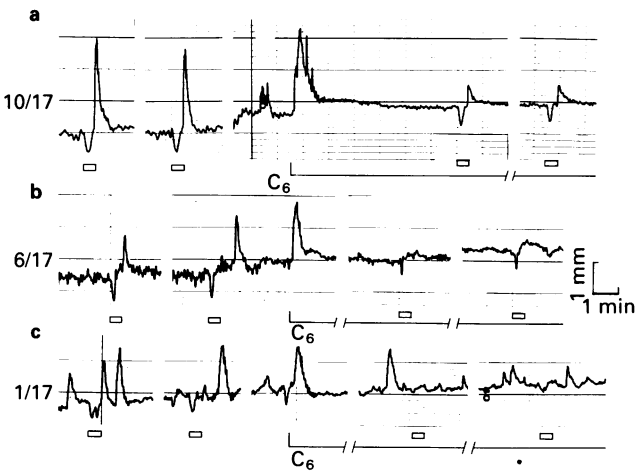

Figure 6 Effect of hexamethonium (C_6, 500 μM) on descending responses of distal colon to balloon inflation. (a) Results from a preparation in which hexamethonium did not affect relaxation. (b) Results from a preparation showing partial inhibition by C_6. (c) Results from a preparation showing complete inhibition by C_6. Ratios are numbers of preparations showing the response to total numbers of preparations tested. For further details, see legends of Figures 2 and 4.

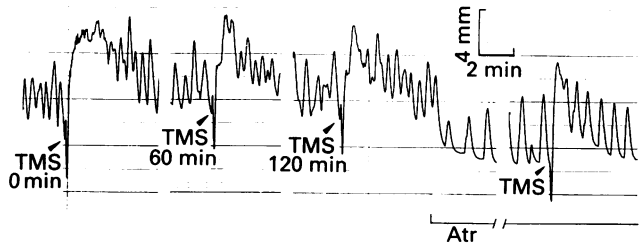

Figure 7 Response of longitudinal muscle of colonic segments to electrical transmural stimulation and effect of atropine (Atr, 1 μM) on the response. A preparation of proximal colon was stimulated transmurally by trains of electrical pulses (TMS) at the point marked by an arrowhead. The time after the beginning of the experiment is shown. For further details see Methods.

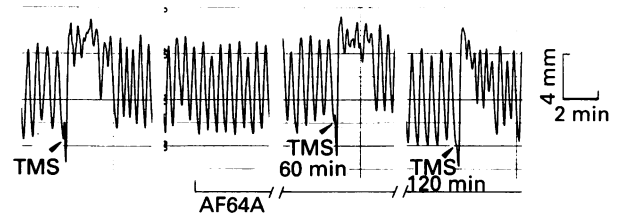

Figure 8 Effect of AF64A treatment on the response to transmural electrical stimulation. A preparation of proximal colon was treated with AF64A (62.5 μM) *in vitro*. The time indicates that after the beginning of the treatment. For further details see legend of Figure 7 and Methods.

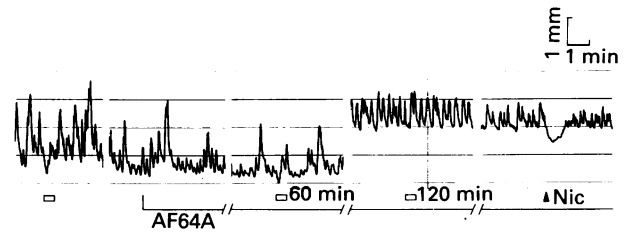

Figure 9 Effect of AF64A treatment on descending relaxation of the proximal colon on balloon distension. A preparation of proximal colon was distended with a balloon before and after AF64A treatment. Nicotine (Nic, 10 μg ml^{-1}) was added in the presence of AF64A. For further details see legend of Figure 7 and Methods.

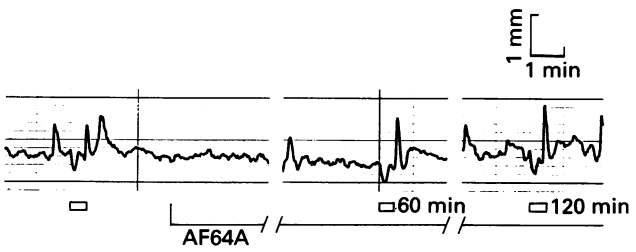

Figure 10 Descending relaxation of a distal colonic segment on balloon distension before and after AF64A treatment. For further details see legends of Figures 7 and 9 and Methods.

within 2 h in all 4 preparations studied. The preparations were still responsive to nicotine when the descending relaxation had been abolished (Figure 9). AF64A had no effect on any of the 4 preparations of distal colon studied (Figure 10).

Discussion

There are differences in the morphology (Christensen *et al.*, 1984) and density (Hukuhara & Neya, 1968) of the myenteric plexus and in its sensitivities to drugs (Fink & Friedman, 1960) in the proximal and distal portions of rat colon. The contents of the intestinal lumen are dehydrated in the proximal colon and pellets of faeces are mainly formed in the distal colon (Ferré & Ruckebusch, 1985). Thus, the two portions of the colon differ greatly in motility and physiological functions.

Rubbing the mucosa with an uninflated balloon did not induce any descending responses. Inflation of the balloon with a volume over 0.1 ml was effective in inducing descending responses in almost all of the segments. By increasing the volume up to 0.2 ml the responses became more distinct without any changes in their shape. With volumes over 0.25 ml, mechanical artifacts were often observed on the record. Hence, we assumed that the inflation of the balloon induced the responses via the mechanoreceptors within the intestinal wall. Paintal (1954a,b) have alluded to the fact that mucosal afferents tend to respond in an 'all or none' manner to a local stimulus while the in-series mechanoreceptors in muscle show a graded response.

Some preparations of proximal colon showed only descending contraction (type A) in response to local distension, whereas others showed descending relaxation (type B, C; Figure 2). However, segments that exhibited the type A response also showed descending relaxation when their tone was increased by treatment with $PGF_{2\alpha}$ (Figure 3). $PGF_{2\alpha}$ is known to induce contraction of the circular muscle of rat colon (Flesher & Bennett, 1969) and increase the resting tone of the colon (Eckenfels & Vane, 1972). Thus the reason why preparations exhibiting the type A response did not show an inhibitory phase in response to local distension was probably that the initial tension of their circular muscle was low. In general, we supposed that inflation of the balloon caused a relaxation followed by contraction. The finding that distal segments showed descending relaxation only after some increase in tone induced by repetitive distensions (Figure 5) supports this hypothesis. Tetrodotoxin increased the spontaneous contractile activity in both the proximal and distal colonic segments. It also significantly increased the tone of circular muscle in the distal segment, but not in the proximal segment. These findings indicate the existence of a tonic inhibition of the spontaneous contractile activity of the colon by inhibitory neurones. Similarly, tonic sympathetic nerve activity exerts an inhibitory effect on proximal and midcolonic motility of the cat (Gillis *et al.*, 1987) and tonic NANC-like activity suppresses the proximal rat colon (Maggi *et al.*, 1987). The above findings also indicate the existence of some neural inhibitory control of the tone of the circular muscle in the distal colon.

Bayliss and Starling (1899) proposed that a local stimulus to the intestine causes contraction on the oral side and relaxation on the anal side of the stimulated region, thereby resulting in transport of the intestinal contents from the oral region to the anus. Some exceptions to descending relaxation have been obtained, such as in the small intestine of the guinea-pig (Yanagiya & Ohkubo, 1958), domestic fowl (Hodgkiss, 1986) and rabbit (Ozaki, 1979). However, there is also an interesting finding that in guinea-pig small intestine local distension produces excitation of descending inhibitory neurones (Hirst & McKirdy, 1974). These and the present findings indicate that excitation of descending inhibitory neurones occurs in the intestine of many species, but that the degree of tone of the tissue may determine whether relaxation occurs.

The inhibitory effect of tetrodotoxin and the absence of effects of atropine and guanethidine on the descending relaxation in the proximal and distal colon of the rat indicate that NANC neurones mediate the relaxation observed in the present study.

Hexamethonium, an antagonist of nicotinic cholinoceptors, inhibited the descending relaxation to local distension in 14 of 17 preparations of the proximal colon, but in only one preparation of the distal colon. The results suggest that there are many cholinergic interneurones of the descending inhibitory pathway in the proximal colon, but few in the distal colon. Crowcroft *et al.* (1971) have shown that cholinergic neurones within the wall of the distal colon extend their axons to the inferior mesenteric ganglion and terminate on the noradrenergic neurones which could depress the activity of excitatory neurones of the colon in guinea-pig. In the present study, the experiments were carried out with the colonic segments of rat. Therefore, the contribution of the nerve pathway proposed by Crowcroft *et al.* (1971) is not relevant to the present experiments on descending relaxation.

AF64A has been shown to cause selective degeneration of cholinergic neurones in the central nervous system (Fisher & Hanin, 1980; Fisher *et al.*, 1982; Mantione *et al.*, 1983b; Sandberg *et al.*, 1984; Walsh *et al.*, 1984). One of its main effects is thought to be inhibition of the high affinity choline uptake system at the nerve terminals (Rylett & Colhoun, 1980; 1984; Mantione *et al.*, 1981; Fisher *et al.*, 1982; Curti & Marchbanks, 1984). There are few studies on the effect of AF64A on the peripheral nervous system (Mantione *et al.*, 1983a; Allen, 1983; Hoyle *et al.*, 1986). We found that AF64A selectively inhibited the small phasic contraction (Figure 8) that could be inhibited by atropine. Therefore, AF64A probably caused dysfunction of cholinergic neurones in the colon *in vitro* as has previously been suggested (Rylett & Colhoun, 1978; Mantione *et al.*, 1983a; Potter *et al.*, 1985). Our finding that AF64A inhibited the descending relaxation in all 4 segments of the proximal colon examined, but not in any of the 4 segments of the distal colon examined, are consistent with findings that hexamethonium abolished the input of signals from cholinergic interneurones by blocking nicotinic cholinoceptors on NANC neurones. The present results, therefore suggest a significant participation of cholinergic interneurones in the proximal, but not the distal portion of the rat colon in the descending neural pathway.

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(Received April 23, 1990
Revised July 10, 1990)
Accepted July 26, 1990)