Characteristics of cholinergic neuroeffector transmission of ganglionic and aganglionic colon in Hirschsprung's disease

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Abstract

Differences in the release and content of acetylcholine and the α_2 adrenoceptor mediated interaction between noradrenergic and cholinergic neurons were investigated by neurochemical and pharmacological methods in aganglionic and ganglionic segments of isolated human colon taken from children suffering from Hirschsprung's disease. Both at rest and during transmural stimulation the release of acetylcholine was significantly higher in the spastic (aganglionic) segment than in the proximal dilated bowel. Significant differences were found in the tissue concentration of acetylcholine between ganglionic and aganglionic specimens. The pattern of response to transmural stimulation was also different in the spastic and dilated bowel. Transmural stimulation induced relaxation and contraction in ganglionic specimens but only contractions in aganglionic specimens. The sensitivity of the smooth muscle in the aganglionic portion to exogenous acetylcholine and to field stimulation was found to be higher than in the ganglionic portion. While noradrenaline added to the organ bath reduced the stimulation-evoked release of acetylcholine from spastic segments, via an α_2 adrenoceptor mediated process, yohimbine did not enhance the release. It is suggested that in Hirschsprung's disease the increased acetylcholine release, the enhanced sensitivity of smooth muscle cells to acetylcholine, and the lack of α_2 adrenoceptor mediated noradrenergic modulation of acetylcholine release from cholinergic interneurons might be responsible for the spasm of aganglionic segments.

Hirschsprung presented the problem of megacolon in his classic paper on 'Constipation in newborn due to dilatation and hypertrophy of the colon' in 1887.¹ The disease discovered by Hirschsprung resulted in many theories but few facts as to its cause. In 1946, however, Ehrenpreis² concluded that the typical megacolon was not congenital but developed secondarily to the constipation. Two years later, Swenson and Bill³ presented conclusive evidence of spasm in the rectosigmoid or rectum as the cause of congenital constipation. In addition, Whitehouse and Kernohan⁴ showed that absence of ganglion cells of the myenteric plexus in the narrow distal segment was the underlying pathology. The bowel dysfunction in Hirschsprung's disease¹⁻³ is now known to be produced by abnormalities of the enteric nerves present in the gut.⁴⁻⁹ The

mechanism of the spasm of the aganglionic segment is not known, however.

Few investigations have been carried out on the storage, release, and metabolism of neurotransmitter substances in Hirschsprung's disease. It was found that the content of acetylcholinesterase⁵⁻⁶ and acetylcholine⁷ were increased in the spastic segment. Preliminary reports⁸⁻⁹ showed that the normal arrangement of adrenergic nerves around myenteric ganglia was absent in the affected segment of Hirschsprung's disease, but that there was a tendency for the numbers of adrenergic nerves in the muscle layers to be increased. An increase, however, in noradrenaline content does not explain the increased tone of the aganglionic segment because sympathetic nerves inhibit intestinal tone and acetylcholine release¹⁰⁻¹² provided noradrenaline is released normally. A similar observation was made on isolated human taenia coli¹³: noradrenaline added to the organ bath reduced the release of acetylcholine evoked by electrical stimulation. Little information exists about the activity of intramural nerves in Hirschsprung's disease with regard to both the relation between the nervous elements and the functioning of the neuroeffector junction.¹⁴ The present paper deals with the acetylcholine content and release in the aganglionic parts as well as with the pattern of longitudinal muscle response during stimulation of the intramural nerve fibres and to different drugs.

Methods

Experiments were carried out on full bowel wall thickness preparations taken from the large intestine of four children, aged 0.5 to 2 years, suffering from typical Hirschsprung's disease. Hirschsprung's is relatively rare in Hungary (1:6000 live births), but the cases we consider here were excellent clinical examples from all viewpoints – medical history, clinical findings (chronic or intermittent constipation, abdominal distension, vomiting, severe intestinal obstruction), and histopathology (an aganglionic segment). The patients were anaesthetised with atropine (0.01–0.02 mg/kg) maintained with nitrous oxide plus halothane, and adequate muscle relaxation was achieved with intravenous d-tubocurarine or succinylcholine (1 mg/kg).

The resected colon was immediately immersed in cold, gassed Krebs solution and removed to the laboratory within 30 min. Strips cut longitudinally, about 2–3 cm long and 4 mm wide, were removed. The strips were suspended in organ baths containing 3.5 ml of Krebs solution at 35°C, gassed with a 95% CO₂ + 5% O₂ mixture,

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and placed under a tension of about 1.5–2.5 g. Changes of bath fluid were made by overflow. To avoid damage to the intramural plexuses no attempt was made to separate the muscle layers. Preparations were taken from both the constricted segment and for comparison from the proximal dilated bowel 5–10 cm above the upper border of the spastic segment. Care was taken not to cut the strips from the cone shaped transitional zone. Since there are structural differences in the intramural plexus between the narrow and the dilated segments,¹⁴ microscopic examination was always performed to verify the aganglionosis in the narrow segment and the presence of normal sized nerve fibres and ganglion cells in the dilated segment.

After the appearance of spontaneous motility the ability of the strips to respond to electrical stimulation was investigated by applying a series of rectangular pulses lasting 1.0 s supramaximal strength 10 V/cm and 1 ms duration at 10 or 20 Hz. Stimuli were delivered by a DISA Multistim stimulator through a pair of platinum electrodes placed at the top and bottom of the organ bath (field stimulation, Paton and Vizi¹⁰). Responses to field stimulation and to drugs added to the organ bath were recorded isometrically with a strain-gauge transducer on a Servogor pen-recorder.

Acetylcholine output was measured both at rest and during transmural stimulation. For this purpose rectangular pulses of 1 ms duration at supramaximal voltage 10 V/cm with a frequency of 1, 10, and 20 Hz were applied throughout successive periods of 15 min, respectively. The total content of acetylcholine in tissue was extracted by the method of Vizi and Pasztor.¹⁵ It was then assayed by the technique described by Paton and Vizi.¹⁰ The resting output, in pmol/g per min, was given by $(R/t) \cdot (1000/W)$, where R is the total output in pmol as measured in the assay, W is the weight of the strip in mg measured after the experiment, excess moisture on the strip being removed by pressing the tissue between two filter papers, and t is the collection period in min. The output per volley is given in pmol/mg per volley by $(S-R/t.f) \cdot (1000/W)$, where S is the total acetylcholine output due to stimulation together with the presumed resting output over the collection period used, R is the resting acetylcholine output in pmol during the period of collection calculated from the control resting output, f is the rate of stimulation per min, W is the weight of the strip in mg, X is duration of stimulation in min. The acetylcholine content was expressed in nmol/g.

Gel filtration was used¹⁵ to identify the substance obtained from human tissue and assayed on guinea pig ileum as acetylcholine. A Sephadex G-10 column of 4 ml (20 cm in length and 5 mm in diameter) was prepared, and the samples collected or extracted were eluted through the column. The void volume measured with dextran blue was 1.5 ml. When a sample had entered the top of the gel bed, the collection of fractions was begun. The eluate fractions of 0.5 ml were then tested on guinea pig ileum or their radioactivity was measured when (¹⁴C) acetylcholine was eluted and the elution profiles were compared. The elution pattern of ¹⁴C-labelled acetylcholine was measured by bioassay plus radioassay taking 0.5 ml fractions of eluate. Because the elution of both biological activity and label occurred in the same 2–3.5 ml where the endogenous substance appeared, it was concluded that the substance assayed on guinea pig ileum was acetylcholine.¹⁶

SOLUTIONS AND DRUGS

The Krebs solution had the following composition (mM) NaCl 113, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25.0, and glucose 11.5. Drugs used were acetylcholine iodide (BDH), atropine sulphate (Biogal), phenolamine (Ciba-Geigy), tetrodotoxin (Sankyo), physostigmine sulphate (BDH), 1-noradrenaline (Sigma), and yohimbine (Chinoin).

Results

RELEASE AND CONTENT OF ACETYLCHOLINE

Due to the difficulty in obtaining samples of normal descending or rectosigmoid colon from healthy children, a comparison of the acetylcholine release at rest and during field stimulation had to be made between samples of the narrow and dilated segments. The results are shown in Table I.

It seems that both during rest (mean (SEM) 4.90 (0.96) pmol/g per min) and during transmural stimulation with 10 Hz (mean (SEM) 70.0 (3.08) pmol/g per min) the release of acetylcholine from the aganglionic specimens was significantly higher than that from the strip taken from the proximal dilated bowel (2.1 (0.10) and 5.9 (0.44) pmol/g per min, respectively). The average ratio between stimulated (10 Hz) and spontaneous release was 14.3 (1.20) in the aganglionic segment and 2.80 (0.18) in the proximal segment, each value being calculated from four

TABLE I Content and release of acetylcholine from isolated strips of spastic and dilated segments taken from human megacolon (Number of experiments in parentheses)

Segment	Acetylcholine Content (nmol/g) (mean (SEM))	Release (pmol/g) (mean (SEM))		Significance <i>p</i>	Ratio between stimulated and resting release of acetylcholine
		Resting*	Stimulated 10 Hz†		
Spastic (aganglionic)	10.8 (0.42) (5)	4.9 (0.96) (5)	70.3 (3.08) (5) }	<0.05‡	14.3
Dilated (ganglionic)	4.45 (0.15) (5)	2.1 (0.10) (5)	5.9 (0.44) (5) }		
Normal§		13.8 (2.28)	12.1 (25.5)		

*Collection period 30 min; †collection period 15 min; ‡paired *t* test, *t* test.

§Data taken from Del Tacca *et al.*¹³ In these experiments healthy muscle was obtained from postoperative specimens removed for carcinoma of the large intestine. Each value represents the mean of eight experiments.

experiments. The rate of acetylcholine release from both spastic and dilated longitudinal muscle was frequency dependent, the higher the frequency used the higher the total amount of acetylcholine released. When output per stimulus was calculated, however, there was an inverse relation between stimulation rate and output per stimulus. The release of acetylcholine during stimulation increased with frequency of stimulation and there was an inverse relation between stimulation rate and output per stimulus (Table I). The extent of the increase in release associated with field stimulation and the changes of acetylcholine output after blockade of nerve impulses could provide some information about the origin of the acetylcholine released into the bath. The mean (SEM) percentage reduction of resting acetylcholine output after tetrodotoxin was 15 (2.4)% ($n=4$) in the proximal segment and 35.2 (6.8)% ($n=4$) in the aganglionic preparations. The difference is significant ($p<0.05$), indicating an increased cholinergic activity in the spastic segment even under resting conditions. In some experiments acetylcholine output in response to 10 Hz stimulation was also measured after tetrodotoxin had been added to the bath fluid in a concentration of 10^{-6} M. Tetrodotoxin inhibited evoked release, as previously found in specimens taken from human normal descending colon.¹³ The increased release associated with transmural stimulation was almost completely inhibited by tetrodotoxin and the average ratio of evoked to spontaneous release in four experiments fell to 1.1 (0.1) ($n=3$) in the dilated segment, and 1.06 (0.05) ($n=3$) in the aganglionic preparations.

The total amount of acetylcholine in tissue was measured in Hirschsprung's specimens and the results are shown in Table I. Significant differences were found between the specimens taken from the aganglionic and the ganglionic regions. Evidence has been obtained¹⁰ to show that noradrenaline reduces the release of acetylcholine from the Auerbach plexus of guinea pig ileum provided a low frequency of stimulation (3 Hz) is used. The effect of noradrenaline on our preparations is shown in Table II. Noradrenaline (10^{-6} M) significantly reduced the release of acetylcholine from the aganglionic portion of the gut evoked by 1 Hz stimulation. Yohimbine, an α_2 blocking drug, in a concentration of 10^{-6} M prevented the effect of noradrenaline. The presence of inhibitory α_2 adrenoceptors in cholinergic fibres in spastic aganglionic portions and dilated, ganglionic segments of the gut is

shown by this finding. While in spastic segments yohimbine (10^{-6} M) did not enhance the release of acetylcholine (Table II), in dilated segments it enhanced the release from 3.92 (0.16) to 6.10 (0.13) pmol/g per min (1 Hz stimulation, $n=4$) ($p<0.05$). These findings indicate that in the aganglionic portion there is no tonic control of acetylcholine released by endogenous noradrenaline.

CONTRACTIONS OF THE LONGITUDINAL MUSCLE

Functional experiments were carried out in both types of preparations. Spontaneous and stimulated motor activities were studied in longitudinal strips dissected from the narrow and dilated segments. There was no difference between the patterns of spontaneous motility. This was fully developed within one hour of incubation in an organ bath and consisted of regular phasic contractions with a range in frequency of 10–24 per min.

Longitudinal muscle from both distended and constricted segments showed tone and spontaneous rhythm. Both muscles were contracted with acetylcholine but the muscle from the constricted segment was about 500 times more sensitive than the muscle from the distended region (Table III).

The pattern of response to field stimulation is also different in the spastic and dilated bowel. Field stimulation (10 Hz, 10 to 40 shocks) usually induced a biphasic response (Figure: top) – relaxation followed by contraction or contraction followed by relaxation in ganglionic specimens. Only contractions were seen in aganglionic specimens (Figure: bottom), however. When the number of shocks was kept constant (40 impulses) and a different frequency of stimulation was applied the maximum contraction (100%) was obtained at 20 Hz. At 1 Hz and 10 Hz the contractions were 23.1 (4.6)% ($n=3$) and 90.1 (8.5)% of those obtained at 20 Hz. At a higher frequency of stimulation (20 Hz) the contraction was smaller than at 2 Hz. The smooth muscle sensitivity to the contractile effect of field stimulation as well as to acetylcholine was higher in the aganglionic segment (Table III).

In each type of preparation the contractile effect of field stimulation could always be reduced by tetrodotoxin (92.5 (4.2)%, $n=3$) and by atropine (98.5 (2.8)%, $n=3$) both at concentrations ranging from 10^{-7} to 10^{-6} M.

Discussion

The much greater acetylcholine output both at rest and during transmural stimulation in the

TABLE II Effect of noradrenaline on the release of acetylcholine from isolated strips of spastic segments of human megacolon (Number of experiments in parentheses)

	Acetylcholine release (pmol/g per min) (mean (SEM))	Significance <i>p</i>
(1) Resting	5.4 (0.12) (9)	
(2) +1–noradrenaline 10^{-6} M	3.7 (0.25) (4)	2:1 $p<0.05$
(3) +yohimbine 10^{-6} M	5.1 (0.12) (4)	
(4) Stimulation 1 Hz	28.7 (1.80) (4)	4:1 $p<0.05$
(5) +1–noradrenaline 10^{-6} M	8.1 (0.90) (4)	5:4 $p<0.05$
(6) +yohimbine 10^{-6} M	32.6 (2.60) (3)	
(7) +yohimbine 10^{-6} M +1–noradrenaline 10^{-6} M	24.6 (4.15) (3)	7:5 $p<0.05$

Note: Collection period during rest 30 min and during stimulation 15 min.

TABLE III Effect of acetylcholine on spastic and dilated segments of megacolon

Segment	Equipotent concentration of acetylcholine* (mean (SEM))
Dilated (ganglionic) ($n=3$)	$8.0 (0.3) \times 10^{-6}$ M
Spastic (aganglionic) ($n=3$)	$1.6 (0.2) \times 10^{-6}$ M

*Concentration needed to produce half maximal contraction. For experimental condition see Methods. Note the difference in sensitivity of longitudinal muscle of colon (500-fold).

Effect of field stimulation and acetylcholine (ACh) on contractions of isolated strips taken from proximal dilated (top) and distal constricted (bottom) segment. Parameters of stimulation are indicated (10 Hz, 10–20–40 impulses: top; 20 Hz, 20–40 impulses: bottom). Arrows show where the tissue was washed out. Note that in the ganglionic segment there is a relaxation in response to electrical stimulation, and there is a difference in sensitivity (100-fold) of the smooth muscles to acetylcholine.

aganglionic preparations compared with those obtained from ganglionic regions suggest that hyperactivity of the intrinsic cholinergic mechanisms occurs. The question needs to be raised as to the origin of the acetylcholine in these preparations, however. Since tetrodotoxin is known to prevent axonal conduction in nerves and it almost completely prevented the release of acetylcholine, it may be concluded that the increased release of acetylcholine associated with field stimulation is nervous in origin. In support of this is the finding that the ratio of the evoked to the spontaneous release was high. We cannot completely exclude damage to the tissue by surgical procedures (including the effect of anoxia and anaesthetic substances). This is an inevitable disadvantage of all experiments

carried out on preparations of human intestine. Nevertheless, Fischlock and Parks^{17,18} have shown that strips of intestine from a spinally anaesthetised patient responded to various drugs in the same way as strips from fully anaesthetised patients. It is remarkable, though, that in our experiments the ratio of evoked to spontaneous release (Table I) was 14.3, closely similar to that found in animal colonic specimens where surgical damage is negligible.¹⁹

The sensitivity of the smooth muscle in the ganglionic portion of acetylcholine and to the contractile effect of field stimulation was found to be much lower than in the spastic portion. Evidence that nerves do exert a predominantly contractile effect on the aganglionic bowel in Hirschsprung's disease was given by Bodian *et al*.²⁰

when they found that a spinal anaesthetic produced relaxation of the distal segment during a barium enema examination in three cases. Therefore, we also suggest that this decreased sensitivity of smooth muscle cells of the dilated segment to cholinergic stimulation is involved in the pathophysiology of Hirschsprung's disease. These data do not agree with those of other workers.²¹⁻²³ The finding that noradrenergic nerves have a non-synaptic but functional^{11 24 25} relation to myenteric cholinergic nerve cells rather than to the muscle cells directly shows that the control of bowel activity is far more complex than is explained by the simple concept of a direct parasympathetic and an inhibitory sympathetic innervation of the muscular tissue.^{12 26 27} Since it is well established^{19 25} that the inhibitory α_2 adrenoceptors are also located distally from the cell body of cholinergic interneurons, noradrenergic modulation could also operate in the absence of ganglia via direct stimulation of inhibitory α_2 adrenoceptors. If such a mechanism were in operation, this should result in a relaxation of spastic gut, but this is not the case in Hirschsprung's disease. The possibility exists that since sympathetic innervation of the aganglionic part of the bowel is very rich, even more adrenergic fibres^{8 28} and noradrenaline content^{9 29} are located there than in the normal bowel. The lack of inhibitory α_2 adrenoceptors on cholinergic neurons cannot be attributed to the functional deficiency of the noradrenergic inhibition on cholinergic hyperactivity, since exogenous noradrenaline was able to reduce acetylcholine release via α_2 adrenoceptor stimulation. Yohimbine, an α_2 adrenoceptor antagonist, failed to enhance the release of acetylcholine, however, indicating that in the spastic portion there is no tonic inhibition of acetylcholine release by noradrenaline, which could have been removed by yohimbine. Therefore, it seems likely that noradrenaline, although present in the gut functionally, is not released, or if it is released it is unable to reach cholinergic varicosities. The effective inhibition of acetylcholine release by noradrenaline which results in relaxation and decreased motility is therefore absent in the affected segment of bowel. The great increase in acetylcholine content in aganglionic segments in conjunction with the absence of a normal sympathetic inhibitory mechanism probably explains the great increase in acetylcholine output both at rest and during field stimulation, thereby the contracted state of the bowel in a typical case of Hirschsprung's disease.

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