J. Pharm. Pharmacol. 1989, 41: 824–828
Received March 16, 1989

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[^{3}H]Acetylcholine Release from the Guinea-pig Distal Colon: Comparison with Ileal [^{3}H]Acetylcholine Release and Effect of Adrenoceptor Stimulation

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Abstract—To study cholinergic function in the guinea-pig colon, resting and electrically evoked ^{3}H release after preincubation with [^{3}H] choline has been compared in colonic and ileal myenteric plexus preparations. Fractional spontaneous colonic ^{3}H release was significantly higher than ileal ^{3}H release, while the reverse was true for electrically evoked ^{3}H outflow. Electrically evoked ^{3}H outflow in the colon was linearly related to stimulation frequency (0·2–3 Hz range) and current intensity (300–600 mA range), while ^{3}H outflow per pulse was inversely related to stimulation frequency. Electrically evoked ^{3}H outflow was prevented in Ca^{2+}-free solution, indicating that it probably mirrored neuronal exocytotic [^{3}H]acetylcholine release. Both noradrenaline and clonidine concentration-dependently inhibited electrically evoked ^{3}H outflow, clonidine being more potent but less efficacious than noradrenaline. For both noradrenaline and clonidine, the potency and efficacy for inhibition of ^{3}H outflow were close to the values previously reported for the inhibition of electrically evoked endogenous acetylcholine output from colonic preparations. In conclusion, these data indicate that ^{3}H release after incubation with [^{3}H]choline is a valid alternative to measurement of endogenous acetylcholine output to study colonic cholinergic neuronal function in the guinea-pig.

In the enteric nervous system, the release of acetylcholine (ACh) has long been studied by measuring endogenous neurotransmitter output, usually estimated by bioassay. However, the technique requires inhibition of esterases, which results in widespread activation of enteric cholinergic neurons by unphysiological high levels of extracellular ACh and in a "resting" ACh output which has been reported to be largely Ca^{2+}-dependent and sensitive to tetrodotoxin, ganglionic blockade or muscarinic autoreceptor activation (Paton et al 1971; Kilbinger & Wagner 1975). On the other hand, ^{3}H recovery in superfusion fluid after depolarization seems to be a sensitive tracer of endogenous neurotransmitter released by the ileal myenteric plexus preparation, whether or not cholinesterases are inhibited (Kilbinger & Wessler 1980; Alberts et al 1982).

While many studies have been carried out on ileal cholinergic neurons by measuring both endogenous and [^{3}H]ACh output, relatively little attention has been devoted to the study of the colon, where cholinergic neuronal function is still usually evaluated by measuring endogenous ACh output (Lecchini et al 1969; Frigo et al 1987).

In the present paper, we describe ^{3}H release after [^{3}H] choline preincubation of the myenteric plexus-longitudinal muscle preparation (MPLM) obtained from the guinea-pig colon in comparison with the ileal MPLM preparation. Moreover, we describe the effect of adrenoceptor agonists, which are well-known inhibitors of cholinergic neuronal activity in the enteric nervous system, on electrically evoked ^{3}H outflow.

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Materials and Methods

Tissue preparation

Male guinea-pigs (300–400 g) were used. Segments of the distal colon and ileum were dissected to obtain myenteric plexus-longitudinal muscle (MPLM) preparations, as described by Paton & Zar (1968). Four preparations from each animal were used.

Equilibration and labelling (Fig. 1A)

Four preparations were kept for 30 min in four 1 mL organ baths, and superfused at the rate of 1 mL min^{-1} with Tyrode solution containing 1 μM choline. The temperature was kept constant at 36·5°C and the solution was continuously bubbled with O_2/CO_2 (95:5).

The preparations were then incubated for 45 min in a 5 mL organ bath containing [^{3}H]choline (final concentration 0·04 μM) and stimulated throughout the incubation period by means of coaxial platinum electrodes (square wave pulses; 0·2 Hz, 1 ms duration, 800 mA). At the end of the labelling period, the preparations were washed three times at 5 min intervals with choline-free Tyrode solution containing hemicholinium-3 (10 μM).

The preparations were then mounted under isometric conditions (weight 1 g) in 1 mL organ baths superfused (1 mL min^{-1}) with Tyrode solution with added hemicholinium-3 (10 μM) and longitudinal muscle movements were recorded.

Sample collection

Samples were collected after a 90 min washout period. In a first series of experiments in colonic and ileal MPLM preparations, samples were collected at 3 min intervals throughout the experiment. To evoke ^{3}H output, transmural stimulation (S) was carried out at intervals of 45–54 min by

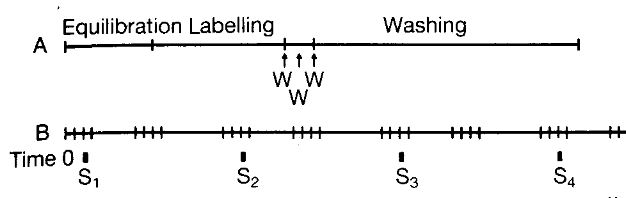

FIG. 1. Time schedule for a standardized ^{3}H release experiment in the guinea-pig colon MPLM preparation. A) Preparations were superfused for 30 min with Tyrode solution containing 1 μM choline (equilibration). Labelling was carried out by incubation (45 min) with [^{3}H]choline (0·04 μM) and transmural stimulation (0·2 Hz, 1 ms, 800 mA). After three washouts (W) at 5 min intervals with choline-free Tyrode solution containing hemicholinium-3 (10 μM) and superfusion for 90 min with Tyrode solution containing hemicholinium-3 (washing), the sample collection was started (time 0). B) Samples or pool samples were collected at the times indicated by bars. Transmural stimulation (■ 1 Hz, 1 ms, 450 mA for 90 s) was applied at t = 6 min (S1), 60 min (S2), 114 min (S3) and 168 min (S4). See text for further details.

means of coaxial platinum electrodes delivering trains of alternate square wave pulses (0·2–3 Hz, 1 ms duration, 300–600 mA), lasting 36–180 s.

In a second series of experiments, to evaluate the effect of adrenoceptor agonists on the colon, the sample collection schedule was standardized and the samples collected as follows (Fig. 1B).

i) resting period (before stimulation): this included two samples (3 min each) collected before stimulation and the 3 min sample obtained immediately after stimulation (this was still basal, because of the dead space of the superfusion system);
ii) stimulation period: the superfusate in the subsequent 15 min period was collected as a single pool from which two 3 mL samples were obtained;
iii) resting period (after stimulation): the subsequent three 3 min samples were collected singly, and then the superfusate in the subsequent 21 min period was collected as a single pool from which two 3 mL samples were obtained.

This stimulation cycle was repeated four times (S1, S2, S3 and S4, respectively at 6, 60, 114 and 168 min after the end of the washout period). The pool corresponding to the resting period after S4 was not collected.

To assess the effect of calcium deprivation, the preparations were superfused with Ca^{2+}-free Tyrode solution, starting at least 20 min before S2, S3 and S4. When the effect of adrenoceptor agonists was evaluated, superfusion with Tyrode solution with added adrenoceptor agonists at various concentrations was started at least 10 min before S2, S3 and S4.

Evaluation of ^{3}H content

Samples were added to 5 mL of scintillation cocktail (Atomlight, NEN Chemical, Boston, MA, USA). The tissue was solubilized overnight in 1 mL Soluene 350 (Packard, Downers Grove, IL, USA) and then added to 15 mL Atomlight.

The ^{3}H contents were determined by liquid scintillation spectrometry. Counting efficiency was (% ± s.e. m., n = 30): 24·1 ± 0·3 and 33·1 ± 0·3 for samples and solubilized tissues, respectively.

Calculations and statistics

The radioactivity of each sample was expressed as a fraction of the initial total radioactivity in the tissue (fractional ^{3}H release). Electrically evoked ^{3}H outflow was obtained by subtracting the spontaneous ^{3}H release from the total radioactivity of the pool obtained after stimulation. Spontaneous ^{3}H release during the stimulation period was estimated by calculating the mean of the two samples preceding the stimulation period and the first two samples of the resting period after stimulation (see Fig. 1B).

The effect of calcium deprivation or adrenoceptor agonists on electrically evoked ^{3}H outflow was measured from the ratios S2/S1, S3/S1 and S4/S1. The ratios obtained in the presence of drugs were then related to the respective ratios obtained under control conditions and are given as percent control. At least one preparation in each experiment was used as a control to determine S2/S1, S3/S1 and S4/S1 ratios in the absence of drugs.

IC35 values (i.e. the concentrations able to reduce the ^{3}H outflow by 35% with respect to the control value) for adrenoceptor agonists were determined from the concentration-response relationships obtained by plotting the percentage inhibition of ^{3}H outflow against log concentration.

E_{max} and K values of adrenoceptor agonists for inhibition of stimulation-evoked ^{3}H outflow were derived by double reciprocal plots obtained from the percentage inhibition of ^{3}H outflow vs the molar concentration. E_{max} was considered as the reciprocal of the intercept on the y axis and was taken as a measure of intrinsic activity. K values, as derived from the product (E_{max}) × (slope), was considered as an estimate of the EC50 (Tallarida 1982).

Concentration-effect relationships were analysed by linear regression analysis (Finney 1978). The best linear fitting for the concentration-response curves was obtained by the least squares method. Statistical significance of the difference among groups was analysed by Student's *t*-test for unpaired data (two-tailed).

Drugs and solutions

The drugs used were: [methyl-^{3}H]choline chloride (80 Ci mmol^{-1}, NEN Chemical, Boston, MA, USA), choline hydrochloride, clonidine hydrochloride, hemicholinium-3

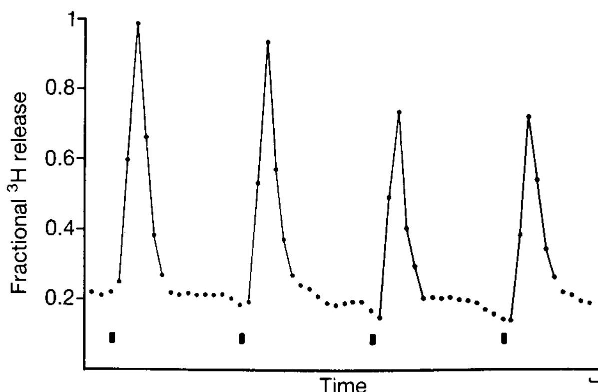

FIG. 2. Time course of spontaneous and evoked ^{3}H release from a guinea-pig ileal MPLM preparation. Transmural stimulation (1 Hz, 1 ms duration, 450 mA) applied for 90 s at ■. Time calibration 3 min.

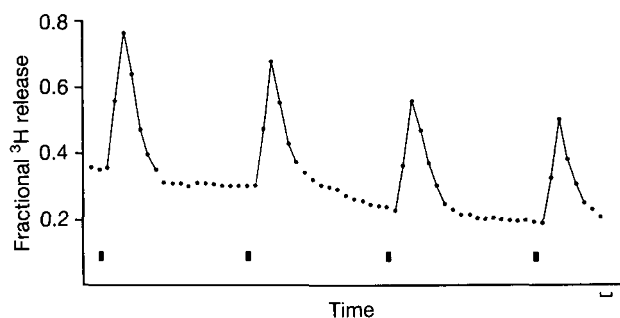

FIG. 3. Time course of spontaneous and evoked ^{3}H release from a guinea-pig colonic MPLM preparation. Transmural stimulation (1 Hz, 1 ms duration, 450 mA) applied for 90 s at ■. Time calibration 3 min.

and (−)-noradrenaline hydrochloride (all from Sigma Chemicals, St Louis, MO, USA). The composition of the Tyrode solution was (mM): NaCl 136·9, KCl 2·7, $CaCl_2$ 1·8, $MgCl_2$ 1·04, $NaHCO_3$ 11·9, NaH_2PO_4 0·4, glucose 11. In Ca^{2+}-free Tyrode solution, $CaCl_2$ was omitted and no osmolar compensation was made.

Results

Spontaneous ^{3}H release

Fractional ^{3}H release in the first three samples was higher ($P<0·05$) in MPLM colonic preparations (0·33±0·006; mean±s.e.m., n=80) than in ileal MPLM preparations (0·23±0·01; n=12) (Figs 2, 3).

Stimulation-evoked ^{3}H outflow

Stimulation-evoked ^{3}H outflow was higher ($P<0·05$) in the ileum than in the colon. S1 (1 Hz, 1 ms, 450 mA for 90 s)-induced ^{3}H outflow was (means±s.e.m.) 1·78±0·08 in the ileum and 1·08±0·03 in the colon (n=6) (Figs 2, 3).

Both in the ileum and in the colon, stimulation-evoked ^{3}H outflow was prevented in Ca^{2+}-free solution, while resting ^{3}H output was not significantly affected (Fig. 4; data not shown for the ileum).

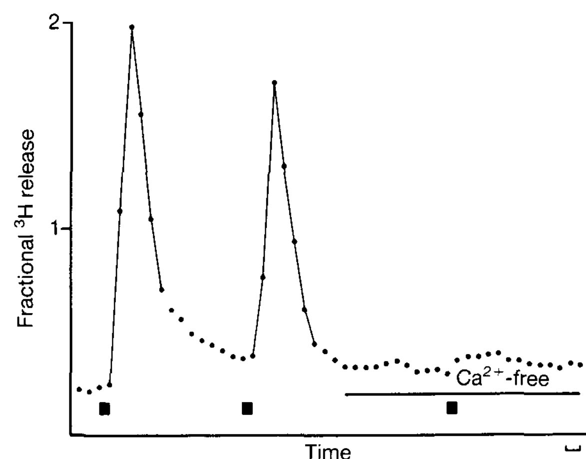

FIG. 4. Effect of calcium deprivation on ^{3}H release from a colonic MPLM preparation. Transmural stimulation (1 Hz, 1 ms duration, 600 mA) applied for 180 s at ■. Time calibration 3 min.

Table 1. Guinea-pig colon MPLM preparation. Linear relationship between stimulation-evoked ^{3}H outflow and both stimulation frequency and current intensity (0·2–3 Hz, 1 ms duration, 300–600 mA for 180 s).

Frequency (Hz)	Current (mA)		
	300	450	600
0·2	NT	0·72±0·03	NT
1	0·5±0·03	2·03±0·18	3·80±0·02
3	NT	4·21±0·037	NT

Values are mean (±s.e.m., n=6) fractional ^{3}H outflow during the first stimulation period (S_1); NT=not tested; correlation coefficient: 0·995 for ^{3}H outflow vs stimulation frequency; 0·999 for ^{3}H outflow vs current intensity.

Table 2. Guinea-pig colon MPLM preparation. Inverse relationship between ^{3}H outflow per pulse and stimulation frequency (0·2–3 Hz, 1 ms duration, 450 mA for 180 s).

Frequency (Hz)	^{3}H outflow/pulse
0·2	0·020±0·001
1	0·012±0·001
3	0·008±0·0007

Values are mean (±s.e.m., n=6) fractional ^{3}H outflow during the first stimulation period (S_1); correlation coefficient 0·908.

In the colon, electrically evoked ^{3}H outflow was linearly related to stimulation frequency (within the 0·2–3 Hz range) and to current intensity (within the 300–600 mA range) (Table 1). A 600 mA current was supramaximal for both mechanical contraction of the longitudinal muscle and ^{3}H outflow. ^{3}H outflow per pulse was inversely related to stimulation frequency (Table 2).

Effect of adrenoceptor agonists

Transmural stimulation was standardized as follows: 1 Hz, 1 ms duration, 450 mA for 90 s. Under these experimental conditions, the S2/S1, S3/S1 and S4/S1 ratios in control

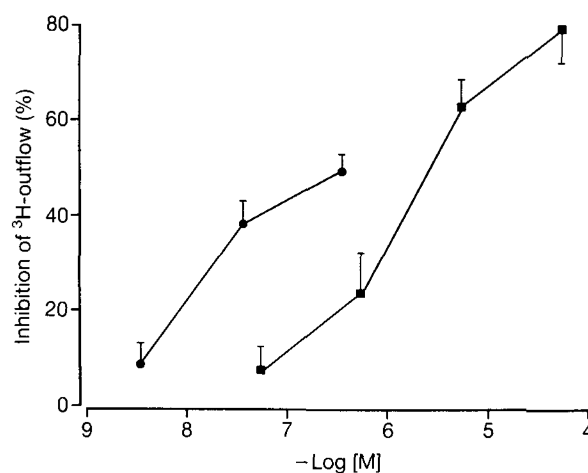

FIG. 5. Inhibition by noradrenaline (■) and clonidine (●) of stimulation-evoked (1 Hz, 1 ms, 450 mA for 90 s) ^{3}H outflow from guinea-pig colonic MPLM preparation. Log concentrations are plotted against % inhibition of ^{3}H outflow compared with the controls. Each point represents the mean of 6 experiments. Vertical bars indicate s.e.m.

Table 3. Inhibition by adrenoceptor agonists of transmurally stimulated endogenous acetylcholine (ACh) and [^{3}H]acetylcholine release from guinea-pig colon.

	Endogenous ACh*		^{3}H outflow	
	Emax	K	Emax	K
Noradrenaline	85	$7{\cdot}78\times10^{-6}$M	79	$1{\cdot}34\times10^{-6}$M
Clonidine	54	$3{\cdot}53\times10^{-8}$M	51	$1{\cdot}95\times10^{-8}$M

Emax and K values were derived from double reciprocal plots.
* Data are taken from Marcoli et al (1985), who evoked ACh output by transmural stimulation (1 Hz, 1 ms duration, supramaximal strength for 10 min).

preparations were respectively (means ± s.e.m., n = 18): 0·89 ± 0·02, 0·80 ± 0·04, 0·63 ± 0·03.

Both noradrenaline and clonidine concentration-dependently inhibited ^{3}H outflow (Fig. 5), clonidine being more potent but less efficacious than noradrenaline. IC35 values for noradrenaline and clonidine in inhibiting ^{3}H outflow were $1{\cdot}03\times10^{-6}$ and $5{\cdot}50\times10^{-8}$ M, respectively, which are similar to the IC35 values obtained when studying inhibition of stimulated endogenous ACh output from colonic preparations ($5{\cdot}86\times10^{-6}$ and $7{\cdot}28\times10^{-8}$ M, respectively; data from Marcoli et al 1985). Moreover, E_{max} and K values for noradrenaline and clonidine correlated well with the values previously obtained for inhibition of endogenous ACh output (Table 3).

Discussion

A technique widely used in the study of ileal cholinergic neuronal function (Szerb 1975; Wikberg 1977) was applied to MPLM preparations from the guinea-pig colon with some modifications.

A low concentration of [^{3}H]choline (0·04 μM) was used when incubating the preparation, to take advantage of the high affinity uptake system of the neurons and to induce a larger synthesis of [^{3}H]ACh relative to other metabolites (Pert & Snyder 1974; Szerb 1975). Moreover, low frequency transmural stimulation was applied during incubation in order to improve the efficiency of labelling (Kilbinger & Wessler 1980; Alberts et al 1982).

The inhibition of esterases, which is required to recover ACh released from enteric preparations in-vitro, could change the transmitter biophase and outflow itself and was avoided. The release experiments were then carried out in the presence of hemicholinium. On the other hand, the addition of hemicholinium, while excluding the interference of possible modification of high affinity choline uptake during the experiment (Antonelli et al 1981), increases ^{3}H loss, thus deviating from physiological conditions. To compensate for store exhaustion, controls were always run in parallel.

Under these conditions, ^{3}H release can be assumed to mirror neurotransmitter outflow, especially after stimulation. In fact, while ^{3}H from choline metabolites, extraneuronal sources or damaged cholinergic neurons can greatly contribute to ^{3}H release in resting tissue (Richardson & Szerb 1974; Wikberg 1977), ^{3}H outflow during stimulation seems to be a good index of release of labelled transmitter. In the ileum, indeed, [^{3}H]choline, as such, is not released either by electrical or ionic depolarization, while the evoked increase in radioactivity in the superfusate is the result of an increased release of ACh and could be used to estimate the evoked secretory response (Szerb 1975; 1976; Wikberg 1977). Although the released [^{3}H]ACh remained intact in the effluent only when ACh-esterase was blocked, the method is assumed to apply both to experiments performed with and without physostigmine (Kilbinger & Wessler 1980; Alberts et al 1982).

In our conditions, it is likely that evoked ^{3}H outflow in the colon originates from the release of labelled ACh, which is subsequently hydrolysed by esterase. Since the uptake of choline was largely prevented, the amount of ^{3}H collected probably reflected the amount released. Moreover, the observation that there was no increase in the release of radioactivity following stimulation in the absence of Ca^{2+} in the superfusion fluid suggested that the most likely source of this increase was the exocytotic release of labelled ACh from the neurons.

We found quantitative differences in ^{3}H release between guinea-pig colon and ileum. In the colon, spontaneous ^{3}H release was higher, while stimulation-evoked ^{3}H outflow was lower than in the ileum. The differences in spontaneous ^{3}H release between ileum and colon may be unimportant, since resting ^{3}H release in our experimental conditions is not likely to reflect neurotransmitter secretion. Accordingly, spontaneous ^{3}H release from either site was unaffected by calcium deprivation. The greater stimulation-evoked ^{3}H outflow in the ileum with respect to the colon parallels the higher endogenous ACh output, as measured by bioassay (unpublished data).

In colonic preparations, the best stimulation parameters for studying the stimulation-evoked ^{3}H outflow appear to be: 90 s trains of pulses, 1Hz, 1 ms duration and 450 mA. This stimulation carried out four times at 54 min intervals gave fairly constant stimulation-evoked ^{3}H outflow, as inferred by S2/S1, S3/S1 and S4/S1 ratios. Stimulation at 1 Hz seems particularly suitable and selective for cholinergic neurons (Waterfield & Kosterlitz 1973).

Both noradrenaline and clonidine inhibited electrically evoked ^{3}H outflow in colonic MPLM preparations and exhibited IC35, K and E_{max} values very close to those previously found for inhibition of endogenous ACh output (Marcoli et al 1985). This clearly indicates that evoked ^{3}H outflow can be modulated by the activation of receptors known to play a role in the physiological regulation of cholinergic neurotransmission in the enteric nervous system and is likely to reflect neurotransmitter release.

In conclusion, the data provided by this study, in particular the calcium dependence of stimulation-evoked ^{3}H outflow and the inhibitory effects of adrenoceptor agonists, indicate that ^{3}H release after incubation with [^{3}H]choline is a valid alternative to measurement of endogenous ACh output to study colonic cholinergic neuronal function in the guinea-pig.

Acknowledgement
The authors thank Mrs Barbara Cantoni for preparing the illustrations.

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