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FACTORS INFLUENCING THE RELEASE OF ACETYLCHOLINE FROM THE MYENTERIC PLEXUS OF THE ILEUM OF THE GUINEA-PIG AND RABBIT

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1 The effects of electrical stimulation, changes in external ion concentrations and various drugs on acetylcholine release from the myenteric plexus were measured by bioassay in the presence of physostigmine and by recording the responses of the longitudinal muscle. In preparations from the guinea-pig, the acetylcholine output per pulse increased with decreasing frequency of stimulation and reached its maximum at a frequency of 0.017 Hz (1/min) and thus ensured that the output per unit of time was constant at frequencies below 0.5 Hz. Spontaneous release was suppressed during stimulation at 0.017 Hz.

2 In the rabbit, the fractional acetylcholine release was lower than in the guinea-pig. The output per pulse increased with decreasing frequency of stimulation but at a lesser rate, with the effect that the output per unit time decreased between 0.5 and 0.017 Hz.

3 In the guinea-pig, reduction of the Ca^{2+} concentration, addition to the bath fluid of Mn^{2+}, ganglion-blocking drugs, morphine and catecholamines reduced output more at low than at high frequencies of stimulation. In the rabbit, acetylcholine output was less sensitive to changes in Ca^{2+} concentration and insensitive to Mn^{2+} and morphine.

4 In the guinea-pig, morphine and catecholamines depressed both the contractile response and acetylcholine output whereas Mn^{2+} in concentrations up to 125 μM, bretylium and ganglion-blocking drugs depressed only acetylcholine output.

5 In preparations from the guinea-pig, drugs blocking noradrenergic neurones or α-adrenoceptors, e.g. bretylium, phenoxybenzamine, thymoxamine and phentolamine, increased acetylcholine output during stimulation at high (1.5 to 10 Hz) but not at low frequencies.

6 The implications of these findings for the release of acetylcholine from different pools in the heterogeneous myenteric plexus are considered. The possible errors, introduced by the effects of physostigmine, on the size of the acetylcholine pools and on the transmission of impulses within the myenteric plexus are discussed.

Introduction

Acetylcholine is the neurotransmitter from the myenteric plexus of the guinea-pig ileum to the cells of the longitudinal muscle layer (Paton, 1957) and also for transmission within the plexus (Nishi & North, 1973).

The fact that acetylcholine is released from nerve terminals with different functions is responsible for the complexity of the factors which regulate acetylcholine output from segments of ileum or the myenteric plexus-longitudinal muscle preparation. This paper is an investigation of the relationship between the frequency of stimulation and the acetylcholine output, first pointed out by Paton (1963), and of the effects of ionic changes and of drugs on this relationship. For an interpretation of the mechanisms involved, these results, obtained in the presence of physostigmine, will be correlated with the effects on the contractions of the longitudinal muscle evoked by electrical stimulation of the preparation.

Preliminary accounts of some of the findings have been given at meetings of the Pharmacological Society (Cowie, Kosterlitz, Lydon & Waterfield, 1970; Greenberg, Kosterlitz & Waterfield, 1970; Kosterlitz & Waterfield, 1970; Kosterlitz & Waterfield, 1972a, b).

Methods

Experimental procedures

The experiments were carried out on the myenteric plexus-longitudinal muscle preparation from male guinea-pigs or rabbits. The terminal portion was used after the 10 cm nearest to the ileo-caecal junction had been discarded because of the presence of excitatory α-adrenoceptors near the ileo-caecal junction (Munro, 1953). The dissection has been described by Kosterlitz, Lydon & Watt (1970). The preparations were folded double to provide more tissue for acetylcholine release in the organ bath (3 ml). For field stimulation two Pt electrodes were used, one at the top and one at the bottom of the organ bath; unless stated otherwise, the parameters were 1.3 times maximal rectangular pulses of 0.5 ms duration at various frequencies. The twitch-like contractions of the longitudinal muscle were recorded isometrically by means of a strain gauge transducer and an ink-writing pen oscillograph.

The fluid bathing the preparation was a modified Krebs solution of the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.20, $NaHCO_3$ 25, glucose 11, containing mepyramine maleate (0.125 μM) and choline chloride (20 μM); it was kept at 36°C and bubbled with 95% O_2 and 5% CO_2.

After pre-incubation for 1 h with physostigmine sulphate (7.7 μM), which was present throughout the experiment, the acetylcholine released either spontaneously or by electrical stimulation at different frequencies was assayed on segments of guinea-pig ileum (Kosterlitz *et al.*, 1970). Collection periods were 4 min for spontaneous release and for release evoked by frequencies of up to 0.33 Hz, 2 min for 1 Hz and 0.5 min for 10 Hz. For the assay of acetylcholine released by single pulses, renewal of the donor bath fluid was followed after 5 s by the application of a single stimulating pulse and after a further 15 s a sample of bath fluid was collected for assay. The samples of fluid from the donor bath were withdrawn into a glass syringe and transferred to a small beaker and two aliquots (50 to 400 μl) of the sample assayed within 15 min. For the investigation of the effects of changes in electrolyte composition of the Krebs solution on acetylcholine release, the donor preparation was exposed to the new medium for 30 min to allow equilibration before samples were collected. Standard solutions of acetylcholine required for the assay were made up in Krebs solution acidified with HCl to give pH 4; otherwise it had the same composition as the fluid bathing the donor preparation. At the end of the experiment the preparation was blotted dry and weighed; the output of acetylcholine was expressed as pmol g^{-1} wet weight of tissue.

The acetylcholine contents of the preparations were determined by a modification (Hutchinson, Kosterlitz & Gilbert, 1976) of the method of MacIntosh & Perry (1950). In principle, the preparation was homogenized in an ice-cold solution of the following composition (mM): NaCl 136, KCl 4.75, $CaCl_2$ 2.54 and CCl_3COOH 610. After removal of proteins by centrifugation, trichloroacetic acid was extracted several times with ether which was removed by bubbling of 95% O_2 and 5% CO_2. Acetylcholine was assayed as above and its identity verified repeatedly by its destruction by alkaline hydrolysis or antagonism by hyoscine.

Drugs

Drugs used were: acetylcholine chloride, (—)-adrenaline bitartrate, choline chloride, manganous chloride (BDH); bretylium tosylate (Burroughs Wellcome); hysocine hydrobromide (Evans Medical); hexamethonium bromide, mepyramine maleate, pempidine tartrate, pentolinium tartrate, physostigmine sulphate (May & Baker); morphine hydrochloride (Macfarlan Smith); phenoxybenzamine hydrochloride (Smith, Kline & French); phentolamine hydrochloride (Ciba); thymoxamine hydrochloride (William & Warner). Stock solutions were prepared in distilled water except for acetylcholine which was made up in 5% (w/v) NaH_2PO_4 solution. The concentrations are expressed as μM base and refer to final bath concentrations.

Results

Frequency-output relationships in the guinea-pig ileum

Spontaneous release of acetylcholine. In the absence of electrical stimulation there was a spontaneous release of acetylcholine at an average rate of 500 pmol g^{-1} min^{-1} (range 110 to 1100; $n = 195$), a value which is in agreement with that reported from this laboratory earlier (Hutchinson *et al.*, 1976) but higher than values found in other laboratories (Paton & Zar, 1968; Paton, Vizi & Zar, 1971). The level of spontaneous release varied between experiments and fluctuated randomly during any one experiment; this is probably due to the fact that 60% of the output is the result of propagated activity in the myenteric plexus and only 40% to true resting release (Paton *et al.*, 1971).

In order to measure the amount of acetylcholine released by electrical stimulation, it was necessary to determine whether or not spontaneous output occurred during the intervals between the electrical stimuli. Since the output evoked by a single stimulus is sufficiently large to make a satisfactory assay poss-

ible, the following design was adopted. In eight experiments the mean rate of spontaneous release was determined for 4 min periods. In the same experiments the outputs due to single pulses applied at 1 min intervals and collected for 15 s were compared with the mean output per pulse when the preparation was stimulated at a rate of 1/min and the output collected for a 4 min period. This latter value would include any acetylcholine released spontaneously between the electrical stimuli in addition to the release evoked by electrical stimulation. There was no significant difference between the mean output from a single pulse collected only for 15 s (1447 ± 248 pmol g^{-1}) and the mean output per pulse (1254 ± 154 pmol g^{-1}) when the output evoked by 4 pulses was collected over a period of 4 min. Since the mean rate of spontaneous output per min was 677 ± 149 pmol g^{-1}, the output per min in the experiments in which acetylcholine was released by 4 stimuli over a period of 4 min should have been about 450 pmol g^{-1} larger than that evoked by single stimuli and collected for 15 s. Since this was not so, it was concluded that spontaneous release is suppressed during stimulation at a frequency as low as 1 per min or 0.017 Hz and that all values of evoked output should be expressed as gross values.

Evoked acetylcholine output. The output of acetylcholine evoked by electrical stimulation varies with the frequency of stimulation, both in a segment of guinea-pig ileum (Paton, 1957; 1963) or the myenteric plexus-longitudinal muscle preparation (Cowie, Kosterlitz & Watt, 1968; Paton & Zar, 1968). The results (Table 1) extend earlier observations to include the low frequency of 0.017 Hz which represents single stimuli. When calculated as output per pulse, the values dropped from 990 pmol g^{-1} tissue at 0.017 Hz to 13.9 pmol g^{-1} tissue at 10 Hz. On the other hand, when calculated as output per min, the values decreased when the stimulation frequency was lowered from 10 to 5 Hz but remained unchanged at frequencies below 0.5 Hz. This relationship has already been shown for segments of guinea-pig ileum stimulated at frequencies between 0.03 and 30 Hz (Paton, 1963). The values at all frequencies may be too high because of the high output due to the first pulse. When the output for the 21st to the 540th pulse was determined, the output per pulse was 30.8 ± 7.5 (n = 4) at 1 Hz and 10.6 ± 2.9 pmol g^{-1} (n = 3) at 10 Hz.

Output evoked by pairs of pulses. These experiments were designed to determine the shortest interval between two pulses necessary to produce the same output from the second or test pulse as from the first or conditioning pulse. First, the output due to a single pulse was measured repeatedly until a constant value was obtained; this was followed by assay of the output due to pairs of pulses in which the interval between the conditioning and test pulse varied from 3 to 180 s. Subtraction of the output evoked by the single pulse from that evoked by pairs of pulses provided the output due to the test pulse alone. The acetylcholine released by the test pulse increased from a very low value at a pulse interval of 3 s to a maximum at intervals between 40 and 60 s (Figure 1). The maximum output per pulse (1139 ± 97 pmol g^{-1}) was not significantly different from that due to a single pulse and was thus restored at an interval of about 50 s after the conditioning pulse. The fact that there was no significant increase in output between 60 and 180 s suggested that there was no spontaneous release of acetylcholine during that time.

Output evoked by trains of 1 to 16 pulses with intervals of 20 ms. Trains of 1, 2, 4, 8 and 16 pulses with intervals of 20 ms between each pulse were repeated

Table 1 The effects of increasing the frequency of stimulation on the gross output of acetylcholine from the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum

Frequency of stimulation (Hz)	*Number of pulses*	*Acetylcholine output per g tissue*	
		pmol pulse^{-1}	*pmol min^{-1}*
0.017	4	990 ± 33	990 ± 33 (181)
0.1	24	191 ± 12	1146 ± 72 (46)
0.25	60	72.5 ± 6.1	1088 ± 92 (24)
0.5	120	46.2 ± 5.0	1386 ± 150 (3)
1	180–540	31.3 ± 3.5	1878 ± 210 (45)
10	300–600	13.9 ± 1.5	8376 ± 916 (36)

The values are the means ± s.e. mean; the number of observations is given in parentheses. Since the values for different lengths of trains at 1 and 10 Hz did not differ significantly, they were pooled.

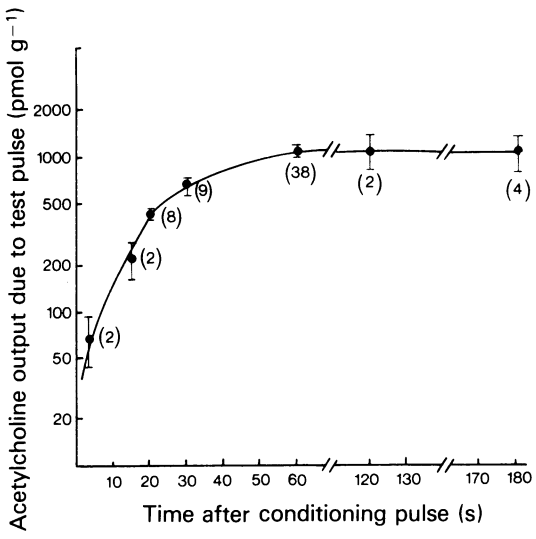

Figure 1 Output of acetylcholine due to a test pulse following a conditioning pulse at varying intervals. Each point represents the mean of the number of observations given in parentheses; vertical lines show s.e. means.

at frequencies of 0.017, 0.033, 0.1 and 0.5 Hz for 4 min. The acetylcholine output was calculated for each train and plotted against the number of pulses in each train (Figure 2). The outputs for the trains of 1 pulse are in fair agreement with those given in Table 1. At train frequencies of 0.033, 0.1 and 0.5 Hz the subsequent pulses evoked additional outputs of acetylcholine. This increase was highest (30 pmol g^{-1} per pulse) at the lowest of these train frequencies and lowest at 0.5 Hz (6.7 pmol g^{-1} per pulse). The acetylcholine outputs of the subsequent pulses appear to be correlated with that of the first pulse of a train (325 pmol g^{-1} at 0.033 Hz and 46 pmol g^{-1} at 0.5 Hz). Increases in the intervals of the pulses from 20 to 100 or 500 ms did not significantly alter this relationship.

When the repetition rate of the train was 0.017 Hz, it was more difficult to establish the effects of the pulses within a train. Under these circumstances, only 4 trains occurred during the stimulation period of 4 min instead of 8, 24 and 120 trains at the other train frequencies. Thus, the amount of acetylcholine available for assay was rather small; therefore, 1, 16 and 64 pulses (Figure 2, abscissa scale at top) were used instead of 1, 2, 4, 8 and 16 pulses (abscissa scale at bottom). The curve for the trains at 0.017 Hz was plotted with both the abscissae and the ordinates scaled down by a factor of 4, in order to compare the slope of this curve with the slopes of the curves for the other train frequencies. Apart from the flat curve for 0.5 Hz, the slopes of the curves are similar.

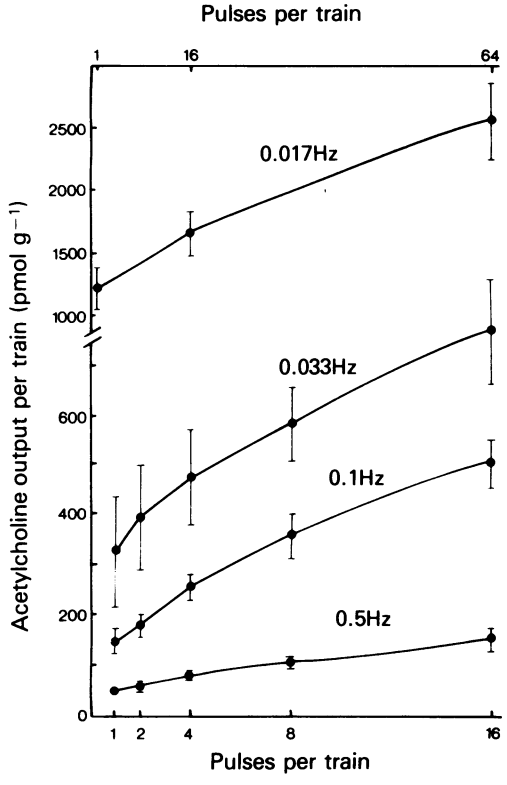

Figure 2 Output of acetylcholine evoked by trains of 1 to 16 or 64 pulses with intervals of 20 ms. The four curves represent the output evoked per train when the trains are repeated at frequencies of 0.017 (n = 5), 0.033 (n = 3), 0.1 (n = 8) and 0.5 (n = 3) Hz. Abscissae, number of pulses per train 1, 16 and 64 for 0.017 Hz and 1, 2, 4, 8 for all other frequencies; ordinate scale, acetylcholine output per train (pmol g^{-1}). Mean values are given; vertical lines show s.e. means.

Frequency-output relationships in the rabbit ileum

In myenteric plexus-longitudinal muscle preparations of the rabbit the spontaneous acetylcholine output per min (41.0 $\pm$ 4.0 pmol g^{-1}; n = 21) was less than 10% of that found in the guinea-pig ileum. Similarly, in rabbit preparations the output evoked by different frequencies and calculated per g tissue was only 5 to 10% of that found in the guinea-pig (Table 2). However, the rate of decrease in the output per pulse with increasing frequency of stimulation was similar in the preparations from the two species with the exception that in the rabbit there was little difference between the outputs due to stimulation at 1 and 10 Hz. The difference between the two species is due

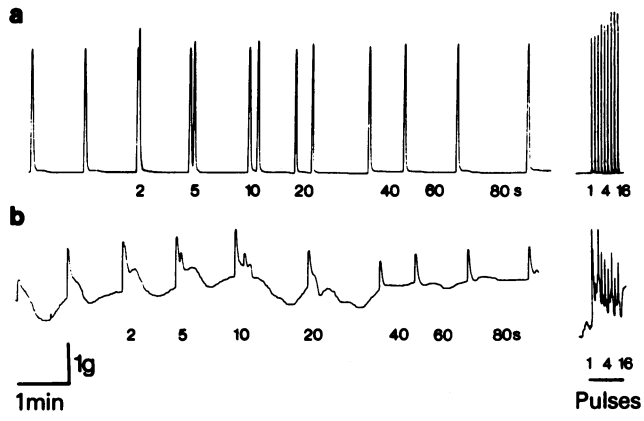

Figure 3 Contractile responses of a myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum to stimulation in the absence (a) and presence (b) of physostigmine (7.7 μM). The traces on the left show responses to single or pairs of supramaximal stimuli with the interval between pulses (s) given below each pair. On the right are shown the responses to stimulation with trains of 1, 4 and 16 pulses (pulse intervals 20 ms) recorded three times at intervals of 1 min.

not only to differences in the ratio of neuronal to other tissues since the fractional release per pulse at a stimulation frequency of 0.017 Hz was in the guinea-pig 0.8% of the acetylcholine content of the tissue (123 ± 15 nmol g^{-1}; $n = 6$) whereas in the rabbit this value was only 0.15% of the tissue content (28.5 ± 3.9 nmol g^{-1}; $n = 3$).

Correlation between contractile responses and acetylcholine output

Since incubation with physostigmine as used for the assay of acetylcholine increased the acetylcholine content of the tissue, prevented its destruction in the extracellular space (Hutchinson *et al.*, 1976) and caused a permanent contraction of the muscle, the contractile responses were observed in the absence of physostigmine. Therefore, direct comparison is not possible. The finding that in trains repeated at 0.017 Hz, subsequent pulses spaced at 20 ms intervals increased acetylcholine output (Figure 2) slightly, is borne out by the increase in the contractions of the longitudinal muscle when trains with 4 and 16 pulses were applied (Figure 3). The record of the contractions of a preparation treated with physostigmine did not show this phenomenon; this was partly due to an increased tone in the absence of stimulation and the very much reduced rate of relaxation subsequent to stimulation.

When pairs of pulses were applied in the absence of physostigmine, the contraction due to the second pulse summated with the first when the pulse interval

Table 2 The effects of increasing the frequency of stimulation on the gross output of acetylcholine from the myenteric plexus-longitudinal muscle preparation of the rabbit ileum

Frequency of stimulation (Hz)	*Number of pulses*	*Acetycholine output per g tissue*	
		pmol pulse^{-1}	*pmol min*$^{-1}$
0.017	4	43.4 ± 5.2	43.4 ± 5.2 (20)
0.1	18–54	10.5 ± 1.5	63 ± 8.9 (12)
1	60–540	1.70 ± 0.34	102 ± 20 (23)
10	60–540	1.29 ± 0.26	774 ± 156 (18)

The values are the means ± s.e. mean; the number of observations is given in parentheses.

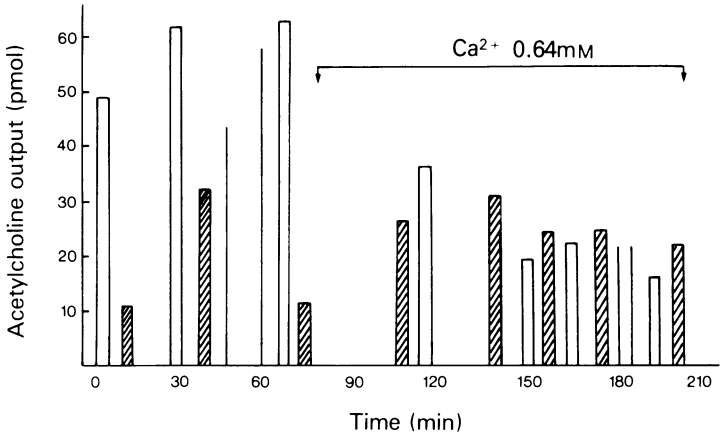

Figure 4 Histogram to show in a typical experiment the spontaneous output of acetylcholine per min (hatched columns), the output per pulse due to stimulation for 4 min at 0.017 Hz (open columns) and due to single pulses with a collection period of 20 s (solid lines) from the myenteric plexus of guinea-pig ileum. The Ca^{2+} concentration was either 2.54 mM (0 to 70 min) or 0.64 mM (70 to 200 min).

was 2 s; from 5 s onwards, the two contractions were discrete and developed about the same tension (Figure 3). This is in contrast to the findings on the acetylcholine output: the second pulse evoked the same output as the first pulse only when the pulse interval was 40 s or more (Figure 2). On the tracings of the preparations treated with physostigmine, the contractions due to the second pulses can be seen but are smaller than those due to the first pulse until the pulse interval is 40 s.

Effects of a decrease in the calcium chloride concentration of the Krebs solution on acetylcholine output

In three preparations from the guinea-pig, the spontaneous release of acetylcholine, the output induced by repeated stimulation at 0.017 Hz and the output due to single pulses were measured in Krebs solutions containing $CaCl_2$ concentrations of 2.54 and 0.64 mM. The sponteneous release and output at 0.017 Hz were collected for 4 min and that due to a single pulse for 20 s. The mean output due to single pulses (Figure 4) was similar to the mean output per min when the preparation was stimulated 4 times in 4 min (0.017 Hz); both outputs were greater than the spontaneous output per min. When the concentration of $CaCl_2$ was reduced to 0.64 mM, the evoked output per pulse due to stimulation at intervals of 1 min or due to single pulses was reduced by almost 60% and was not as low as the rate of spontaneous release. That there was still an evoked release during stimulation was shown by the fact that the output due to a single pulse with a collection period of only 20 s was similar to that due to one of the four pulses applied during a collection period of 4 min. It follows that no spontaneous output of acetylcholine was discernible at $CaCl_2$ concentrations of either 2.54 or 0.64 mM for at least 1 min after a stimulus had been applied.

In view of the differences in the evoked fractional release of acetylcholine from the myenteric plexuses of the guinea-pig and rabbit, it was important to compare the effects of reduction in the $CaCl_2$ concentration of the Krebs solution in the two species. The preparations were stimulated at 0.017, 1 and 10 Hz in the presence of 2.54, 1.27, 0.64 and 0.32 mM $CaCl_2$ (Figure 5). At all frequencies, a decrease in the calcium concentration had a greater effect on preparations from the guinea-pig than on those from the rabbit. In both species, a change from 2.54 to 1.27 mM $CaCl_2$ either raised the acetylcholine output or caused no change. At a $CaCl_2$ concentration of 0.32 mM, the maximal acetylcholine output was reduced in the guinea-pig by 83, 82 and 84% at the three frequencies of 0.017, 1 and 10 Hz and in the rabbit by 31, 52 and 66%. The corresponding values at 0.64 mM $CaCl_2$ were 72, 53 and 38% in the guinea-pig and 0, 25 and 42% in the rabbit. It follows that in the guinea-pig the output at 0.017 Hz was more susceptible to a reduction in $CaCl_2$ concentration than the outputs at the higher frequencies whereas in the rabbit the relationship was reversed.

Effects of manganese ions on acetylcholine output

Since Mn^{2+} can compete with Ca^{2+} in the processes responsible for transmitter release (Meiri & Rahamimoff, 1972), the effects of Mn^{2+} on the output of acetylcholine were determined. From dose-response

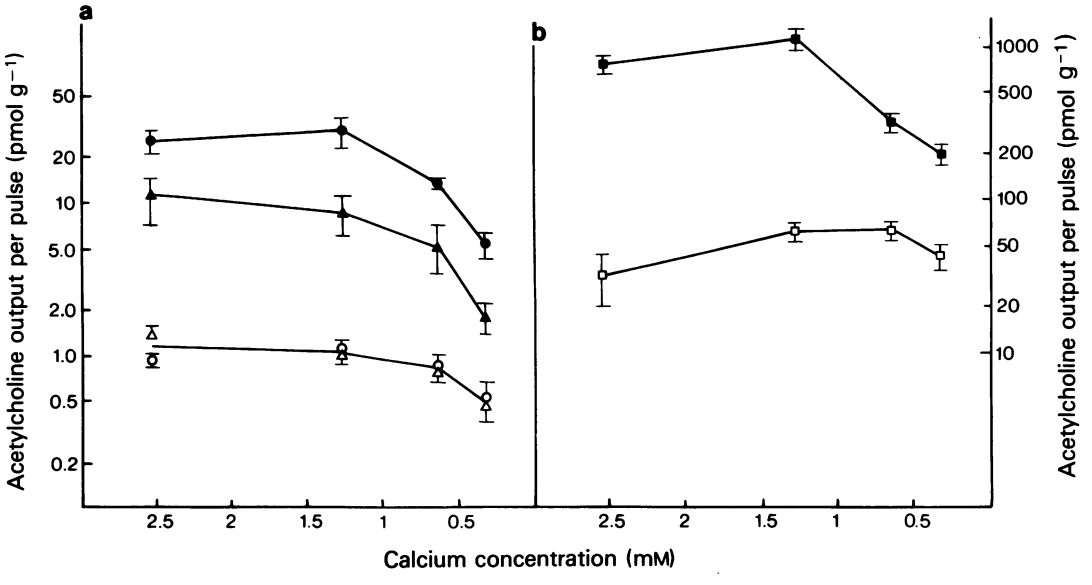

Figure 5 The acetylcholine output evoked by stimulation at 0.017, 1 and 10 Hz at Ca^{2+} concentrations of 2.54, 1.27, 0.64 and 0.32 mM. Abscissae, calcium chloride concentration (mM); ordinate scales, acetylcholine output per pulse (pmol g^{-1} tissue) plotted on a logarithmic scale. (a) Guinea-pig: (●) 1 Hz; (▲) 10 Hz; rabbit: (○) 1 Hz; (△) 10 Hz. (b) 0.017 Hz: (■) guinea-pig; (□) rabbit. The points are the means of 3 expts; vertical lines show s.e. means.

curves (Figure 6) for the effects on spontaneous output and on that evoked by stimulation at 0.017 Hz, the concentrations inhibiting the output by 50% (IC_{50}) were 40 and 80 μM, respectively.

When the effects of a supramaximal concentration of Mn^{2+} (1 mM) were examined in the rabbit, no effect was found at any of the frequencies tested, 0, 0.017, 1 and 10 Hz (Figure 7). In the guinea-pig ileum, Mn^{2+} (1 mM) had no effect at 1 and 10 Hz, whereas both spontaneous output and that evoked by stimulation at 1/min were reduced by 80% (Figure 7). In another series of experiments on preparations from the guinea-pig ileum, the output evoked by stimulation at 0.5 Hz was reduced by Mn^{2+} (1 mM) from 94.6 ± 12.1 to 12.1 ± 3.9 pmol g^{-1} per pulse ($n = 3$).

In the guinea-pig, when the contractile responses to electrical stimulation at 0.017, 0.1, 1.0 or 10 Hz were recorded in the absence of physostigmine, there was no significant effect of 125 μM Mn^{2+}. This contrasts with the effects of Mn^{2+} on acetylcholine release evoked by stimulation at 0.017 Hz where 125 μM Mn^{2+} caused an inhibition of about 70% (Figure 6). When the concentration of Mn^{2+} was increased to 1 to 2 mM, the contractile responses were reduced, presumably due to a postjunctional effect since the contractions caused by acetylcholine added to the bath fluid were also depressed.

The effects of morphine on acetylcholine output

Variation in frequency of stimulation. When the guinea-pig preparation was stimulated at a frequency of 0.017 Hz, the IC_{50} of morphine was about 120 nM (Figure 11) and this effect was antagonized by naloxone (Lees, Kosterlitz & Waterfield, 1972). When the acetylcholine output at different frequencies was plotted as output per min, the constancy of this output over the low frequencies of 0.017 to 0.5 Hz was abolished by morphine (Figure 8), a phenomenon also brought about by Mn^{2+} ions and hexamethonium. Morphine changed the relationship of output per min to frequency of stimulation in the guinea-pig to one similar to that found in preparations from the rabbit, although the levels of acetylcholine output were lower in the rabbit than in the guinea-pig.

When trains of 10 or 100 pulses with pulse intervals of 100 ms were applied at a frequency of 0.017 Hz, the acetylcholine output was increased by 1330 pmol for 100 pulses over and above the output produced by single pulses at 0.017 Hz (Table 3). The output

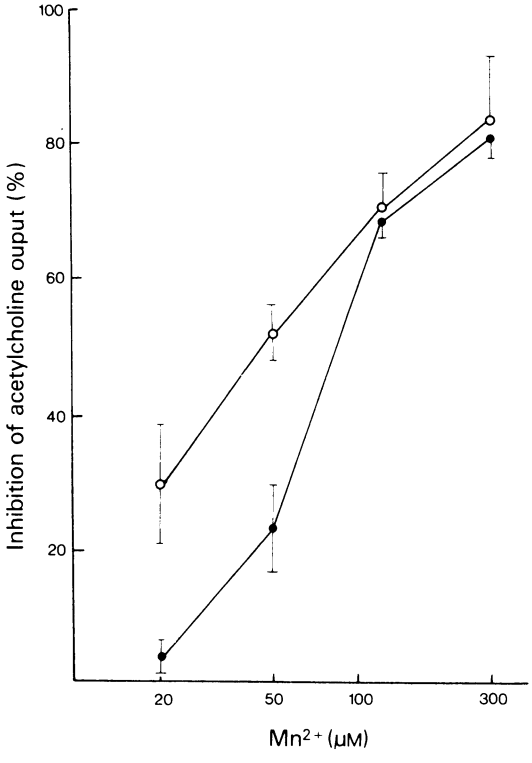

Figure 6 Dose-response curve for the effect of Mn^{2+} on the spontaneous output (○) and the output evoked by stimulation at 0.017 Hz (●) in the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum. The points indicate the means of 6 expts; vertical lines show s.e. means. Control spontaneous release was 481 ± 62 and control evoked output 1085 ± 133 pmol g^{-1} tissue min^{-1}.

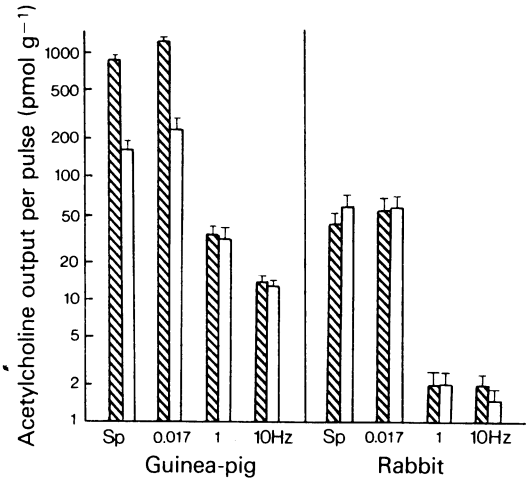

Figure 7 The effects of Mn^{2+} (1 mM) on acetylcholine output from the myenteric plexus of the ilea of guinea-pig and rabbit. Collection periods for spontaneous release (Sp) and stimulation at 0.017 Hz were 4 min and for stimulation at 1 and 10 Hz 10 and 1 min, respectively. Note that the acetylcholine output is plotted on a logarithmic scale; therefore in the guinea-pig the depression of spontaneous release and that at 0.017 Hz is 80%. The number of expts was 4 for the rabbit and, for the guinea-pig, 3 for Sp and 1 Hz, and 6 for 0.017 and 10 Hz.

per pulse was similar to that found after prolonged stimulation with single pulses spaced 100 ms apart, i.e. 10 Hz (Table 1). Whereas morphine (1 μM) depressed the output evoked by the first pulse of a train, the output due to the next 9 or 99 pulses was not decreased (Table 3).

Table 3 The effect of morphine on the output of acetylcholine evoked by trains of 1, 10 and 100 pulses

Pulses in train	*Acetylcholine output per train (pmol g^{-1})* *Control*	*Morphine (1 μM)*	*Difference due to morphine*
1	1892 ± 337	1057 ± 133	835 ± 269
10	1847 ± 347	1148 ± 219	699 ± 170
100	3220 ± 735	2729 ± 707	491 ± 98

The values are the means ± s.e. mean of 4 experiments. The pulses were applied at intervals of 100 ms. The trains were repeated at a rate of 1 min^{-1} (0.017 Hz) for 4 min. While morphine depressed the output evoked by the 1st pulse of each train ($P < 0.05$), the increase in output caused by the 2nd to 100th pulses was not significantly affected by morphine: in the absence of morphine the output increased by 1328 ± 423 pmol g^{-1} and in its presence by 1672 ± 590 pmol g^{-1}, the difference not being significant (344 ± 193; paired analysis).

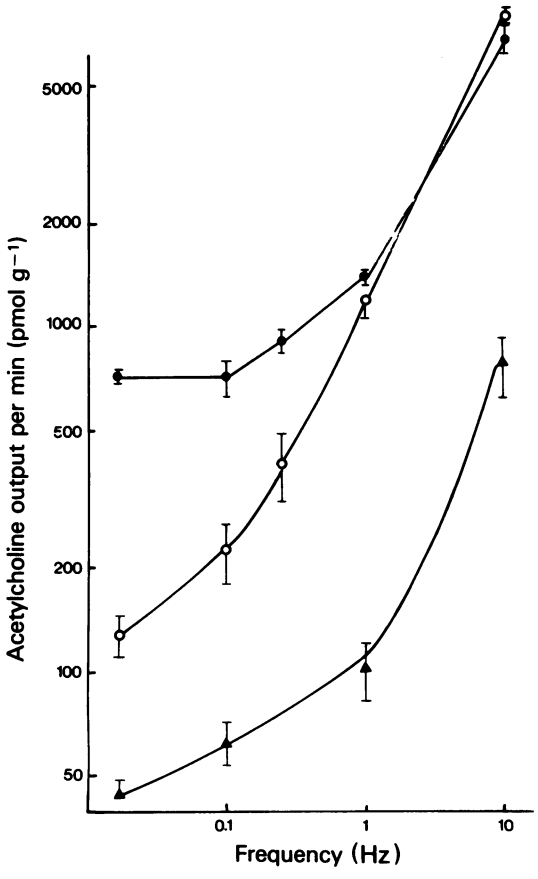

Figure 8 The effect of morphine on the electrically evoked output of acetylcholine from myenteric plexus-longitudinal muscle preparations. Abscissa scale, frequency of stimulation (Hz); ordinate scale, output of acetylcholine per min (pmol g^{-1}). Mean values are shown; vertical lines give s.e. means. (●) Control output and (○) output in presence of morphine (1 μM) in 3 preparations from the guinea-pig; (▲) output in 12 to 23 preparations from the rabbit.

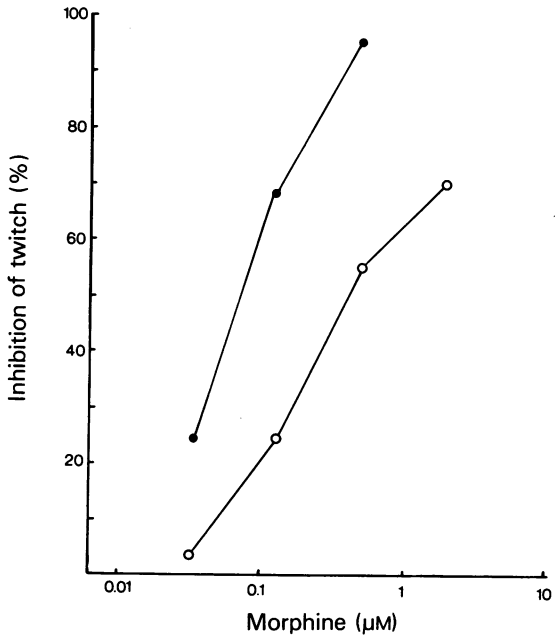

Figure 9 The depressant action of morphine on the contractions of the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum. Stimulation at 0.1 Hz; (●) submaximal stimulation (40 V); (○) maximal stimulation (70 V).

The spontaneous release of acetylcholine from the myenteric plexus is due to varying levels of electrical activity within the plexus (Paton *et al.*, 1971). Since this release can be depressed by morphine, the reduction in output during periods of stimulation might be due to the reduction in spontaneous release between pulses. In three preparations there was no significant difference between the reduction by morphine of the output induced by four pulses applied at 1 min intervals during a 4 min collection period (54.7 ± 7.2%) and the reduction of the output due to single pulses again applied at 1 min intervals when the acetylcholine released by each pulse was collected individually over a period of 20 s (52.6 ± 9.7%). In the same experiments, the spontaneous release per min was reduced by 69.1 ± 9.7% from 503 ± 65 to 144 ± 37 pmol g^{-1} min^{-1}. Therefore the depression of the evoked output at 0.017 Hz could not be attributed to the depression of spontaneous release.

Variation in strength of stimulus. The sensitivity of segments of guinea-pig ileum to the depressant action of morphine is dependent on the strength of stimulus, the contractile response due to submaximal stimuli being more depressed by a given concentration of morphine than those due to supramaximal stimuli (Cox & Weinstock, 1966). This relationship also holds for the myenteric plexus-longitudinal muscle preparation; for instance, in a typical experiment the IC_{50} of morphine for submaximal stimulation at 0.1 Hz was found to be 50 nM whereas at maximal stimulation this value was increased almost tenfold to 490 nM (Figure 9). In many experiments the value of IC_{50} due to maximal stimulation decreased considerably during the course of 4 to 6 h.

The output of acetylcholine evoked by submaximal stimulation was lower than that obtained by maximal

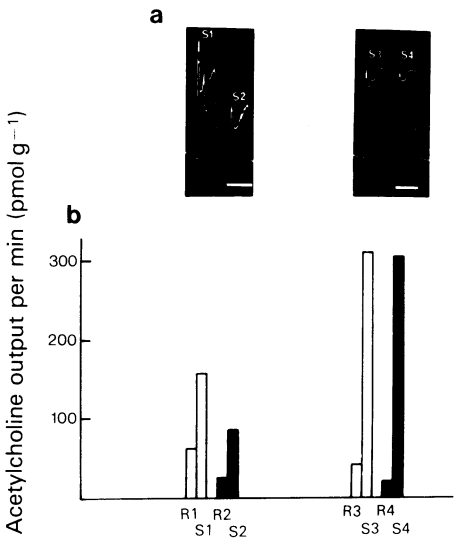

Figure 10 Effects of morphine (1 μM) on the contractile responses and the acetylcholine output of the myenteric plexus-longitudinal muscle preparation stimulated for 2 min at 5 Hz at submaximal and supramaximal stimulus strengths. (a) Tracings of contractile responses; (b) acetylcholine output per min (pmol g^{-1}). R_1 and R_3, control spontaneous acetylcholine outputs; R_2 and R_4, spontaneous outputs in presence of morphine. S_1 and S_2, contractile responses and evoked outputs due to stimulation at 70 mA, S_2 in the presence of morphine; S_3 and S_4, contractile responses and evoked outputs due to stimulation at 150 mA, S_4 in the presence of morphine.

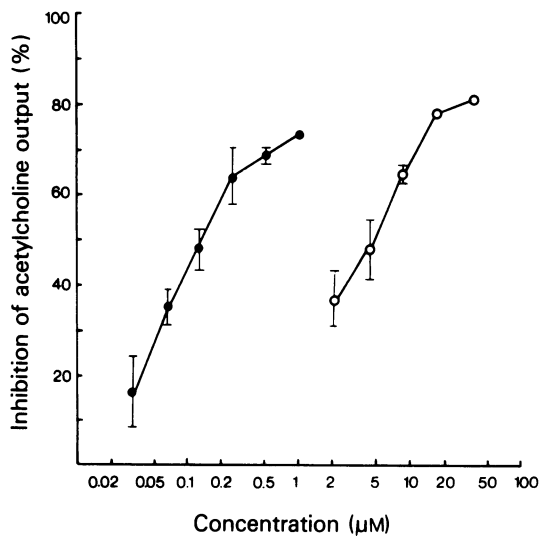

Figure 11 Dose-response curves for the inhibitory effects of morphine and hexamethonium on the output of acetylcholine from the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum. Rate of stimulation, 0.017 Hz. Abscissa scale, concentration of morphine or hexamethonium (μM); ordinate scale, inhibition of acetylcholine output. Each point is the mean of 2 to 11 observations, vertical lines show s.e. means. (●) Morphine; (○) hexamethonium.

stimulation. This phenomenon was more easily demonstrated at higher frequencies, e.g. 5 Hz (Figure 10). At the lower stimulation current (70 mA) morphine depressed both the contractile response and the acetylcholine output whereas at a current of 150 mA morphine had no depressant effect on either. Even at 10 Hz, morphine was still able to depress acetylcholine output, provided the stimulus strength was submaximal. In a series of three experiments, the control output due to a stimulus giving a just submaximal response was 27.0 ± 1.7 pmol min^{-1} and was reduced by morphine (2.1 μM) to 11.6 ± 0.6 pmol min^{-1}; on the other hand, the corresponding outputs due to 1.5 times maximal stimuli were 39.6 ± 7.7 and 38.0 ± 7.2 pmol min^{-1}.

Other inhibitors of acetylcholine release

Ganglion-blocking drugs. In the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum, hexamethonium depressed the output in a dose-dependent manner, with an IC_{50} of 4.4 μM at a stimulation rate of 0.017 Hz (Figure 11). Similar effects were obtained with pentolinium (IC_{50}, 0.8 μM) and pempidine (IC_{50}, 0.15 μM). The inhibitory effect of a maximal dose of hexamethonium (140 μM), pentolinium (21 μM) or pempidine (10 μM) decreased as the frequency of stimulation was increased (Table 4), in a manner similar to that found for morphine. In three experiments, the maximal inhibitory effects of morphine (1 μM) and hexamethonium (140 μM) were determined in the same preparation. The acetylcholine output due to stimulation at 0.017, 1 and 10 Hz was measured in the absence and presence of hexamethonium, which was then washed out. When the output had recovered to normal, the depressant effect of morphine was determined. At no frequency was there a statistically significant difference between the depressant effects of hexamethonium and morphine (Table 5).

From these results it follows that, at low frequencies, the output of acetylcholine was reduced by morphine and the ganglion blocking drugs to 15 to 45% of the control value. If the sites of action were identical, a concentration of one of the drugs would be

Table 4 Comparison of the effects of maximal concentrations of hexamethonium (140 μM), pentolinium (21 μM) and pempidine (10 μM) on the electrically evoked output of acetylcholine (pmol g^{-1} per pulse) from the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum at different frequencies

Frequency of stimulation (Hz)	*Control*	*Hexamethonium*	*Decrease*	*Control*	*Pentolinium*	*Decrease*	*Control*	*Pempidine*	*Decrease*
0.017	940 ± 136	243 ± 42	697 ± 142**	1082 ± 161	211 ± 21	871 ± 141*	864 ± 198	268 ± 49	596 ± 151*
0.066	250 ± 25	112 ± 11	138 ± 28**	—	—	—	—	—	—
0.1	—	—	—	154 ± 37	46 ± 10	108 ± 36*	160 ± 30	68 ± 9	92 ± 22*
0.25	76 ± 7	40 ± 4	36 ± 8.3*	—	—	—	—	—	—
1	34.2 ± 4.0	23.0 ± 1.3	11.2 ± 3.7*	30.8 ± 8.3	23.5 ± 2.6	7.3 ± 5.8	23.7 ± 2.5	16.7 ± 2.1	7.0 ± 1.0**
10	16.7 ± 3.3	14.3 ± 2.3	2.4 ± 1.0	11.8 ± 1.3	13.2 ± 1.7	0	12.3 ± 1.6	13.4 ± 1.1	0

The values are the means ± s.e. mean of 5 experiments with hexamethonium and 3 experiments each with pentolinium and pempidine. The results for pentolinium and pempidine have been provided by Dr I. F. Russell. *$P < 0.05$; **$P < 0.01$ (paired analysis).

Table 5 A comparison of the effects of maximal concentrations of hexamethonium (140 μM) and morphine (1 μM) on the electrically evoked acetycholine output (pmol g^{-1} per pulse) from the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum at different frequencies

Frequency stimulation (Hz)	*Control*	*Hexamethonium*	*Decrease*	*Control*	*Morphine*	*Decrease*
0.017	897 ± 87	308 ± 86	589 ± 84**	842 ± 93	336 ± 86	506 ± 6***
1	42.0 ± 5.2	28.8 ± 9.6	13.2 ± 4.9	38.4 ± 7.3	27.7 ± 7.3	10.7 ± 2.5*
10	16.7 ± 3.3	14.3 ± 2.3	2.4 ± 1.0	16.0 ± 3.9	16.7 ± 2.0	0

The values are the means ± s.e. mean of three experiments. After the determination of the effects of hexamethonium, the preparation was washed free of drug until the output values had recovered; then the effects of morphine were determined. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (paired analysis).

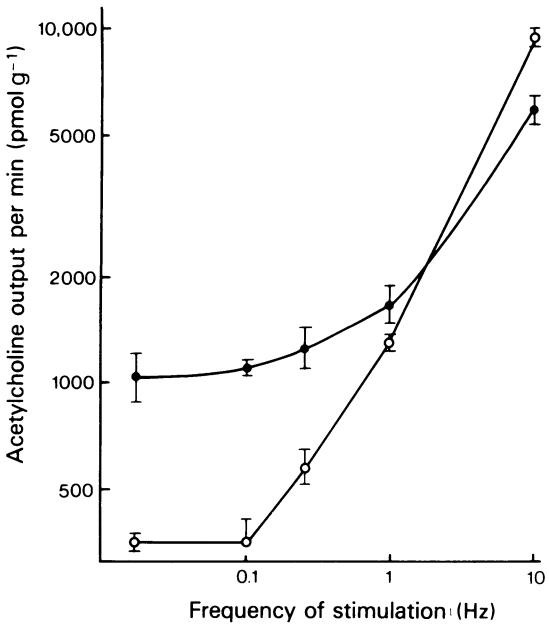

Figure 12 The effects of bretylium (20 μM) on the output of acetylcholine from the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum. Abscissa scale, frequency of stimulation (Hz); ordinate scale, acetylcholine output per min (pmol g^{-1}). The points are the means of 3 expts; vertical lines show s.e. means. (●) Control values; (○) values 20 min after the addition of bretylium (20 μM).

ineffective in the presence of a concentration of the other giving a maximal depressant effect. In three experiments, the effect of morphine (1 μM) was determined on the output of acetylcholine from the myenteric plexus after the output had been maximally depressed by hexamethonium (Table 6). The output of acetylcholine evoked by stimulation at frequencies of up to 1 Hz was reduced further by morphine; at 10 Hz, morphine had no effect. In these experiments 72.4% of the output was inhibited apparently by hexamethonium; morphine then reduced the output by a further 14.3% whereas the remaining 13.3% were neither hexamethonium- nor morphine-sensitive. Thus, morphine did not exert its effect at the same site as hexamethonium.

Catecholamines also depress the output of acetylcholine from the myenteric plexus of the guinea-pig ileum (Cowie *et al.*, 1968; Paton & Vizi, 1969; Kosterlitz *et al.*, 1970). In two experiments, adrenaline (0.5 μM) caused a further reduction of acetylcholine output at 0.017 Hz but not at 1 or 10 Hz after maximal depression by hexamethonium.

Drugs blocking adrenergic neurones or α-adrenoceptors. The depressant effects of adrenaline or noradrenaline on acetylcholine output may be modified by the action of endogenous noradrenaline released during stimulation. To resolve this problem two approaches were used: prevention of the release of noradrenaline from adrenergic nerve terminals by bretylium, and the use of the α-adrenoceptor blocking agents phenoxybenzamine, thymoxamine and phentolamine. In three preparations from the guinea-pig, the rate of spontaneous release of acetylcholine and the output at various frequencies was determined before and 20 min after bretylium (20 μM). At the low frequencies of 0.017 to 0.26 Hz, bretylium decreased the output per min of acetylcholine (Figure 12) and also the rate of spontaneous release (643 ± 179 to 161 ± 14 pmol g^{-1} min^{-1}). The effect on spontaneous release was irreversible but the depressant effect on the output at 0.017 Hz and 0.1 Hz was partially reversed when bretylium was washed out. As the frequency of stimulation was further increased (1 Hz) bretylium no longer had an inhibitory

Table 6 The effect of morphine (1 μM) on the electrically evoked acetylcholine output (pmol g^{-1} per pulse) of the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum at different frequencies, after maximal depression of the output by hexamethonium (280 μM)

Frequency stimulation (Hz)	*Control*	*Hexamethonium*	*Decrease*	*Hexamethonium and Morphine*	*Decrease*
0.017	1252 ± 289	348 ± 79	904 ± 297*	167 ± 41	181 ± 46*
1	49.5 ± 5.8	23.7 ± 7.0	25.8 ± 4.1**	15.3 ± 4.1	8.4 ± 2.9
10	22.8 ± 5.9	20.2 ± 5.8	2.6 ± 1.8	14.9 ± 3.7	5.3 ± 2.1

The values are the means ± s.e. mean of 3 experiments. *$P < 0.05$; **$P < 0.0125$. The acetylcholine outputs in the presence of 140 μM hexamethonium were 308 ± 94, 20.9 ± 4.4 and 14.9 ± 5.5 at 0.017, 1 and 10 Hz, respectively.

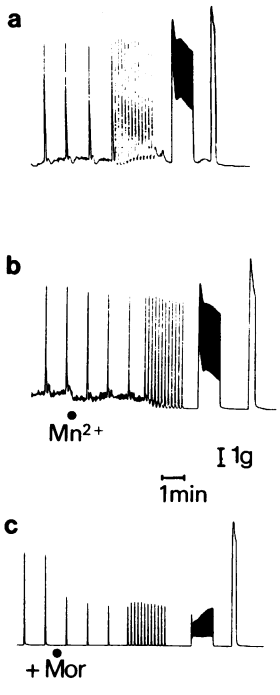

Figure 13 The contrast between the effects of Mn^{2+} and morphine on the contractile responses of the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum at stimulation frequencies of 0.017, 0.1, 1 and 10 Hz. Isometric recording. (a) Control responses; (b) effect of Mn^{2+} (125 μM) applied at dot; (c) effect of morphine (Mor, 1 μM) in presence of Mn^{2+} at dot. Calibrations: horizontal, 1 min; vertical 1 g.

effect and, at 10 Hz, it caused a significant increase ($P < 0.05$) in output, the increase persisting after the drug had been removed from the bathing medium. A similar result was found for phenoxybenzamine (3 μM) which depressed the spontaneous release and the output during stimulation at 0.017 Hz and 0.1 Hz whereas the output during stimulation at 1 Hz and 10 Hz was increased. Thymoxamine, on the other hand, did not cause a decrease in acetylcholine output at low frequencies.

Since the release of noradrenaline from the myenteric plexus increases steeply with increasing frequency of stimulation (Henderson, Hughes & Kosterlitz, 1975), any effect of blocking drugs may be expected to be present at the higher frequencies of stimulation. Such an effect has been shown for bretylium, phenoxybenzamine, phentolamine and thymoxamine (Table 7).

Comparison of drug effects on contractile responses and acetylcholine output

It has been known since the introduction of the model of the coaxially stimulated ileum (Paton, 1955) that with maximal stimulation hexamethonium does not depress the longitudinal muscle contraction of this preparation. On the other hand, the results of experiments presented in this paper showed that hexamethonium reduced the output of acetylcholine evoked by stimulation at frequencies between 0.017 and 0.25 Hz. A similar discrepancy was found for the effect of Mn^{2+} ions in a concentration which maximally inhibited acetylcholine output. This absence of a depressing effect of Mn^{2+} is shown in Figure 13, which also demonstrates that, in the presence of Mn^{2+} (125 μM),

Table 7 The effects of drugs blocking adrenergic neurones or α-adrenoceptors on the electrically evoked acetylcholine output (pmol g^{-1} per pulse) from the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum at different frequencies

Frequency stimulation (Hz)	*Control*	*Bretylium*	*Control*	*Phenoxybenzamine*
1	28.4 ± 3.4	21.8 ± 1.2	26.1 ± 3.2	31.8 ± 3.1
10	10.0 ± 0.9	15.8 ± 2.4*	11.0 ± 0.8	18.2 ± 1.8***
	Control	*Phentolamine*	*Control*	*Thymoxamine*
1.5	29.2 ± 5.5	48.4 ± 11.6*	28.1 ± 3.9	35.2 ± 4.4
4.5	16.5 ± 2.8	25.9 ± 4.4**	13.8 ± 1.1	17.6 ± 1.7*

The values are the means ± s.e. mean of 3 experiments each with bretylium (20 μM), phentolamine (0.31 μM) and thymoxamine (0.054 μM) and 7 expts. with phenoxybenzamine (3 μM). Significance of difference from controls obtained by paired analysis: *$P < 0.05$, **$P < 0.025$, ***$P < 0.0025$.

morphine (1 μM) exerts its usual depression of the contraction. Lack of agreement between the effect on contractile responses and on acetylcholine output was also observed for other ganglion-blocking drugs and for bretylium which at frequencies below 1 Hz reduced and above 1 Hz increased output but had no effect on the corresponding contractile responses.

Discussion

Although the depressant effects of opiates and opioid peptides on the electrically evoked contractions of the myenteric plexus-longitudinal muscle preparation are widely used for the characterization of these compounds, the underlying mechanisms have not been analyzed satisfactorily. While the original observations of Paton (1957) that the inhibition of the contractions by opiates is associated with a decrease in acetylcholine output has been confirmed, the fact that there is cholinergic transmission between neurones in the myenteric plexus (Paton & Zaimis, 1949; Feldberg, 1951; Nishi & North, 1973) makes it difficult to correlate drug effects on the contractile responses with those on acetylcholine output. Moreover, the presence of the anticholinesterase physostigmine when acetylcholine output is determined, introduces complications because it causes a marked increase in free cytoplasmic acetylcholine which is readily releasable (Hutchinson *et al.*, 1976). Labelling of the acetylcholine stores with [^{3}H]-choline (Szerb, 1975; 1976) avoids this difficulty but choline uptake has to be blocked since acetylcholine output is measured by the appearance of hydrolysed [^{3}H]-choline.

Since 60% of the spontaneous release is due to propagated activity in the myenteric plexus and only 40% to true resting release (Paton *et al.*, 1971), it is not surprising that factors which affect the output at low frequencies often produce similar effects on the spontaneous release of acetylcholine. It was therefore important that the spontaneous release between pulses was suppressed at a rate of stimulation as low as 0.017 Hz, and that for this reason spontaneous output should not be deducted from the total output evoked by stimulation.

When single stimuli were applied, the output of acetylcholine was very large, amounting to 0.8% of the tissue content. Since in the presence of physostigmine ganglion cells of the myenteric plexus remain depolarized for a prolonged period during which they may produce repetitive action potentials (North & Nishi, 1974), the question arises whether this large output is due to a single impulse or a train of impulses arriving at the nerve terminals. Szerb (1976) has calculated that, in the absence of physostigmine, the fractional release per pulse at 0.1 Hz is 40 times greater than at 16 Hz while with physostigmine we find a fractional release 15 times greater at 0.1 than at 10 Hz and 70 times greater at 0.017 Hz than at 10 Hz. These values do not support the view that the large acetylcholine output in the presence of physostigmine is due to repetitive discharge of ganglion cells in the myenteric plexus.

During continuous stimulation of the guinea-pig myenteric plexus at frequencies from about 0.5 Hz upwards, the output of acetylcholine per unit of time is greater at the higher than at the lower frequencies. In contrast, the output per unit of time remains constant when the frequency of stimulation is lowered from 0.5 to 0.017 Hz. This inflection of the curve showing output per unit of time against frequency of stimulation in the guinea-pig ileum has already been noted by Paton (1963); it is absent from the frequency-output relationship in the rabbit. Furthermore, the inflection is removed in conditions in which the acetylcholine output is depressed much more at low than at high frequencies, e.g. by lowering the bath temperature (Cowie *et al.*, 1968) or by Mn^{2+} ions, hexamethonium, catecholamines (Paton & Vizi, 1969; Kosterlitz *et al.*, 1970) and morphine (Paton, 1957, 1963; Cox & Weinstock, 1966; Cowie *et al.*, 1968; Paton & Zar, 1968).

It is unlikely that these agents act in the same manner since the contractile responses in the absence of physostigmine were depressed by catecholamines and morphine and also by strychnine, cyclic adenosine 3′,5′-monophosphate (cyclic AMP) and dibutyryl cyclic AMP (Takagi & Takayanagi, 1966; 1972) but not by hexamethonium, pentolinium, pempidine or Mn^{2+} ions. The action of hexamethonium on acetylcholine output could be explained by the prevention of repetitive discharge in the myenteric plexus caused by physostigmine (North & Nishi, 1974) whereas this effect would be absent when the contractile responses are examined in the absence of physostigmine. Two points may make this explanation less acceptable. First, as has been shown above, the repetitive discharge may not be responsible for the high acetylcholine output and secondly, the blocking action of hexamethonium in a sympathetic ganglion is more effective at 8 than at 2 Hz (Riker & Kamalahiranya, 1962) whereas in the myenteric plexus the greatest effect of hexamethonium is found at frequencies below 1 Hz. The difference could possibly be due to the fact that in the experiments in the sympathetic ganglia, the block was measured by the action potentials in the postganglionic axon while in the output studies the frequency relationship may be modified by the processes involved in the release of acetylcholine triggered by the arrival of the action potential in the nerve terminals.

No information is available as to the mechanism of the depression of acetylcholine output by Mn^{2+} which may be related to the reduction of output by

a decrease in the Ca^{2+} concentration of the Krebs solution.

As far as the mechanism of action of morphine and some other opiates is concerned, evidence has been adduced for a hyperpolarization of the membrane of the soma and the cellular processes which, it is suggested, may prevent invasion of the nerve terminal and thus lower acetylcholine output (North & Tonini, 1977).

The discussion so far has developed possible interpretations of the available experimental data. Several difficulties remain. Clearly, in the guinea-pig myenteric plexus several stores of acetylcholine are available for release (Hutchinson *et al.*, 1976). The release of large amounts of acetylcholine depends on the interval between two stimuli, 40 s being the shortest time which will permit a maximum output by the second pulse; this fact would indicate that this readily releasable store is relatively small, an interpretation which agrees with the findings with labelled acetylcholine stores (Szerb, 1976). The rate of release from this small store is faster than that from the larger stores and it is also more sensitive to the inhibitory action of morphine (Down & Szerb, personal communication). Nothing is known about the sites of the various pools of acetylcholine although circumstantial evidence suggests that the nerve terminals responsible for the release of acetylcholine to the longitudinal muscle layer contain readily releasable stores which are sensitive to the depressant actions of morphine and catecholamines.

It is likely that the release of acetylcholine towards the muscle is mediated by more than one class of nerve terminals because when a submaximal stimulus is used the release is more sensitive to morphine than at higher stimulus strengths; this holds for both contractile responses and acetylcholine output. Similar findings were also obtained by Down & Szerb (personal communication) with labelled acetylcholine stores.

For the interpretation of the action of the agents which depress acetylcholine output it is important to note that hexamethonium, Mn^{2+}, morphine or noradrenaline do not affect the acetylcholine content of preparations during stimulation for periods of up to 60 min (Hutchinson *et al.*, 1976). It is of interest that, in the presence of physostigmine, Mn^{2+} ions, hexamethonium, pentolinium, pempidine, morphine, adrenaline and noradrenaline all depress the acetylcholine output due to stimulation at 0.017 Hz to a similar extent (55 to 85%). This observation implies that at least in part a common mechanism is involved. This mechanism is probably elicited by an action at different sites of the same neurones. Thus, even after maximal inhibition of the output by hexamethonium, morphine causes a further depression. As a corollary, a concentration of hexamethonium which maximally depresses acetylcholine output, has no effect on the contractile response while addition of morphine promptly depresses this response.

The effects of bretylium and phenoxybenzamine on acetylcholine release during stimulation at varying frequencies are complex; these drugs reduce acetylcholine output at low frequencies and increase it at high frequencies. The former effect of bretylium may be correlated to its ganglion-blocking effect in the myenteric plexus (Kosterlitz & Lees, 1961). However, a more interesting finding is the fact that all drugs which either reduce noradrenaline release or block α-receptors result in an increase in acetylcholine release at frequencies greater than 1 Hz. Since noradrenaline release from the myenteric plexus increases steeply with an increase in frequency of stimulation (Henderson *et al.*, 1975), it may be assumed that the noradrenaline released by stimulation at high frequency acts on presynaptic α-adrenoceptors to reduce acetylcholine release, an effect which is reduced by drugs blocking adrenergic neurones or α-adrenoceptors. The depression of release of acetylcholine by endogenous noradrenaline does not appear to be responsible for the lack of effect of morphine stimulation at high frequency since pretreatment of preparations with phenoxybenzamine does not render the output at high frequencies morphine-sensitive.

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