J. Pharm. Pharmacol. 1986, 38: 310–312
Communicated November 6, 1985

Assessment of acetylcholine release from myenteric plexus of guinea-pig colon

S. J. TROUT, *Department of Pharmacology and Therapeutics, The London Hospital Medical College, Turner Street, London E1 2AD, UK*

Due to a complex mechanical tissue response to electrical stimulation, a colonic smooth muscle-myenteric plexus (SMMP) preparation, combined with radiolabelling techniques for assessing the acetylcholine release, has been developed to investigate intrinsic cholinergic nerve activity in the guinea-pig colon. Electrical field stimulation of the preparation gave reproducible release of label which was inhibited by tetrodotoxin. Release increased proportionally with the strength of stimulus (130, 150, 195 and 250 mA), but was inversely proportional to frequency of stimulation for a fixed number of pulses, 1 Hz releasing more than 10 Hz. Electrical stimulation of the tissue during incubation with [^{3}H]choline enhanced the release by subsequent field stimulation. Release of label increased progressively towards the distal end of the colon.

It has been reported that excitation of intrinsic nerves, in segments of guinea-pig colon, by field stimulation resulted in complex mechanical responses (Costa & Furness 1972). The aim of this study was to develop a reliable preparation for investigating the intrinsic cholinergic nerve activity in that tissue. The longitudinal muscle-myenteric plexus (LMMP) preparation of the guinea-pig ileum is well established for studying radiolabelled acetylcholine release (Wikberg 1977). To study acetylcholine release in the large intestine, a similar nerve/smooth muscle preparation from the guinea-pig colon has been investigated.

Materials and methods

The preparation is similar to that described for the LMMP preparations from the rabbit ileum (Ambache 1954) and the guinea-pig ileum (Rang 1964; Paton & Zar 1968). Gabella & Juorio (1975) have also used this technique on proximal colon to study the histology of the myenteric plexus. Guinea-pigs of either sex were killed by a blow to the neck and exsanguinated. The entire colon was cut free, placed in a beaker of warm Krebs solution and cut into five segments of approximately equal length (10 cm). Remaining mesentery was removed. A segment of colon was pulled over a glass pipette and the muscularis externa separated from the underlying submucosa and mucosa by stroking tangentially with a wisp of wet cotton wool, starting at the broken border of mesenteric attachment. This was repeated for the remaining segments.

Preliminary histological investigation has shown this preparation to contain longitudinal muscle, myenteric plexus and some circular muscle, therefore the term 'smooth muscle-myenteric plexus (SMMP)' preparation is used here as 'colonic LMMP' would be inaccurate. Each tissue was folded twice and set up in a 1 ml organ bath under a force of 2·5 mN for isotonic recording of muscle activity. Krebs fluid at 37 °C, gassed with 5% CO_2 in oxygen, was superfused by displacement at a rate of approximately 2·5 ml min^{-1}. Responses were obtained to electrical field stimulation (EFS) using a Grass S88 square-wave stimulator connected to parallel Pt wire electrodes mounted in the organ baths.

For radiolabelling cholinergic transmitter stores, superfusion was stopped and the tissues incubated with 4 μCi ml^{-1} [^{3}H]choline (15 Ci mmol^{-1}, Amersham). For some experiments the tissue was field-stimulated (0·5 ms, 10 Hz, 195 mA) for the first 30 min of incubation. After incubation, the tissues were superfused with fresh Krebs solution containing hemicholinium-3 (HC-3, 34·8 μM). HC-3 was present from the beginning of the washout period to the end of the experiment. The superfusate was collected every 2 min using a Gilson FC-220 fraction collector. Three 2-min collections were made before each stimulus to establish basal release and 2-min collections were then made during the stimulation period, and for a further 6 min after, to measure stimulated release. A further two 2-min basal collections were made at the end of the experiment.

Radioactive overflow was measured by taking 0·5 ml aliquots of the superfusate for liquid scintillation counting. The data were automatically adjusted for quench, determined using the external standard channels ratio method. All samples were counted to an accuracy of 5% of 2 sigma error. At the end of the experiment the tissue was weighed, dissolved in hyamine hydroxide and an aliquot counted to establish the amount of radioactivity remaining in the tissue. Drugs used were: hemicholinium-3 and tetrodotoxin (Sigma). The Krebs fluid had the following composition (mM): Na^+ 140·0, K^+ 5·9, Cl^- 112·0, $H_2PO_4^-$ 1·2, Ca^{2+} 2·5, Mg^{2+} 1·2, SO_4^{2-} 1·2, HCO_3^- 25·0 and (+)-glucose 11·5.

Calculation of release. Radioactivity in all samples was computed in terms of d min^{-1} mg^{-1} of tissue. Expected basal outflow (spontaneous) during each peak was determined by fitting a regression line through all basal values measured before and after each stimulus. The calculated basal values were then subtracted from the

FIG. 1. Qualitative regional variation of tissue response to electrical field stimulation in the 'SMMP' preparation. Isotonic recordings of responses to electrical field stimulation (0·5 ms, 1 Hz, 150 mA, 240 pulses; solid bar) from different regions of the colon.

observed outflow during a stimulation, the difference being evoked radiolabel release. The total evoked release for a given stimulus was expressed as a fraction of the radioactivity present in the tissue at the beginning of that stimulus (i.e. fractional release). Two stimuli were given at a fixed interval; fractional release values of the first stimulus were used to assess the effect of various stimulation parameters, whereas fractional release ratios (fractional release of the second stimulus divided by the first) were used to assess the effects of drugs introduced between the stimuli. Data are expressed as medians and semi-quartile ranges. Statistical comparisons were made with the Mann-Whitney U-test (2-tailed) and Spearman's rank correlation coefficient.

Results

Tissue responses to electrical field stimulation were complex and variable (Fig. 1) so a method of radiolabelling acetylcholine was chosen to study cholinergic nerve activity. In this preparation, fractional release of the first stimulus was proportional to the current strength in the range tested ($P < 0·01$; Table 1); however, the fractional release of the first stimulus was less at 10 Hz than at 1 Hz ($P < 0·01$). Tissues receiving electrical field stimulation during incubation released more labelled material when stimulated subsequently with EFS ($P < 0·05$; Table 1). A fractional release ratio of 0·84 (0·83–0·85) was obtained to EFS at 250 mA. When tetrodotoxin (TTX, 1·57 μM) was introduced 16 min before the second stimulation, the fractional release ratio fell to 0·010 (0·004–0·014; $P < 0·01$), suggesting that the evoked release of labelled material was neuronal in origin.

Closer examination of the fractional release values revealed a trend where release of labelled material was greater towards the distal end of the colon (Fig. 2; $P < 0·01$).

Table 1. All values represent the median fractional release of the first stimulation period (with inter-quartile ranges) to electrical field stimulation (0·5 ms 240 pulses) where the tissues received EFS during incubation.

Stimulation parameters		Fractional release ($\times 10^3$)		
1 Hz	130 mA	3·98	(3·79– 4·61)	n = 5
1 Hz	150 mA	5·73	(3·70– 7·96)	n = 40
1 Hz	195 mA	9·19	(6·56–13·87)	n = 40
1 Hz	250 mA	12·72	(9·17–19·49)	n = 7
There was a significant increase in release with increasing current ($r_s = 0·57$, $P < 0·01$)				
1 Hz	150 mA	5·73	(3·70– 7·96)	n = 40
10 Hz	150 mA	3·72	(2·35– 5·00)	n = 40
There was significantly less release at 10 Hz ($P < 0·01$)				
1 Hz	195 mA	9·19	(6·56–13·87)	n = 40
1 Hz	195 mA*	5·68	(3·12– 8·25)	n = 5

* No electrical field stimulation during incubation. Release was higher to subsequent stimuli following EFS during incubation ($P < 0·05$)

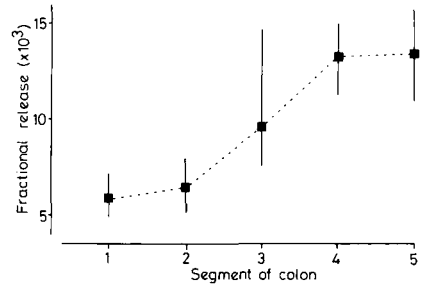

FIG. 2. Variation in release of labelled material with region of colon. Fractional release of the first stimulus (median ■ with inter-quartile ranges, n = 8) against segment of colon (each colon cut into five segments of approximately equal length, numbered from proximal (1) through to distal (5), $r_s = 0·75$, $P < 0·01$).

Discussion

Tissue responses to electrical field stimulation were complex in this preparation. This emphasizes the advantage of using labelled acetylcholine to study cholinergic activity rather than tissue response, which may be due to a variety of contributing factors. Evoked radiolabel release probably represents release of [^{3}H]acetylcholine from nerve endings (Wikberg 1977), especially as the low [^{3}H]choline concentrations used for incubation would be selectively accumulated by the high-affinity uptake system in cholinergic nerve terminals (Pert & Snyder 1974). The SMMP preparation was chosen in preference to whole segments of colon as it

was thought to offer the same advantages that the ileum LMMP has over the ileum segment: absence of a mucosal layer (Wikberg 1977), higher transmitter output to tissue mass ratio (Paton & Zar 1968) and a thin strip releasing relatively large amounts of acetylcholine (Paton et al 1971).

Fractional release represents an absolute amount of tritiated material and was used to compare the evoked release to various electrical stimulation parameters. It is conventional to examine successive stimuli in the same tissue as a ratio of the second peak divided by the first (Vizi et al 1981). The effect of TTX introduced between the two stimuli was assessed by its effect on this ratio. The EFS-induced release of labelled transmitter from the colonic SMMP preparation was comparable to that from guinea-pig ileum LMMP in this laboratory (Burleigh & Trout 1985). Release from the SMMP preparation was directly proportional to the current within the range tested, however, it was inversely proportional to the frequency of the stimulus, as observed in the guinea-pig ileum LMMP preparation using bioassay (Paton 1963; Paton & Zar 1968; Knoll & Vizi 1971; Cowie et al 1978). Negative feedback mechanisms may therefore operate more effectively at the higher frequency (James & Cubeddu 1984).

The ability of TTX to reduce markedly the output of labelled transmitter evoked by EFS, suggests that the release is the result of a propagated action potential, presumably in cholinergic neurons. Field stimulation of the tissue for the first half of the 60 min incubation period enhanced the subsequent evoked release of labelled transmitter. This phenomenon has also been described in the guinea-pig ileum LMMP, and is probably due to enhanced incorporation of labelled material into endogenous acetylcholine stores (Szerb 1975). The variation in the amount of labelled material released from proximal to distal colon may contribute to the different tissue responses observed along the colon and may be due to the presence of more cholinergic nerves in the distal colon or perhaps greater negative-modulatory influences in the proximal colon.

This SMMP preparation from the guinea-pig colon shares some of the characteristics of the guinea-pig ileum LMMP preparation. Both are well suited to studying cholinergic activity by radiolabelling techniques. The colonic SMMP preparation provides a direct method for investigating cholinergic events where the tissue response is too complex and variable for accurate analysis.

I would like to thank Dr D. E. Burleigh for his helpful comments and encouragement during this study; Dr M. Hathorn for his statistics program and help with the radio-release program and Janssen Pharmaceutica n.v., Beerse, Belgium, for financial support.

REFERENCES

Ambache, N. (1954) J. Physiol. 125: 53P–55P

Burleigh, D. E., Trout, S. J. (1985) Br. J. Pharmacol. 84: 165P

Costa, M., Furness, J. B. (1972) Ibid. 45: 151–152P

Cowie, A. L., Kosterlitz, H. W., Waterfield, A. A. (1978) Ibid. 64: 565–580

Gabella, G., Juorio, A. V. (1975) J. Neurochem. 25: 631–634

James, M. K., Cubeddu, L. X. (1984) J. Pharmacol. Exp. Ther. 229: 98–104

Knoll, J., Vizi, E. S. (1971) Br. J. Pharmacol. 41: 263–272

Paton, W. D. M. (1963) Can J. Biochem. Physiol. 41: 2637–2653

Paton, W. D. M., Zar, M. A. (1968) J. Physiol. 194: 13–33

Paton, W. D. M., Vizi, E. S., Zar, M. A. (1971) Ibid. 215: 819–848

Pert, C. B., Snyder, S. H. (1974) J. Pharmacol. Exp. Ther. 191: 102–108

Rang, H. P. (1964) Br. J. Pharmacol. 22: 356–365

Szerb, J. C. (1975) Can. J. Physiol. Pharmacol. 53: 566–574

Vizi, E. S., Somogyi, G. T., Magyar, K. (1981) J. Autonom. Pharmacol. 1: 413–419

Wikberg, J. (1977) Acta. Physiol. Scand. 101: 302–317