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MODULATION BY ACETYLCHOLINE OF THE ELECTRICALLY-EVOKED RELEASE OF [^{3}H]-ACETYLCHOLINE FROM THE ILEUM OF THE GUINEA-PIG

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1 Acetylcholine (ACh) stores within neurones of the myenteric plexus of the guinea-pig were labelled with [^{3}H]-choline and the influence of unlabelled ACh, atropine, or atropine and unlabelled ACh on the electrically-evoked output of [^{3}H]-ACh was evaluated.

2 Electrical transmural stimulation (5 Hz) of the ileum led to an increase in the output of [^{3}H]-ACh over that released spontaneously. Superfusion with unlabelled ACh (6.8 μM) caused a marked reduction in the release of [^{3}H]-ACh which was reversed by atropine (3.5 μM). Atropine itself had no effect on the electrically-evoked [^{3}H]-ACh.

3 These experiments provide further evidence for the existence in the guinea-pig ileum of neuronal muscarinic receptors for ACh subserving an inhibitory role on transmitter release.

Introduction

Twitch responses of the guinea-pig ileum to low frequency field stimulation are inhibited when their sequence is interrupted by acetylcholine (ACh; Fosbraey & Johnson, 1978; 1980). The post-ACh inhibition appears to result from the activation of prejunctional muscarinic receptors subserving an inhibitory role on transmitter release. Evidence for the existence of such receptors in the guinea-pig ileum has also been presented by Kilbinger & Wagner (1975), Kilbinger (1977) and Dzieniszewski & Kilbinger (1978) who showed that the muscarinic agonist, oxotremorine, inhibited the spontaneous, electrically-evoked and dimethylphenylpiperazinium (DMPP)-induced release of ACh. Sawynok & Jhamandas (1977) found that oxotremorine did not inhibit the electrically-induced release of ACh from the guinea-pig ileum but it reduced the increased output of ACh caused by atropine.

Apart from the work of Fosbraey & Johnson, the presence of pre-junctional inhibitory cholinoceptors on the guinea-pig ileum has been shown only by means of the muscarinic agonist, oxotremorine, and the crucial question remained as to whether ACh itself inhibits the release of transmitter ACh. In the present experiments, cholinergic nerve terminals were labelled with [^{3}H]-choline and the influence of unlabelled ACh on the electrically-evoked outflow of tritium has been evaluated.

Methods

All experiments were carried out on ileum isolated from male guinea-pigs over 250 g. Segments 1 cm in length and proximal to the terminal 15 cm were freed of mesentery and cut along the mesenteric border to give sheets of tissue, each approx. 1 cm square. Four such tissues were transferred to a conical flask containing 25 ml Krebs solution at 37°C to which was added [^{3}H-methyl]-choline (99 nM; 10.1 Ci/mmol, Radiochemical Centre, Amersham). The Krebs was bubbled with 5% CO_2 in oxygen. The tissues were incubated for 2 h and were then washed in a large volume of tritium-free Krebs for 30 min. Each piece of ileum was cut in two and half from one piece was placed with half from another on the lower of a pair of platinum grid electrodes spaced 2 mm apart. This was placed in a heated superfusion chamber in which the tissue was superfused for 120 min with Krebs solution at 37°C flowing at a rate of 2 ml/min (Watson-Marlow MHRE flow inducer). The remaining two half pieces of tissue were dried between filter papers, weighed and solubilized in Soluene-350 (Packard). The remaining two ileal squares were similarly divided to serve as control preparations. From the time that the tissues were washed in tritium-free Krebs solution, the biological fluid contained physostigmine (180 nM) and hemicholinium-3 (HC-3, 17 μM). Initial experiments in which physostigmine, HC-3 or both were omitted yielded significantly lower outputs of [^{3}H]-ACh.

Spontaneous release of [^{3}H]-acetylcholine

In experiments to determine the spontaneous efflux of tritium from the ileum, the superfusate was collected

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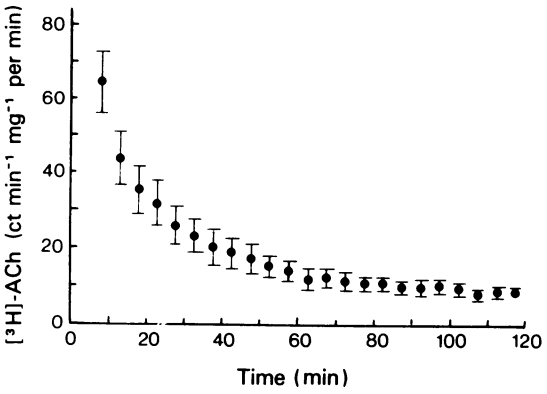

Figure 1 The spontaneous rate of release of [^{3}H]-acetylcholine ([^{3}H]-ACh) (ct min^{-1} mg^{-1} per min) from the guinea-pig ileum (n = 11) at 5 min intervals for 120 min.

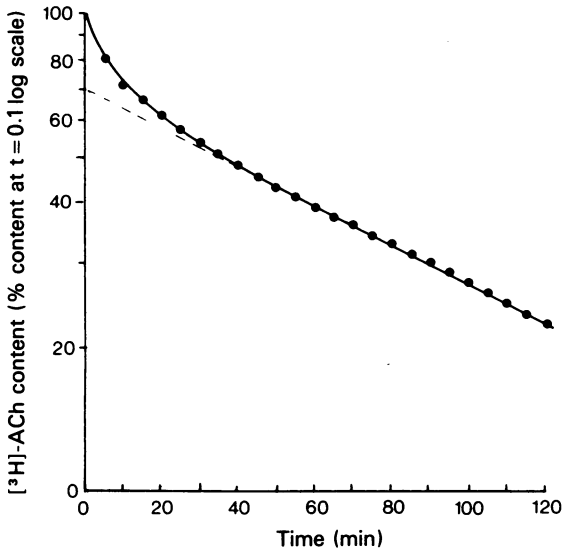

Figure 2 The ileal content of [^{3}H]-acetylcholine ([^{3}H]-ACh) (% content at t = 0 min) plotted on a log scale (ordinates) against the corresponding time (abscissae). The resultant curve is characteristic of a two compartment system, the β-phase of which has a half-life of 75 min.

in 5 min (i.e. 10 ml) samples for the duration of the experiment. At the end of superfusion, the pieces of ileum were removed from the electrodes, dried, weighed and solubilized in 5 ml Soluene-350. The amounts of tritium in 0.2 ml aliquots of the solubilized tissue and superfusate samples were determined by liquid scintillation spectrometry after the addition of appropriate scintillators and the outputs were expressed as ct min^{-1} mg^{-1} tissue. Counting efficiency was determined by the use of internal standards. The tissue content of tritium at time t was determined by adding to the tissue content at the end of the experiment (t = 120), all the tritium lost from time t onwards.

Electrically-evoked release of [^{3}H]-acetylcholine

During the stimulation experiments, 5 min samples were collected over the first 50 min, thereafter each preparation was stimulated for 10 min with rectangular pulses derived from an SRI stimulator at a frequency of 5 Hz and a voltage of 25 to 30 V depending on tissue resistance to give a current of 500 mA. Samples of superfusate were collected for 1 min during stimulation and the subsequent 5 min before the resumption of 5 min collections for the rest of the experiment.

To determine the effects of unlabelled ACh on tritium output, one of the pair of tissues was superfused with Krebs containing unlabelled ACh, ACh plus atropine or atropine alone. The concentration of ACh used (6.8 μM) was that which gave approximately 60% inhibition of the first post-ACh twitch in the experiments of Fosbraey & Johnson (1980).

Drugs

The following drugs were used: acetylcholine chloride (BDH); atropine sulphate (Sigma); hemicholinium-3 (Aldrich); physostigmine salicylate (BDH). All drugs were dissolved in Krebs solution of the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.5. Aliquots of solubilized tissue or superfusate were added to scintillation fluid of the following composition: toluene 38.5%; 1,2-dioxan 38.5%; methanol 23%; 2.5 diphenyl-oxazole (PPO) 5 g/l; 1,4 di-2-(5-phenyl oxazolyl) benzene (POPOP) 0.1 g/l and naphthalene 80 g/l.

Results

Spontaneous release of [^{3}H]-acetylcholine

The spontaneous output of [^{3}H]-ACh fell progressively throughout the experiment. The means of 11 experiments are presented in Figure 1 from which it can be seen that the rate of release declined rapidly over the first 30 min and thereafter at a slower rate. When each of the outputs during the experiment were added to the tissue content at the end of the experiment, values for the tissue content at all time points during the experiment were obtained. A semi-logarithmic plot against time yielded a curve characteristic of a two compartment system with an initial rapid distribution phase followed by a straight line elimin-

ation component (β) whose half-life ($T_{\frac{1}{2}}\beta$) was 75 min (Figure 2). The point of commencement of the straight line component (β phase) indicates the point from which all tritium is derived from a single tissue compartment; this point corresponded to t = 37.5 min and therefore it was decided not to undertake electrical stimulation within the first 50 min of superfusion.

Electrically-evoked release of [^{3}H]-acetylcholine

During electrical transmural stimulation (5 Hz) of the ileum, an increase in the output of [^{3}H]-ACh over that released spontaneously was recorded. The output returned to the equivalent spontaneous level by the end of the experiment. The output of [^{3}H]-ACh on electrical stimulation was calculated as the % increase over the spontaneous output at t = 47.5 min

$$\left(\text{output, x, at time t} = \frac{x_t - x_{47.5}}{x_{47.5}} \times 100\%\right).$$

In all, 14 control stimulation experiments were made, the results of which are compared with those from 14 experiments in which unlabelled ACh (6.8 μM) was added to the superfusate. The means (±s.e. mean) for the pooled results are shown in Figure 3. The addition of ACh caused a decrease in [^{3}H]-ACh output which was statistically significant from the 4th minute of stimulation onwards ($P < 0.05$). The spontaneous outputs presented in Figure 1 have also been calculated by the method used for the stimulated outputs and their means (±s.e. mean) are included in Figure 3 for comparison.

The lack of statistical significance for the pooled results until after the fourth minute of stimulation largely reflected the variation in response to electrical stimulation between different tissues used on different days. For this reason, all subsequent experiments were designed to show the difference between matched pairs of tissues removed from the same animal. In this way, an analysis by the paired *t* test showed that there was a significant difference between the outputs in the presence and absence of exogenous ACh by the second minute of stimulation, the ACh producing a reduction in [^{3}H]-ACh output ($P < 0.05$; $n = 7$; Table 1A).

The inhibitory action of added ACh on [^{3}H]-ACh output was prevented by the inclusion of atropine (3.5 μM) in the superfusion fluid containing ACh, there being a statistically significant difference between the [^{3}H]-ACh output in the presence of ACh and the output in the presence of ACh and atropine by the second minute of stimulation (Table 1B). There was no significant difference in [^{3}H]-ACh output on electrical stimulation in the presence and absence of atropine at any time point (Table 1C).

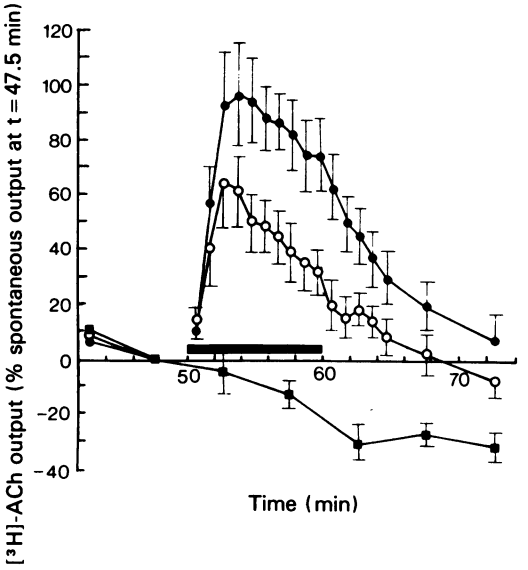

Figure 3 Electrically-evoked release of [^{3}H]-acetylcholine ([^{3}H]-ACh) in the presence (○; $n = 14$) and absence (●; $n = 14$) of unlabelled ACh (6.8 μM). The outputs have been calculated as % increase over the spontaneous output at t = 47.5 min (see text). The spontaneous outputs (■; $n = 11$) presented in Figure 1 have been included for comparison. The horizontal bar indicates the period of electrical stimulation; the vertical lines give s.e. means.

Discussion

The presence of cholinoceptors involved in the modulation of ACh release has been suggested for brain (Polak, 1971; Szerb & Somogyi, 1973; Rospars, Lefresne, Beaujouan & Glowinski, 1977) and guinea-pig ileum (Kilbinger & Wagner, 1975; Kilbinger, 1977; Sawynok & Jhamandas, 1977; Fosbraey & Johnson, 1978; 1980). Apart from Fosbraey & Johnson, most of these investigators base their evidence on the reduction of ACh output evoked by oxotremorine or physostigmine and its reversal by atropine. The present study was undertaken to determine whether ACh itself would inhibit the release of transmitter ACh whose stores had been previously labelled with tritium.

It is now well-established that all the ACh released from the guinea-pig ileum at rest and during electrical stimulation has its origin in the nervous tissue (Johnson, 1963; Paton & Zar, 1968). When the ileum is preincubated with [^{3}H]-choline, the output of [^{3}H]-ACh accounts for nearly all the tritium released by electrical stimulation (Szerb, 1976). This justified taking the evoked output of tritium in the present

experiments as a measure of ACh release in order that the effect of unlabelled ACh could be observed: such observations would be impossible if the evoked ACh were measured in any other way.

The spontaneous output of [^{3}H]-ACh followed first order kinetics, the distribution component being clearly separable from the equilibrium phase after 40 min. Electrical stimulation was therefore not carried out within the first 50 min of superfusion to ensure that all the ACh evoked was derived from a single tissue component. A significant increase ($P < 0.001$, Figure 3) in the output of [^{3}H]-ACh, calculated as a percentage increase over the spontaneous output at t = 47.5 min, was clearly demonstrated during electrical stimulation. The addition of unlabelled ACh (6.8 μM) to the superfusate led to a 40% decrease in the amount of [^{3}H]-ACh released on stimulation. This inhibitory action of added ACh on [^{3}H]-ACh output was prevented by the addition of atropine (3.5 μM) to the superfusate containing ACh. This result serves to demonstrate that the reduced output of [^{3}H]-ACh in the presence of unlabelled ACh was not due to the dilution of the labelled stores with unlabelled ACh, a possibility also precluded by the use of HC-3 and physostigmine.

These results are generally consistent with those of Kilbinger (1977) who showed that oxotremorine (1 μM) reduced the output of ACh at 0.1 Hz stimulation by 54%. With increasing frequencies of stimulation, the inhibitory effects of oxotremorine became less and at 3 Hz, the output in response to a 10 min stimulation period was not modified by oxotremorine in concentrations up to 0.1 mM. The findings in the present experiments, using 5 Hz stimulation for a period of 10 min, indicate that ACh is more effective in inhibiting transmitter ACh release than oxotremorine at higher frequencies. It was found during initial experiments that a significant increase in the amounts of [^{3}H]-ACh released on electrical stimulation was obtained only when the frequency exceeded 1 Hz, due most probably to the low concentration of [^{3}H]-choline in the incubation medium.

With such high frequencies of stimulation, the biophase concentration of endogenous ACh is likely to be high. If the myenteric plexus contains inhibitory muscarinic receptors, then the ACh released on electrical stimulation should activate them with a consequent reduction in output; thus the addition of exogenous ACh could not be expected to suppress the 5 Hz output maximally. Bearing this in mind, the atropine-

Table 1 Differences (means ± s.e. means) in the outputs of [^{3}H]-ACh evoked by electrical transmural stimulation (5 Hz; 25–30 V, 0.5 ms, 500 mA) of the guinea-pig ileum: (A) in the absence and presence of unlabelled ACh (6.8 μM) in the superfusate (control − ACh; $n = 7$); (B) in the presence of ACh (6.8 μM) and atropine (3.5 μM) compared with in the presence of ACh (6.8 μM) alone (ACh and atropine − ACh; $n = 7$); (C) in the presence and absence of atropine (3.5 μM; atropine − control; $n = 7$)

Time (min)	*A* *Mean difference in output Control − ACh*	*B* *Mean difference in output ACh and Atropine − ACh*	*C* *Mean difference in output Atropine − control*
50.5	−4.76 ± 2.41	63.6 ± 26.57	7.08 ± 9.5
51.5	22.90 ± 7.78*	94.39 ± 28.24*	20.85 ± 28.16
52.5	30.21 ± 12.2*	50.81 ± 14.87*	22.17 ± 43.81
53.5	33.69 ± 14.15	40.39 ± 13.39*	15.53 ± 44.84
54.5	42.98 ± 10.59**	48.57 ± 13.15*	6.2 ± 35.78
55.5	41.17 ± 14.14*	54.03 ± 14.4**	7.92 ± 23.79
56.5	52.76 ± 13.04**	73.87 ± 28.84*	20.17 ± 25.64
57.5	60.77 ± 20.1*	100.86 ± 43.98	26.17 ± 29.14
58.5	57.7 ± 19.67*	102.76 ± 45.68	19.75 ± 16.62
59.5	47.21 ± 20.51	74.99 ± 29.81	2.63 ± 19.89
60.5	29.01 ± 22.35	61.96 ± 20.04	−0.37 ± 22.63

The output of [^{3}H]-ACh on electrical stimulation was calculated as % increase over the spontaneous output at t = 47.5 min

$$\left(\text{output, x at time t} = \frac{x_t - x_{47.5}}{x_{47.5}} \times 100\%\right).$$

Statistical significance was determined by means of a t test using the method of paired comparisons (Bailey, 1975: *$P < 0.05$; **$P < 0.01$).

sensitive inhibitory effect of added ACh was, therefore, particularly marked.

Sawynok & Jhamandas (1977) found that the muscarinic agonist oxotremorine did not inhibit ACh release from the ileum stimulated at 0.1 Hz but antagonized the ability of atropine to increase release. Fosbraey & Johnson (1980) provided evidence for nicotinic receptors for ACh which subserve an excitatory role on transmitter release which tended to oppose the inhibition due to activation of muscarinic receptors. This action might be expected to be more evident with higher concentrations of endogenous ACh released by high frequency electrical stimulation. If endogenous ACh is producing an inhibition of transmitter output at high frequencies of electrical stimulation, then atropine should lead to an increase in the release of transmitter. In the present experiments, no significant increase in transmitter output was obtained when the preparations were superfused with Krebs containing atropine, a finding which may possibly indicate that with 5 Hz stimulation the output of ACh is maximal.

In conclusion, the experiments on the release of [^{3}H]-ACh have confirmed the findings of Fosbraey & Johnson (1980) that receptors for ACh subserving an inhibitory role on transmitter release are present in the guinea-pig ileum.

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