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# Effect of frequency of stimulation on the inhibition by noradrenaline of the acetylcholine output from parasympathetic nerve terminals

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**Summary**

1. The relationship between the number of shocks delivered, the frequency of stimulation and the acetylcholine output per volley from nerve terminals of the longitudinal muscle strip of the guinea-pig ileum was studied.

2. There was an inverse correlation between acetylcholine output per volley and the frequency of stimulation when the same number of shocks was applied in each train.

3. With sustained stimulation, the volley output declined more rapidly the higher the frequency of stimulation. There was no decrease in volley output (11·7 (ng/g)/volley) when the frequency applied was 0·1 Hz or less.

4. Noradrenaline ($10^{-6}$ g/ml) reduced the acetylcholine output per volley to the level produced by sustained stimulation at 10 Hz (1·4–1·9 (ng/g)/volley). The acetylcholine output following application of the first shocks of a train at high frequency stimulation was much reduced by noradrenaline as the output was higher than 1·4–1·9 (ng/g)/volley. This action of noradrenaline was antagonized by phentolamine (2 $\mu$g/ml for 20 min).

5. Amphetamine and methylamphetamine, which release NA from sympathetic nerve terminals, were active in reducing the acetylcholine output to low frequency parasympathetic nerve stimulation. Reserpine and $\alpha$-methyl-*p*-tyrosine pretreatment prevented the effect of amphetamine and reduced that of methylamphetamine.

6. The fact that addition or release of noradrenaline, reduced acetylcholine output when the firing rate was high but short in duration, suggests that noradrenaline plays a general modulator role in controlling the output of acetylcholine from the parasympathetic nerve terminals.

## Introduction

The finding that, in the longitudinal muscle strip of guinea-pig ileum, all the acetylcholine (ACh) is contained in the nerve network (Paton & Zar, 1968), provides an opportunity for studying the release of ACh of nervous origin in response to electrical stimulation. When the preparation is given a train of stimuli there is an initial rapid fall in output per volley, followed by a relatively constant output (Paton, 1963 ; Paton & Vizi, 1969). Noradrenaline and adrenaline reduce the acetylcholine output per volley at low frequencies of stimulation (0·1–2 Hz) and this action is prevented by $\alpha$-adrenoceptor blocking agents (Paton & Vizi, 1969 ;

Kosterlitz, Lydon & Watt, 1970); sympathetic stimulation also inhibits acetylcholine release from guinea-pig colon (Beani, Bianchi & Crema, 1969) and from rabbit jejunum (Vizi, 1970).

The experiments described in this paper deal with the inhibitory effect of noradrenaline and of sympathetic stimulation on acetylcholine release as a function of the duration (number of shocks delivered) and the frequency of parasympathetic nerve stimulation.

Some of the findings described in the paper have been presented at a meeting of the British Pharmacological Society (Knoll & Vizi, 1970).

## Methods

### *Preparation of longitudinal muscle strip with attached nerve elements*

Longitudinal muscle strips of the guinea-pig ileum were prepared according to the method of Rang (1964) as modified by Paton & Vizi (1969). They weighed 68 (45–90) mg.

Muscle strip was set up in an organ bath of 3·5 ml capacity in Krebs solution at 36° C, bubbled with 95% oxygen and 5% carbon dioxide. The composition of the Krebs solution was (mM): NaCl, 113; KCl, 4·7; $CaCl_2$, 2·5; $KH_2PO_4$, 1·2; $MgSO_4$, 1·2; $NaHCO_3$, 25 and glucose, 11·5.

'Field' stimulation was used (Paton & Zar, 1968). This method excites postganglionic fibres and is effective in the presence of hexamethonium, in producing acetylcholine release and contraction. Square wave pulses of 1 ms duration were applied through platinum electrodes at the top and bottom of the organ bath, at a frequency of 0·05–30 Hz. In some experiments physostigmine sulphate ($2 \times 10^{-6}$ g/ml) was added to the Krebs solution in order to prevent enzymic hydrolysis of acetylcholine. Since physostigmine sulphate requires some time for its complete action to develop, a period of preincubation of 60 min was allowed. The strips were repeatedly washed with Krebs solution containing physostigmine before any samples were collected for acetylcholine assay. The acetylcholine obtained in this way was assayed on a length of guinea-pig ileum suspended in 5 ml Krebs solution at 36° C. A polythene cannula was inserted into the lower end of the gut in order to drain the intraluminal contents; this improved the stability of the assay preparation, which was stored for several hours at 4° C before use, in order to increase its sensitivity and decrease spontaneous movement. Morphine sulphate ($5 \times 10^{-6}$ g/ml) was also added to reduce spontaneous movements. During the assay, control responses to standard solutions of acetylcholine were obtained in the presence of the same concentration of the drug under test as was produced by adding the test sample to the assay bath.

In order to collect sufficient acetylcholine for assay, intermittent stimulation was used. Trains of one–ten shocks at frequencies of 1–20 Hz were repeated at intervals of 10 s until enough acetylcholine had been released for assay. It was assumed that the volley output of the first shock of each train was equal to the volley output when only one shock was delivered every 10 seconds. From this value, and the output per train of two shocks, the volley output for the second of the two shocks could be calculated. It was thus possible to calculate the volley output for all of the successive shocks in the train, and to determine the rate of decline of the volley output as a function of frequency of shocks during the train.

TABLE 1. *Inhibitory action of* (−)*-noradrenaline* ($10^{-6}$ *g/ml*) *on acetylcholine output per volley at different frequencies*

| Rate of stimulation | No. of shocks | Duration of stimulation | Total ACh output ((ng/g)/min) mean ± S.E. | (No. of expts) | Volley output ((ng/g)/volley*) | Volley output in the presence of (−)-NA ($10^{-6}$ g/ml) | % reduction |
|---|---|---|---|---|---|---|---|
| 0·05 Hz | 60 | 20 min | 83·5 ± 8·6 | (3) | 11·6 ±1·7 | 1·85±0·1 | 84·0 |
| 0·1 Hz | 60 | 10 min | 109·6 ± 6·5 | (10) | 11·7 ±0·5 | 2·0 ±0·12 | 83·0 |
| 1·0 Hz | 1200 | 20 min | 229·5 ± 18·5 | (5) | 3·0 ±0·2 | 1·75±0·09 | 41·7 |
| 3·0 Hz | 1200 | 6·6 min | 470·8 ± 57·2 | (3) | 2·45±0·1 | 1·9 ±0·11 | 22·5 |
| 10·0 Hz | 1200 | 2·0 min | 1106·0 ± 98·0 | (5) | 1·75±0·12 | 1·85±0·12 | −10·5 |
| 20·0 Hz | 1200 | 1·0 min | 1170·0 ± 140·6 | (3) | 0·93±0·08 | 0·88±0·07 | 5·4 |
| 30·0 Hz | 1200 | 40 s | 980·0 ± 65·0 | (5) | 0·52±0·03 | 0·60±0·03 | −11·5 |

* The output per volley is calculated as described by Paton & Vizi (1969).

The acetylcholine output during stimulation was calculated after subtracting the resting output, and expressed as (ng/g)/volley.

In several investigations, the mechanical responses to parasympathetic postganglionic stimulation were also recorded, using an isometric strain gauge system.

### *Drugs*

Drugs used were: (−)-noradrenaline bitartrate (Koch-Light Lab. Ltd.); (−)-adrenaline bitartrate (G. Richter); acetylcholine iodide (BDH); physostigmine sulphate (Macarthys Ltd.); atropine sulphate (Biogal); phentolamine methanesulphonate (CIBA); amphetamine phosphate (Chinoin); methylamphetamine tartrate (Chinoin); α-methyl-*p*-tyrosine (Merck); reserpine (G. Richter). LB/46 [dl-4-(2-hydroxy-3-isopropylaminopropoxy)-indol, Sandoz]. The drugs were dissolved in 0·9% W/V NaCl solution. Concentrations of the drugs are expressed in terms of their salts, and sometimes given also in molar concentration.

## Results

When a given number of shocks was delivered at different frequencies, the acetylcholine output per volley varied inversely with the frequency of stimulation applied (Table 1). The highest volley output, at a frequency of 0·1 Hz, was 11·7 (ng/g)/volley (42·6 (pmol/g)/volley). This was not increased by further reducing the frequency. The minute output changed little between rates of 0·05 and 1·0 Hz, and increased sharply between 1·0 and 10 Hz, but did not increase further at higher frequencies. The volley output given in Table 1 represents the volley output during sustained stimulation for 1200 shocks. (−)-Noradrenaline ($10^{-6}$ g/ml) reduced the volley output at frequencies of 0·05–3 Hz, but not at higher frequencies.

The indirectly acting sympathomimetic amines, amphetamine and methylamphetamine also reduced the acetylcholine output at a frequency of 0·1 Hz (Table 2). Reserpine and α-methyl-*p*-tyrosine pretreatment prevented the effect of amphetamine and reduced that of methylamphetamine, but not that of noradrenaline at a concentration of $5 \times 10^{-7}$ g/ml.

### *Correlation between acetylcholine output per volley and number of shocks delivered at different frequencies*

Using intermittent stimulation with different numbers of shocks in each train (see **Methods**), it was possible to measure and calculate the output per volley for the $n^{th}$

TABLE 2. *Effects of indirectly acting sympathomimetic amines on acetylcholine release before and after reserpine and α-methyl-*p*-tyrosine*

| Drug added to the bath | Concentration (g/ml) | % reduction of acetylcholine volley output mean ± S.E. | |
|---|---|---|---|
| | | Control | Pretreated with reserpine + α-methyl-*p*-tyrosine |
| (−)-Noradrenaline | $5 \times 10^{-7}$ | 74·5 ± 5·4 (5) | 68·6 ± 6·7 (3) |
| (±)-Amphetamine | $2 \times 10^{-4}$ | 62·0 (2) | −15·1 ± 4·6 (3) |
| (±)-Methylamphetamine | $5 \times 10^{-5}$ | 68·8 ± 9·2 (3) | 31·6 ± 2·5 (4) |

The eserinized strip was stimulated for 10 min at 0·1 Hz. The number of experiments is indicated in brackets. Reserpine (1·5 mg/kg i.p.) was administered on 2 consecutive days; α-methyl-*p*-tyrosine (60 mg/kg i.p.) was given on the third day. The guinea-pigs were killed 6 h after α-methyl-*p*-tyrosine and 18 h after the last injection of reserpine.

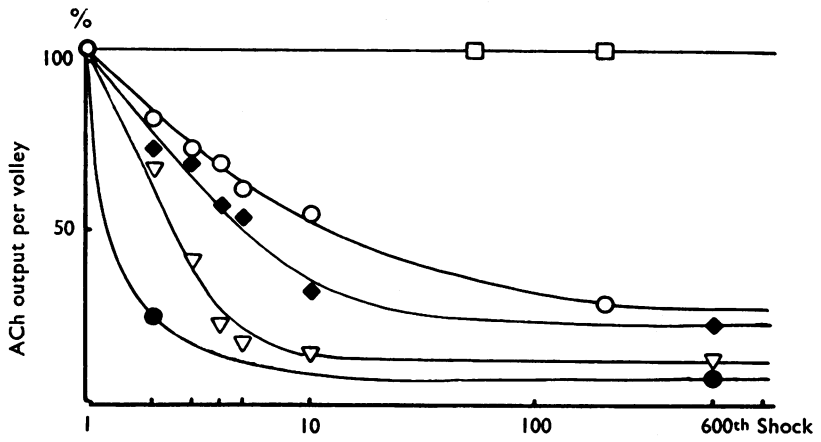


FIG. 1. Relation between the output per volley of acetylcholine, number of stimuli and frequency of stimulation. Eserinized longitudinal muscle strip of guinea-pig ileum. Field stimulation. The acetylcholine output per volley produced by the first shock of each train is taken as being equal to the volley output when only one shock was delivered every 10 s, and set as 100%. Trains of one–ten shocks at frequencies of 1–20 Hz were repeated at 10 s intervals. For the calculation of volley output by $n^{th}$ shock see **Methods.** Mean of two experiments. Sixty trains were used. (□——□), 0·1 Hz; (○——○), 1 Hz; (◆——◆), 3 Hz; (▽——▽), 10 Hz; (●——●), 20 Hz.

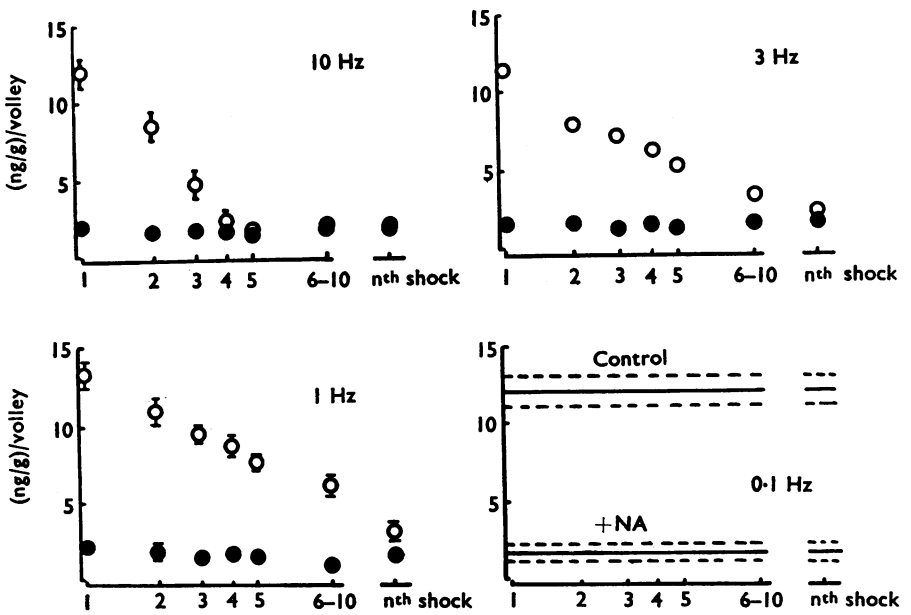


FIG. 2. Inhibitory action of (−)-noradrenaline on acetylcholine output per volley. Longitudinal muscle strip of guinea-pig ileum. For technique of intermittent train stimulation and calculation of output by $n^{th}$ shock see **Methods.** The graphs show the acetylcholine output (ng/g)/volley elicited by the $n^{th}$ shock of the train at different frequencies. Control output (○) and in the presence of (−)-noradrenaline $10^{-6}$ g/ml (●). The number of experiments at 10 Hz was five; at 3 Hz, two; at 1 Hz, four; at 0·1 Hz, five. The standard errors of the means are indicated; where they are not they were less than the size of the circle. The acetylcholine output per volley by sustained stimulation (mean of acetylcholine volley output by 1200 shocks at 1, 3 and 10 Hz and by 120 shocks at 0·1 Hz) is indicated by $n^{th}$ shock.

TABLE 3. *Inhibitory effect of* (−)-*noradrenaline* ($10^{-6}$ *g/ml*) *on the output per volley of acetylcholine induced by electrical stimulation at different frequencies. Intermittent train stimulation for five shocks*


| Rate of stimulation | No. of trains *n* | No. of shocks *5n* | Total ACh output (ng/g) | Presumed resting output during stimulation period* | Calculated ACh output by train of shocks (ng/g) | Volley output‡ (ng/g per volley) | % Inhibition by (−)-noradrenaline $10^{-6}$ g/ml | % Inhibition by (±)-methylamphetamine $5\times10^{-5}$ g/ml |
|---|---|---|---|---|---|---|---|---|
| 0·1 Hz | | | | | 68·5† | 13·7±1·8 | 84·1±6·2 | 52·2±4·9 |
| 1·0 Hz | 60 | 300 | 3191 | 634·5 | 42·6 | 8·5±1·4 | 73·1±5·1 | |
| 3·0 Hz | 60 | 300 | 2668 | 477·9 | 36·5 | 7·3±1·0 | 66·2±4·6 | |
| 10·0 Hz | 60 | 300 | 2094 | 439·9 | 27·4 | 5·4±0·4 | 60·2±3·8 | 44·5±2·5 |
| 20·0 Hz | 60 | 300 | 1700 | 433·6 | 21·1 | 4·2±0·3 | 49·4±8·0 | |

* Calculated from the preceding resting period. † Calculated by multiplying the volley output obtained in the same experiment at 0·1 Hz stimulation for 15 min ($n=10$). ‡ Mean volley output of five shocks in one train. Note the effectiveness of (−)-noradrenaline on acetylcholine output per volley at higher frequencies (10, 20 Hz) of stimulation.

shock of each train. When trains of two–ten shocks, with intervals of 100–1,000 ms between consecutive shocks, were applied, the mean volley output decreased as the length of the trains was increased. At 0·1 Hz the volley output did not decline with time, but at higher frequencies there was an initial rapid fall in volley output followed by a relatively stable output. This is shown in Fig. 1, where the acetylcholine output per volley evoked by the first shock at any stimulation frequency was assumed to be equal to the volley output at 0·1 Hz stimulation in the same experiment. The higher the frequency of stimulation the more rapid was the

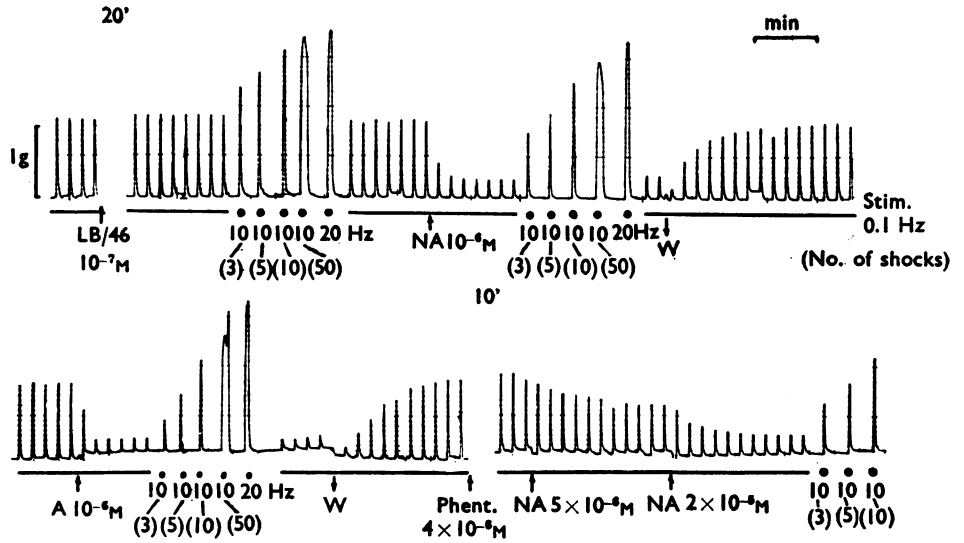


FIG. 3. Inhibitory action of (−)-noradrenaline on the responses of longitudinal muscle strip of guinea-pig ileum to electrical stimulation at 0·1, 10 and 20 Hz. At 10 Hz stimulation, three, five, ten and fifty shocks were delivered as indicated in brackets. At 20 Hz stimulation, fifty shocks were given. Field stimulation. Krebs solution. Isometric recording. LB/46 ($10^{-7}$M; $2{\cdot}2\times10^{-8}$ g/ml) was present throughout the experiment. NA, (−)-noradrenaline ($10^{-6}$M, $3\times10^{-7}$ g/ml); A, adrenaline ($10^{-6}$M, $3{\cdot}33\times10^{-7}$ g/ml); Phent., phentolamine ($4\times10^{-6}$M, $1{\cdot}5\times10^{-6}$ g/ml). The interval between the first and second parts of the trace was 20 min, and between the second and third, and third and fourth parts was 10 minutes. Note that phentolamine reduced the inhibitory action of (−)-noradrenaline (DR=25).

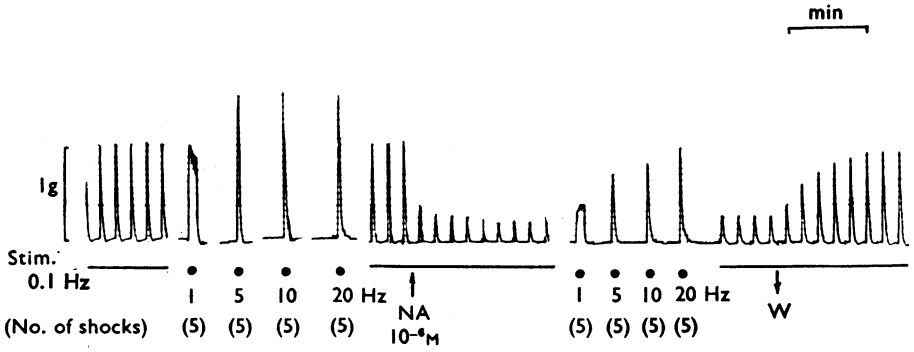


FIG. 4. Inhibitory action of (−)-noradrenaline on the responses of longitudinal muscle strip of guinea-pig ileum produced by electrical stimulation at different frequencies but with the same number of shocks. Field stimulation. Isometric recording. Krebs solution. Five shocks were used at 1, 5, 10 and 20 Hz stimulation, as indicated in brackets. NA, (−)-noradrenaline ($10^{-6}$M, $3\times10^{-7}$ g/ml); W, wash.

decline in volley output of acetylcholine. The effect of noradrenaline on acetylcholine volley output as a function of the duration of the train and of frequency of stimuli is shown in Fig. 2. At all frequencies of stimulation, a fraction of the volley output (1·4–1·9 (ng/g)/volley) was resistant to noradrenaline; this was identical with the volley output evoked by continuous stimulation at 10 Hz (see Table 1 and Fig. 2). Noradrenaline ($10^{-6}$ g/ml) reduced the output of acetylcholine produced by the first shocks of high frequency trains (Fig. 2, Table 3). Methylamphetamine ($5 \times 10^{-5}$ g/ml) also reduced the acetylcholine volley output by 44·5% when trains of five stimuli at 10 Hz were repeated every 10 s (Table 3). The inhibitory action of noradrenaline on acetylcholine release was blocked by phentolamine ($2 \times 10^{-6}$ g/ml).

In order that the acetylcholine released could be assayed, a cholinesterase-inhibitor (physostigmine sulphate) was used. Might physostigmine influence the effect of noradrenaline on acetylcholine release? Figures 3 and 4 show the results obtained in longitudinal muscle strip which had not been treated with anticholinesterase using mechanical contraction as a measure of the acetylcholine released by stimulation. The contractions were recorded isometrically. A $\beta$-adrenoceptor blocking agent, LB-46, which lacks a $\beta$-agonist action (Saameli, 1967) was used to exclude any possibility of direct action of noradrenaline or adrenaline on smooth muscle influencing the contractile force recorded. Noradrenaline ($3 \times 10^{-7}$ g/ml ($10^{-6}$M)), and adrenaline ($3{\cdot}3 \times 10^{-7}$ g/ml ($10^{-6}$M)), reduced the contraction due to stimulation at 0·1 and 10 Hz, when a short train was used. The lower the frequency of stimulation, the greater the inhibitory action of noradrenaline; or at a given frequency, the fewer shocks applied, the higher the inhibitory effect (Figs. 3 and 4). Noradrenaline released by methylamphetamine also reduced the contractions (Fig. 5). The contraction produced by adding acetylcholine to the bath was not affected, indicating that the reduction observed was not due to a postsynaptic action of methylamphetamine in the presence of a $\beta$-adrenoceptor blocking agent. The contraction caused by stimulation at 10 Hz for ten shocks was hardly influenced. The results obtained using an isometric recording system are thus in good agreement with those from direct measurement of acetylcholine release.

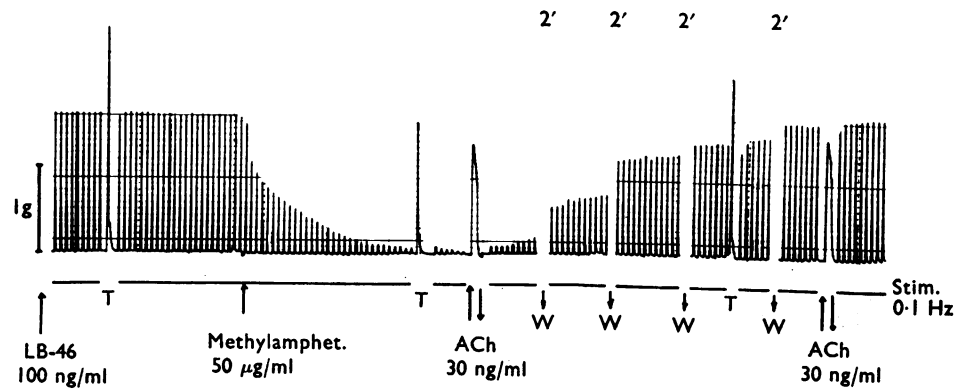


FIG. 5. Inhibition by (±)-methylamphetamine of the responses of longitudinal muscle strip of guinea-pig ileum to electrical stimulation (0·1 Hz). Field stimulation. Krebs solution. Organ bath, 3·5 ml. The contractions are recorded isometrically. Contractile force is indicated in g. T, tetanus (10 Hz, ten shocks); W, wash.

**Discussion**

Attempts to estimate the amount of acetylcholine released by a single nerve impulse have been made (Feldberg & Vartiainen, 1935 ; Perry & Talesnik, 1953 ; MacIntosh, 1959 ; Paton, 1963 ; Emmelin & MacIntosh, 1956 ; Paton & Zar, 1968 ; Paton & Vizi, 1969) by collecting the transmitter released in sufficient quantities to be assayed. The release of acetylcholine during sustained stimulation has been studied by Paton (1963) on whole gut ; he found that the volley output fell with time, faster with higher rate of stimulation ; and Paton & Zar (1968) and Paton & Vizi (1969) have found with the longitudinal strip that a single stimulus released less acetylcholine at high frequencies of stimulation than at low frequencies.

Perry (1957), studying the volley output from ganglia, argued that an explanation of decline could be the exhaustion of the stock available for release and that synthesis failed to keep pace with release. This is excluded in these experiments, since using intermittent stimulation with a fixed number of shocks arranged in trains of short duration (for example, one to five shocks) the total output per train also declined with increasing frequency. Using the refined technique of intermittent stimulation of short duration it has been established that the volley output falls from the first shock to a final constant output per volley. This constant output also depended on the frequency applied.

At high frequencies the minute output of acetylcholine released ((1,100–1,300 ng/g)/min) becomes limited by the rate at which the stores can be made available. Presynaptic failure may exist so that not all the impulses reach the nerve endings, therefore limiting the output.

Noradrenaline (Paton & Vizi, 1969 ; Kosterlitz *et al.*, 1970 ; Vizi, 1968) and adrenaline (Paton & Vizi, 1969 ; Vizi, 1968) can inhibit the acetylcholine output during stimulation at low frequencies (up to 2 Hz) using sustained stimulation. When a fixed number (1,200) of shocks was given at different frequencies, the inhibitory action of noradrenaline varied inversely with the frequency. With stimulation at high frequencies (1–20 Hz), provided short trains of pulses were used, noradrenaline also reduced acetylcholine output per volley. The fewer the shocks delivered and the lower the frequency applied, the higher was the volley output in the control period and the greater was the inhibition by noradrenaline. This indicates that the inhibitory action of noradrenaline depends on the number of shocks delivered and the frequency of stimulation. The inhibition, by noradrenaline, of the acetylcholine volley output declined in parallel with the decline of acetylcholine volley output, noradrenaline being able only to reduce the acetylcholine output per volley to that level produced by sustained stimulation of 10 Hz.

The indirectly acting sympathomimetic amines, amphetamine and methylamphetamine, also reduced acetylcholine output. It is suggested that this action is due to the endogenous, cytoplasmic noradrenaline released by the amines, an explanation which is supported by the findings that amphetamine and methylamphetamine were not effective in preparations previously depleted of noradrenaline by reserpine and $\alpha$-methyl-*p*-tyrosine treatment. However, there is some difference in their actions, the prevention being incomplete in the case of methylamphetamine. The fact that amphetamine and methylamphetamine reduced the acetylcholine output is evidence for the possibility that noradrenaline is released in the intestine, reaches

the presynaptic nerve terminals and prevents the acetylcholine release in response to nerve activity.

Paton (1957), Paton & Zar (1968), and Cowie *et al.* (1968) have shown that morphine depresses acetylcholine release when the myenteric plexus-longitudinal muscle preparation is stimulated at low frequencies (0·1–1 Hz). Cowie, Kosterlitz, Lydon & Waterfield (1970) have, in addition, found some evidence that morphine is active at high frequencies (10 Hz), inhibiting the acetylcholine output per volley evoked by the early pulses of a train, as did noradrenaline in our experiments.

Our finding that noradrenaline, either added to the organ-bath or released from sympathetic nerve terminals, reduced the acetylcholine output due to nerve activity even at high rates of firing, supports the idea that noradrenaline may play a general modulator role in controlling the output of acetylcholine from the parasympathetic nerve terminals. This seems to be one type of presynaptic inhibition.

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