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Evidence for a functional cholinergic deficit in human colonic tissue resected for constipation

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Abstract—There is evidence to suggest an abnormality of the colonic myenteric plexus in severe chronic constipation. The present study investigates whether this abnormality involves functional changes in the cholinergic innervation of human colon. Human taenia coli muscle strips (taenia), previously incubated with [^{3}H]choline to radiolabel neuronal stores of acetylcholine, were subjected to electrical field stimulation (1 Hz or 10 Hz, 1 ms, 480 pulses at 200 mA). The stimulation evoked release of tritiated material, shown previously to accurately represent neural [^{3}H] acetylcholine release, was depressed in tissue from constipated compared with non-constipated patients. Evoked release of tritiated material was reduced by storage of the taenia at 4 °C or by increasing the frequency of stimulation, but increased by stimulation during incubation with [^{3}H]choline. The results indicate that reduced activity of cholinergic nerves may occur within the bowel wall of colon removed for severe chronic constipation.

Patients with severe chronic constipation may or may not respond to intraluminal bisacodyl with onset of peristaltic activity (Preston & Lennard-Jones 1985; Shouler & Keighley 1986). One possible interpretation of this observation is that non-responders have a disorder of the myenteric plexus which prevents a response to bisacodyl. Abnormality of the myenteric plexus has been observed in some young women with severe constipation who have severe clinical disability (Krishnamurthy et al 1985). The present study investigates whether the disorder of severe chronic constipation affects functional activity of cholinergic nerve fibres within human isolated colonic tissue. A radiolabelling technique (Wikberg 1977) was employed to assess the activity of intrinsic cholinergic nerves.

Materials and methods

Source of material. Specimens resected for constipation were obtained from six female patients (aged 20 to 74 years), undergoing colectomy with ileo-rectal anastomosis. Control colonic tissue was obtained from 25 female or male patients, 23 at sigmoid colectomy and 2 at right hemicolectomy (20 of the specimens were resected for carcinoma, two for carcinoma with diverticular disease and three for diverticular disease). macroscopically normal human ascending and sigmoid taenia coli muscle strips (2 × 20 mm), henceforth referred to as taenia, consisting of muscularis externa without attached mucosa and submucosa, were suspended in 1·5 mL organ baths containing Krebs bicarbonate buffer, maintained at 37 °C and gassed with 5% CO_2 in O_2. The composition of the Krebs fluid was (mM): Na^+ 140, K^+ 5·9, Ca^{2+} 2·5, Mg^{2+} 1·2, Cl^- 122, HCO_3^- 25, SO_4^{2-} 1·0, $H_2PO_4^{2-}$ 1·2, glucose 11·5.

Radiolabelling and measurement of [^{3}H]acetylcholine overflow. Wikberg (1977) has shown that radioactive tracers accurately quantitate acetylcholine (ACh) release. The evoked release of tritiated ([^{3}H]) material by electrical field stimulation, after previous incubation of the tissue with [^{3}H]choline, accurately estimates the [^{3}H]ACh release from taenia muscle strips (Burleigh & Trout 1986).

Taenia muscle strips were placed under a tension of 0·5 g and changes in motility were recorded with SRI isotonic transducers and displayed on a Rikadenki potentiometric pen recorder (8× magnification). Some muscle strips were stored overnight at 4 °C before use. The preparations were incubated for 60 min, at 37 °C, in Krebs fluid containing 4 μCi mL^{-1} of [^{3}H]choline chloride (spec. act. 15 Ci mmol^{-1}, Amersham) after which they were superfused at 2·2–2·4 mL min^{-1} with Krebs fluid containing hemicholinium (34·8 μM). Some muscle strips were stimulated (10 Hz, 1 ms, 200 mA) during the first 30 min of the incubation period. Incubation and superfusion (by displacement overflow) of individual taenia preparations were carried out in the same 1·5 mL volume baths which were fitted with vertical platinum wire electrodes (0·5 mm diameter) to allow electrical field stimulation (EFS) of the tissue from a Hugo Sachs constant current stimulator. After 90 min equilibration, the strips were stimulated (1 or 10 Hz, 1 ms, 480 pulses at 200 mA). Superfusion fluid was collected for 4 min periods, and 0·5 mL aliquots removed from each sample for liquid scintillation counting. Three sample collections were made immediately before stimulation (basal release), four sample collections were made during and after stimulation (stimulated release) and finally a further three samples were collected (basal release). Efficiency of counting was determined by the automatic external standard channels ratio method. Radioactive content of the superfusion samples was expressed as disintegrations min^{-1} mg^{-1} of tissue (d min^{-1} mg^{-1}) according to the formula:

$$\text{Radioactivity per sample} = \frac{\text{d min}^{-1} \times \text{FR} \times \text{T}}{\text{W} \times \text{V}}$$

where d min^{-1} = disintigrations per minute (this value supplied by the spectrometer), FR = superfusion flow rate, T = sample collection time, W = taenia muscle strip weight, and V = volume of sample added to scintillation fluid.

The evoked release of radioactive material, collected during or after EFS was calculated from the difference between the 'calculated' basal release and the stimulated release (stimulated release = calculated basal release + evoked release). Calculated basal release was obtained by fitting a regression line through observed basal values. The sum of evoked values for a given challenge was expressed as the % of radioactivity present in the tissue at the beginning of the challenge (i.e. % radioactivity present released per stimulation period = % RPR).

Data analysis

Data are expressed as medians, with interquartile ranges, and the number of observations is given in parentheses. Statistical comparisons were made on the number of strips using the Mann-Whitney U-test (2-tailed).

Results

Cholinergic component of motor response. A cholinergic motor response of taenia to EFS can be revealed. Thus, in the presence of physostigmine (0·31 μM) responses to EFS (1 Hz, 1 ms, 60 pulses at 200 mA) were converted from biphasic to a large contraction with almost no relaxation. The contraction,

in the presence of physostigmine, was reversibly prevented by tetrodotoxin (3·14 μM). After removal of tetrodotoxin, but not physostigmine, the contraction recovered. Hyoscine (2·28 μM) reduced the contraction and a larger relaxation was unmasked (Fig. 1).

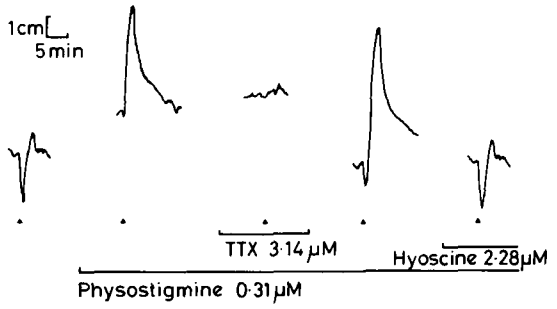

FIG. 1. Response of a human sigmoid taenia coli muscle strip to electrical field stimulation (1 Hz, 1 ms 60 pulses at 200 mA). In the absence of drugs a biphasic response was produced. Physostigmine converted the biphasic response to a large contraction with almost no relaxation. The contraction in the presence of physostigmine, was reversibly prevented by tetrodotoxin. After removal of tetrodotoxin, but not physostigmine, the contraction recovered. Hyoscine reduced the contraction and a larger relaxation was unmasked.

Release of tritiated material by electrical field stimulation. The effects of constipation, overnight storage of muscle strips at 4 °C, stimulation of muscle strips during incubation with [^{3}H]choline (10 Hz, 1 ms, 200 mA for 30 min) and stimulation frequency (1 or 10 Hz) on the release of tritiated material by EFS (shown previously to be equivalent to [^{3}H]ACh release) are summarized in Table 1. Taenia resected for constipation released less [^{3}H]ACh than taenia resected for local malignancy. Release was also reduced by overnight storage and stimulation at the higher frequency of 10 Hz, but increased by stimulation during incubation. Regional differences in transmitter release were not observed in constipated tissue. Thus in four specimens of constipated colon ascending taenia gave a % RPR value of 0·82 (0·61–1·03, 8 muscle strips) while sigmoid taenia gave a % RPR value of 0·78 (0·70–0·98, 12 muscle strips; $P > 0·5$).

Table 1. Release of tritiated material from human taenia coli muscle strips by electrical field stimulation (1 or 10 Hz, 1 ms, 480 pulses at 200 mA). Release expressed as percentage of radioactivity present in tissue at beginning of stimulation period. n = number of muscle strips with number of specimens in parentheses.

Non-constipated tissue stimulated at:			1 Hz + stim during [^{3}H]-choline incubation	Constipated tissue stimulated at:
1 Hz	10 Hz	1 Hz + storage at 4 °C		1 Hz
0·95 (0·92–1·33) (n = 9)	0·68[2] (0·64–0·87) (n = 4)	0·43* (0·42–0·52) (n = 6)	1·51 (1·09–1·85) (n = 6) 1·54 (1·47–1·85)[1] (n = 5)	0·75* (0·69–0·85) (n = 6)

*$P < 0·05$.
[1] $P < 0·05$ when 1 value of 0·38 omitted.
[2] $P < 0·05$ using t-test for 2 independent samples (2-tailed).

Pathology of colonic specimens resected for constipation. There was no consistent gross abnormality in any of the six specimens investigated. Three specimens had a darkened mucosal surface (melanosis coli) one of which also showed localized evidence of diverticular disease. One specimen had scattered polyps, one was slightly dilated proximally and in another there was marked dilatation of the distal colon. Histological sections stained by haematoxylin and eosin were prepared from all regions of each specimen. The mucosa, submucosa and muscle coats appeared normal, apart from melanosis coli in three cases. The complement of nerves and ganglion cells within the myenteric and submucosal plexuses appeared normal, though no special techniques for their identification were performed.

Discussion

Overnight storage of human gut tissue at 4 °C is an accepted procedure and one reported not to affect motor responses to EFS (Bennett & Stockley 1975). However, radiolabelling studies reveal a significant depression of [^{3}H]ACh release due to this procedure; henceforth stored tissue was not used for radiolabelling experiments. The applicability of the radiolabelling technique to human taenia is demonstrated by the findings that [^{3}H]ACh release increased when the tissue was stimulated during incubation but decreased if the frequency of stimulation, used to release labelled material, was increased. These findings are well documented using animal tissue and are due, respectively, to enhanced turnover (and therefore radiolabelling) of neuronal ACh store (Szerb 1975) and activation of presynaptic autoinhibitory receptors by raised levels of synaptic ACh (Cowie et al 1978; James & Cubeddu 1984).

The reduction in [^{3}H]ACh release from constipated tissue was probably not restricted to one area of the colon. In the four specimens where taenia were cut from both ascending and sigmoid regions it was not possible to detect a significant difference in neurotransmitter release between the two regions. This finding is supported by histological evidence using silver staining techniques, where abnormalities in the myenteric plexus were found in all regions of colons resected for constipation (Krishnamurthy et al 1985). The same authors failed to reveal any abnormalities of the myenteric plexus when conventional haematoxylin and eosin staining of serial section was used. The possibility of abnormal cholinergic innervation in severe constipation would not be surprising considering the established role of acetylcholine as a neurotransmitter at neuro-neuronal and neuro-effector synapses in the enteric nervous system (Kosterlitz & Lees 1964; Paton & Zar 1968; North 1982; Furness et al 1983).

At present there appears to be no consensus on the pathophysiology of constipation (Read et al 1986), although abnormal colonic motility may be involved. The abnormality of the colonic myenteric plexus found in individuals with severe constipation (Krishnamurthy et al 1985) was unlikely to be due to laxative abuse (Krishnamurthy et al 1985; Badiali et al 1985). In the dog, pelvic nerves enter the wall of the rectum and travel up to a maximum of 50% of the way round the colon as intramural pelvic nerves (Fukai & Fukuda 1984). These nerves, which have been recognized in human colon, probably convey the extrinsic parasympathetic supply to the colon and may mediate colonic reflexes. Damage to such nerves could lead to constipation (Christensen & Schultz-Delrieu 1985). Cholinergic nerve activity may be supressed in severe constipation by other causes. The depression, by morphine, of acetylcholine release from cholinergic nerves in human isolated colonic muscle (Burleigh & Trout 1986) and the beneficial effects of naloxone in chronically constipated individuals indicates that excessive activity of endogenous opioid mechanisms could contribute to chronic constipation (Kreek et al 1983, 1984).

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Myoclonic seizures in the mouse induced by alphaxalone and related steroid anaesthetics

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Abstract—The anaesthetic steroids alphaxalone. 5β-alphaxalone and pregnanolone each caused myoclonic jerks in mice in a dose-related manner between 4 and 16 mg kg^{-1} i.v. There was no loss of righting reflex at these doses. The veterinary product Saffan, which contains alphaxalone and alphadalone, also caused myoclonic jerks at 2 mg kg^{-1} i.v., and a loss of righting reflex at doses of 4 mg kg^{-1} and above. These effects appear to be unrelated to the wide spectrum of potencies at the $GABA_A$ receptor complex of the three individual steroids as potentiators of muscimol, or as attenuators of picrotoxin.

The steroidal anaesthetic, alphaxalone, potentiates responses to the $GABA_A$ receptor agonist, muscimol, in the rat cuneate nucleus (Harrison & Simmonds 1984) and prolongs the open times of Cl^- channels operated by the $GABA_A$ receptor (Barker et al 1987). These actions are similar to those of barbiturates. However, the barbiturates differ as to whether they possess anticonvulsant as well as anaesthetic actions, e.g. pentobarbitone is not anticonvulsant at sub-anaesthetic doses, whereas phenobarbitone is anticonvulsant at doses that are not excessively sedative. These two barbiturates have different profiles of interaction with the $GABA_A$ receptor complex (Harrison & Simmonds 1983). Their relative potencies as potentiators of muscimol correlate well with their relative anaesthetic potencies. But, at equi-effective concentrations for a small potentiation of muscimol, phenobarbitone reduced the antagonism of muscimol by picrotoxin, whereas pentobarbitone did not (see Simmonds 1986). It was therefore suggested that this latter phenomenon might be relevant to the anticonvulsant action of phenobarbitone.

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Similar studies of the effects of a series of steroids related to alphaxalone on the rat cuneate nucleus have shown an analogous distinction between the structure/activity profile for potentiation of muscimol, and that for the reduction in the potency of picrotoxin as a muscimol antagonist (Turner 1987). Therefore we selected for study as potential anticonvulsants three steroids that were distinctly different from each other in terms of their interactions with the $GABA_A$ receptor complex. They were alphaxalone (3α-hydroxy-5α-pregnane-11,20-dione), pregnanolone (3α-hydroxy-5β-pregnane-20-one) and 5β-alphaxalone (11-keto-pregnanolone). Their potencies as potentiators of muscimol were alphaxalone > pregnanolone > 5β-alphaxalone (Simmonds & Turner 1987). At equi-effective concentrations for a small potentiation of muscimol, alphaxalone had no effect on picrotoxin potency, whereas pregnanolone caused a large reduction that was substantially greater than that previously reported for phenobarbitone (Turner 1987). 5β-Alphaxalone also caused a large reduction in picrotoxin potency at concentrations that produced little or no potentiation of muscimol.

We therefore started with the aim of comparing in-vivo the sedative and anticonvulsant actions of these steroids. A decrease in spontaneous locomotor activity is a sensitive measure of sedative drug effects and this was measured in a holeboard (File & Wardill 1975). Pilot experiments were conducted to determine the time at which the effects of alphaxalone (1 mg kg^{-1} i.v.) were maximal in the holeboard.