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# The effects of sympathetic nerve stimulation and guanethidine on parasympathetic neuroeffector transmission; the inhibition of acetylcholine release

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The effect of noradrenaline released either by sympathetic nerve stimulation or guanethidine added to the organ bath has been studied on acetylcholine release from parasympathetic nerve terminals and compared with the effect of exogenous noradrenaline. Sympathetic nerve stimulation, guanethidine and noradrenaline reduced the release of acetylcholine from resting rabbit intestine by up to 70%. Sympathetic stimulation and guanethidine failed to reduce acetylcholine release in preparations previously depleted of noradrenaline. Noradrenaline added to the bath still remained effective. The fact that noradrenaline released is capable of inhibiting acetylcholine release supports the concept that noradrenaline physiologically controls the release of acetylcholine.

It has been shown by Paton & Vizi (1969) and Vizi (1968) that noradrenaline and adrenaline inhibit the acetylcholine release from the parasympathetic nerve terminals of longitudinal muscle strip of guinea-pig ileum, particularly during rest periods or when stimulation frequency was low (0·1 to 2·0 Hz) and that this inhibitory action is mediated through α-adrenoceptors. It has further been shown (Paton & Vizi, 1969; Vizi, 1968) that the reduction of sympathetic outflow by reserpine or guanethidine pretreatment increases the acetylcholine which thus indicates a continuous sympathetic control on parasympathetic transmitter release. Norberg (1964), Jacobowitz (1965), and Norberg & Sjöqvist (1966) presented evidence that the adrenergic fibres embrace the ganglia in gut without directly innervating the smooth muscle. The inhibitory action of noradrenaline on acetylcholine release was also observed by Kosterlitz, Lydon & Watt (1970). In addition, Beani, Bianchi & Crema (1969) presented evidence that noradrenaline reduced the release of acetylcholine from colon. It was confirmed (Kosterlitz & others, 1970; Beani & others, 1969) that the inhibitory effect of noradrenaline prevails when the cholinergic fibres were stimulated at low rates. However, Knoll & Vizi (1970, 1971), using intermittent (trains of 2–10 shocks, with intervals of 50–1000 ms, repeated at intervals of 10 s) high rate stimulation of parasympathetic nerves of the longitudinal muscle strip of guinea-pig ileum, have established that under these conditions noradrenaline is able to reduce the acetylcholine release also.

The experiments now described were made to study the effect of noradrenaline released either by sympathetic nerve stimulation or by guanethidine on the release of acetylcholine due to parasympathetic nerve stimulation of different frequency and at resting condition.

Some of the present findings have been communicated to Hungarian Physiological Society (1968 Meeting; Vizi, 1970).

## METHODS AND MATERIALS

The preparation of the rabbit isolated intestine was essentially similar to that described by Finkleman (1930). Rabbits, 2 to 3 kg, were killed by blow on the head and as long a length as possible of periarterial sympathetic nerve was dissected out together with 3–4 cm of the intestine. The preparation was suspended in Krebs solution at 36° in a 10 ml organ bath, aerated with 5% carbon dioxide in oxygen. 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. Mesenteric artery was drawn through an insulated platinum ring electrode as far away from the intestine as possible. This was to reduce current spread that might cause acetylcholine release and so overshadow the effect of sympathetic nerve stimulation on acetylcholine release. The pulse duration was 0·3 ms and the strength of stimulation 5 to 10 V, was supramaximal. In other experiments the preparation was also stimulated by a square-wave pulse of 1 ms duration through two platinum electrodes one in the top and one in the bottom of the organ bath (Field stimulation) giving a potential drop of 8–12 V/cm. This also was supramaximal. The stimulation was so arranged that one electrode was used for stimulation, the other was without earth connections. Except during actual stimulation, the electrodes were short-circuited. During single field stimulation the simultaneous sympathetic stimulation was stopped for 1 s to avoid any passing of current.

Contractions of the intestine were recorded auxotonically (Paton, 1957) using a frontal writing lever with a magnification of 10-fold and exerting a tension of 1 g.

Longitudinal muscle strip of guinea-pig ileum prepared according to Paton & Vizi (1969) was set up in a 3·5 ml organ-bath filled with Krebs solution, bubbled with 5% carbon dioxide in oxygen at 37°. Supramaximal field stimulation (8–12 V cm), was used. The contractions were recorded either by means of an auxotonic writing lever and kymograph or by an isometric recording system.

The output of acetylcholine from rabbit intestine and guinea-pig ileum was collected in the presence of eserine sulphate ($2 \times 10^{-6}$ g/ml) and assayed using a guinea-pig ileum suspended in 3·5 ml Krebs solution at 36°. A polythene cannula was inserted into the distal end of the gut to drain off intraluminal contents and in this way to maintain a gut highly reliable for the assay. Control responses to a standard solution of acetylcholine were obtained in the presence of the same concentration of test drug that was produced when the test sample was added to the assay bath. In few experiments "intermittent" train stimulation was used (Knoll & Vizi, 1970).

The resting acetylcholine output and the output per volley were calculated according to Paton & Vizi (1969) and expressed in ng/g min, and ng/g per volley, respectively. Drugs used were: acetylcholine iodide (BDH), (−)-noradrenaline bitartrate (Koch-Light Laboratories Ltd.), guanethidine sulphate (CIBA), physostigmine sulphate (Macarthys Ltd.), cocaine HCl, phentolamine methane sulphonate (CIBA) tetrodotoxin (Sankyo). The drugs were dissolved in distilled water or distilled water saline. Concentrations are expressed in terms of the drug salts or in molar concentration.

Statistical calculations were made according to conventional procedures.

## RESULTS

Table 1 shows the inhibitory effect of noradrenaline, sympathetic nerve stimulation and guanethidine on acetylcholine output from the Finkleman preparation. The

Table 1. *Reduction of acetylcholine release from rabbit jejunum by endogenous and exogenous noradrenaline.*

| Expt No. | Condition | Rate of field stimulation or collection period in min | No. of shocks applied | Control ACh-output ng/g min s.e. | Change in ACh-output during treatment or sympathetic stimulation | Change in ACh-output reduction in percent s.e. | P |
|---|---|---|---|---|---|---|---|
| 1. | Resting (23) | 5–10 | — | 5·1 ± 0·9 | | | |
| 2. | Resting (4) | 5 | — | 8·6 ± 1·1 | sympathetic stim. 10 Hz for 5 min | 40·2 ± 4·0 | <·001 |
| 3. | Resting (3) | 10 | — | 4·8 ± 0·7 | (−)-NA $1·5 \times 10^{-6}$M | 57·1 ± 6·0 | <0·01 |
| 4. | Resting (3) | 10 | — | 4·3 ± 0·6 | guanethidine $4 \times 10^{-5}$M | 41·3 ± 5·5 | <0·05 |
| 5. | Stimulated (15)* | 0·5 Hz | 600 | 8·9 ± 0·7 | — | — | — |
| 6. | Stimulated (3) | 0·5 Hz | 600 | 10·2 ± 1·9 | (−)-NA $1·5 \times 10^{-6}$M | 66·5 ± 9·0 | <0·01 |
| 7. | Stimulated (3) | 0·5 Hz | 600 | 7·1 ± 1·1 | guanethidine $4 \times 10^{-5}$M | 57·2 ± 6·2 | <0·01 |
| 8. | Stimulated (5) | 10 Hz | 1200 | 26·5 ± 3·6 | — | — | 8:5 <0·10 |
| 9. | Stimulated (2) | 10 Hz | 1200 | 29·1 | (−)-NA $3 \times 10^{-6}$M | 5·5 | n.s. |

* The corresponding resting output is 5·0 ± 0·3 ng/g min and the difference is significant, $P < 0·01$. Number in brackets indicate the number of experiments.

Table 2. *The inhibitory action of noradrenaline released by guanethidine on acetylcholine output from the Auerbach plexus of longitudinal muscle strip of guinea-pig ileum.*

| Expt No. | Condition | Rate of stimulation or collection period in min | Type and duration of stimulation | No of shocks | Total ACh output ng/g min s.e. | Volley output ng/g min | Change in ACh output reduction % | P |
|---|---|---|---|---|---|---|---|---|
| 1. | Resting (25) | 15 | — | — | 41·2 ± 2·0 | — | — | — |
| 2. | Resting + (−)NA $2 \times 10^{-6}$M | 15 | — | — | 10·7 ± 2·1 | — | 74·3 | 2:1<0·01 |
| 3. | Resting (5) + guanethidine $4 \times 10^{-5}$M | 15 | — | — | 18·7 ± 4·1 | — | 55·7 | 3:1<0·01 |
| 4. | Stimulated (3) | 0·1 Hz | continuous 10 min | 60 | 114·0 ± 5·6 | 10·6 | — | — |
| 5. | Stimulated (3) + (−)-NA $2·9 \times 10^{-6}$M | 0·1 Hz | continuous 10 min | 60 | 21·2 ± 5·6 | 1·8 | 81·4 | 5:4<0·01 |
| 6. | Stimulated (3) + guanethidine $4 \times 10^{-5}$M | 0·1 Hz | continuous 10 min | 60 | 50·7 ± 7·1 | 4·9 | 55·6 | 6:4<0·01 |
| 7. | Stimulated (5) | 10 Hz | continuous 1 min | 600 | 1025·0 ± 41·6 | 1·6 | — | — |
| 8. | Stimulated (5) + (−)-NA $2·9 \times 10^{-6}$M | 10 Hz | continuous 1 min | 600 | 1003·0 ± 60·1 | 1·6 | — | no change |
| 9. | Stimulated (3) + guanethidine $4 \times 10^{-5}$M | 10 Hz | continuous 1 min | 600 | 1102·0 ± 74·0 | 1·8 | — | n.s. |
| 10. | Stimulated (3) | 10 Hz | inermittent* | 300 | 1489† ± 92·4 ng/g 10 min | 4·8 | — | — |
| 11. | Stimulated (3) + guanethidine $4 \times 10^{-5}$M | 10 Hz | intermittent* | 300 | 735·2† ± 65·2 ng/g 10 min | 2·3 | 46·2 | 11:10<0·05 |

* Intermittent stimulation (5 shocks of 100 ms intervals in every 10 s = one train; 60 trains = 60 × 5 = 300 shocks).
† Total ACh output during the 10 min period. Numbers in parentheses indicate the number of experiments.

resting output, 5·1 ng/g min (18·8 pmol/g min), showed a large deviation, probably dependent on the density of sympathetic innervation. This is supported by the fact that the acetylcholine output in intestine from the rabbit pretreated with guanethidine (20 mg/kg, s.c., 6 h before testing), was higher (13·6 ng/g min; n = 3). The concomitant stimulation of the sympathetic nerve (10 Hz; 0·3 ms; for 5 min) reduced the resting output on average by 45%. The higher the control resting output, the

more effective was the sympathetic stimulation in reducing acetylcholine release. The data in Table 1 also show that when the sympathetic nerve stimulation was applied simultaneously with noradrenaline for 5 min, the increased acetylcholine output in response to the field (parasympathetic) stimulation was abolished. Noradrenaline ($1{\cdot}5 \times 10^{-6}$M) and guanethidine ($4 \times 10^{-5}$M), reduced the acetylcholine output both at resting and at 0·5 Hz stimulation. However, the sympathetic stimulation and guanethidine proved to be ineffective in rabbit intestine pretreated with guanethidine (20 mg/kg, s.c., 6 h before dissection); moreover in one experiment the sympathetic stimulation increased the acetylcholine output by 16%. Noradrenaline, $1{\cdot}5 \times 10^{-6}$M, inhibited both the resting and stimulation (0·5 Hz) output, but failed to have an effect at a high rate of stimulation (10 Hz; see Table 1). There is an inverse relation between stimulation rate and output per volley. At a stimulation rate of 0·5 Hz the volley output was 0·13 ng/g per volley while at 10 Hz the output was only 0·04 ng/g per volley. This is in agreement with the findings of Paton (1963), Paton & Zar (1968), Paton & Vizi (1969) and Knoll & Vizi (1971), who observed this phenomenon in parasympathetic nerve terminals of longitudinal muscle strip of guinea-pig ileum.

Tetrodotoxin, $4 \times 10^{-5}$M, inhibited the increase of acetylcholine output produced by field stimulation, indicating the neural origin of acetylcholine.

Table 2 shows the effect of guanethidine on acetylcholine output from nerves of the longitudinal muscle preparation of guinea-pig ileum. Guanethidine ($4 \times 10^{-5}$M), like noradrenaline, reduced the output both of resting gut and of gut stimulated at low frequency. At 0·1 Hz the amount of acetylcholine released per impulse was reduced from 10·6 to 4·9 ng/g per volley. Acetylcholine release produced by continuous stimulation of 10 Hz was not affected by guanethidine. However, using intermittent stimulation, where the trains of 5 pulses with intervals of 100 ms (10 Hz) were repeated once every 10 s, guanethidine, $4 \times 10^{-5}$M reduced the acetylcholine output from 4·8 to 2·3 ng/g per volley (Table 2).

In the longitudinal muscle strip of ileum from the guinea-pig pretreated with guanethidine (15 mg/kg, s.c. 6 h previous to preparation), guanethidine ($2\text{–}4 \times 10^{-5}$M) added to the organ bath, was less effective in reducing the acetylcholine volley output.

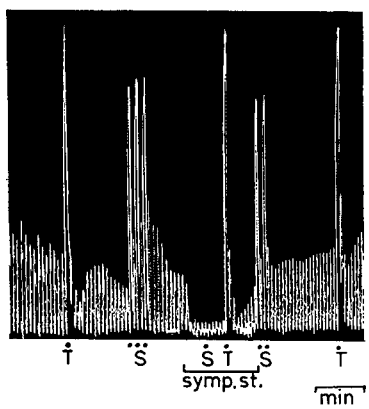


FIG. 1. Effect of sympathetic nerve stimulation on the contraction of rabbit jejunum evoked by "field" stimulation. Rabbit isolated jejunum prepared according to Finkleman. Sympathetic nerve stimulation: 10 Hz, 0·3 ms, 5 V. T = "field" stimulation of 10 Hz, 1 ms, 30 shocks, 8 V/cm. S = "field" stimulation with a single shock, 1 ms, 8 V/cm. Auxotonic recording. Krebs solution bubbled with a gas mixture of 95% $O_2$ + 5% $CO_2$. Organ bath, 10 ml.

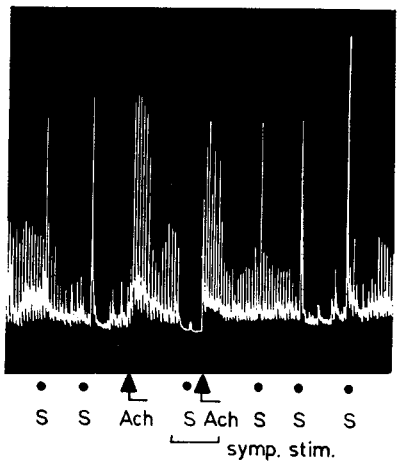


FIG. 2. Effect of sympathetic nerve stimulation on the responses to single "field" stimulation of rabbit jejunum and to acetylcholine. Finkleman preparation. Sympathetic stimulation (symp. stim.) = 10 Hz, 0·3 ms, 5 V. S = "field" stimulation with a single shock, 1 ms, 8 V/cm. ACh = acetylcholine iodide, 30 ng/ml. Contractions are recorded by auxotonic writing lever. Krebs solution. 95% $O_2$ + 5% $CO_2$. Organ bath, 10 ml.

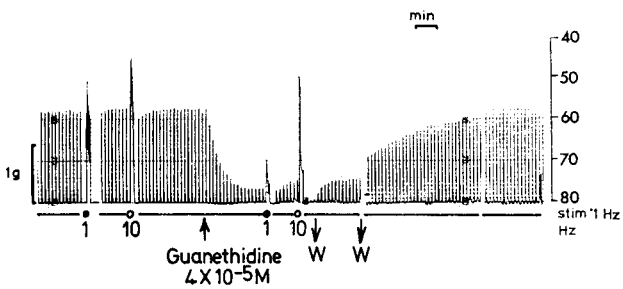


FIG. 3. The inhibitory action of guanethidine on the responses of longitudinal muscle strip of guinea-pig ileum to parasympathetic nerve stimulation with different frequencies. "Field" stimulation, 1 ms, 8 V/cm. The contractions were recorded isometrically. At 1 Hz 10 shocks, at 10 Hz 100 shocks were delivered. W = wash out. Krebs solution. 95% $O_2$ + 5% $CO_2$. Organ bath, 3·5 ml. "Overflow"-technique. The interval between first and second and second and third parts of trace was 5 min. Note the inhibitory action of guanethidine on the responses of longitudinal muscle strip of guinea-pig ileum to 0·1 and 1 Hz stimulation.

The above problems were also examined in the absence of a cholinesterase-inhibitor, the responses to the acetylcholine released were recorded auxotonically or isometrically. Fig. 1 shows the inhibitory action of sympathetic nerve stimulation on the response to single "field" stimulation. The contraction caused by high frequcncy of stimulation (10 Hz; 30 shocks) was not influenced. Noradrenaline ($10^{-6}$ to $10^{-5}$M) behaved similarly when added to the organ bath. The effect of acetylcholine added to the bath to increase pendular movement and to cause contraction of rabbit jejunum was not affected by sympathetic nerve stimulation; the contraction to single "field" stimulation, however, was still reduced (Fig. 2).

In longitudinal muscle strip of guinea-pig ileum, guanethidine ($2\text{–}4 \times 10^{-5}$M), added to the organ bath, like noradrenaline, reduced the twitch caused by stimulation of 0·1 and 1 Hz, without markedly reducing the contraction produced by 10 Hz stimulation (30 shocks were delivered; see Fig. 3). The effect was rapid in onset. After washing out, a fast recovery was observed. But if the guanethidine was left in

the bath for 15–45 min, a full recovery, and sometimes even an increase in size of contraction was observed. In any experiment in which repeated administration occurred, the strip at low frequency stimulation, developed tachyphylaxis to guanethidine. Exposing the strip to cocaine, $4{\cdot}7 \times 10^{-4}$M, or to phentolamaine $2{\cdot}2 \times 10^{-5}$M, for 10 min, reduced the effectiveness of guanethidine to abolish response to stimulation by 30–40%.

## DISCUSSION

In the presence of the cholinesterase inhibitor, eserine sulphate, it was possible to measure the acetylcholine output from nerve elements of rabbit jejunum. The resting output varied from preparation to preparation (1·5–12·1 ng/g min), perhaps because of the density of sympathetic innervation. Noradrenaline released by sympathetic nerve stimulation reduced the acetylcholine output.

However, in Gershon's experiments (1967), stimulation of the sympathetic nerve at a frequency sufficient to produce relaxation (10 Hz) in an eserine-treated muscle failed to decrease the release of acetylcholine from rabbit jejunum. The discrepancy between these data and our results is probably due to the differences in stimulation. Gershon stimulated the sympathetic nerve for alternate 1 min periods for 10 min while we used continuous stimulation. Now Paton & Vizi (1969) have observed in longitudinal muscle strip of guinea-pig ileum an "overshoot" in acetylcholine release after withdrawal of noradrenaline i.e. the release exceeded the control level. Del Tacca, Soldan & others (1970) also found an "overshoot"-phenomenon in human isolated taenia coli. The relation of "overshoot" observed by Paton & Vizi (1970) to the response to noradrenaline was also inversely related to the time of exposure to noradrenaline (Paton & Vizi, 1970). This could explain the negative result obtained by Gershon. Nevertheless, Gershon did observe a reduction in acetylcholine output with sympathetic nerve stimulated at 30 and 40 Hz. In spite of the alternate stimulation, this reduction was probably due to release of an excess of noradrenaline, the effect of which was preserved between stimulation periods thus preventing the "overshoot"-phenomenon which would otherwise have overshadowed the depression of acetylcholine output.

In the Finkleman preparation the motor response to field stimulation is due to acetylcholine release since its output increased after stimulation.

Guanethidine interferes with the mechanism for noradrenaline-storage by releasing the amine (Cass, Kuntzman & Brodie, 1960) and, as Paton & Vizi (1969) have previously shown, it also effectively interferes with the release of acetylcholine by reducing noradrenaline content and output. In the present experiments guanethidine added to the organ bath reduced both the resting and the low rate stimulation ouptut of acetylcholine. This effect of guanethidine is probably due to release of noradrenaline. This explanation is supported by the fact that guanethidine proved to be ineffective in preparations previously depleted of noradrenaline. In addition, its release by guanethidine (Cass & others, 1963) is backed by the finding of Garret & Sousa (1963) and Harrison, Chidsey & others (1963) who provided evidence for the view that some acute biological responses, i.e. positive inotropic and chronotropic effects on isolated atria to guanethidine depend on the presence of releasable noradrenaline. Any effect on nerve conduction can also be excluded since the contractions of gut evoked by stimulation of low frequency (0·1 Hz) recovered in time in spite of the presence of the drug and since the response to sustained stimulation with a frequency greater than

5 Hz remained almost always unchanged. Moreover, nerve conduction in the vagus nerve of the rabbit was not influenced by guanethidine in concentrations of up to $10^{-3}$M (Vizi & Knoll, unpublished observations). Chang, Chen & Cheng (1967) also failed to observe any change in nerve action potential with guanethidine. However, there is also no evidence that noradrenaline affects impulse transmission in the axon (Paton & Thompson, personal communication).

The evoked acetylcholine release per volley was reduced by guanethidine at the sustained stimulation of 0·5 Hz, however, at 10 Hz there was no reduction. Using high rate stimulation (10 Hz), but trains short in duration (5 shocks) and 10 s intervals between two consecutive trains, guanethidine reduced the volley output significantly. This result indicates that at high rate of stimulation the acetylcholine output caused by the first shocks is also sensitive to noradrenaline like that produced by low rate stimulation. Recently, the same effect was observed by Knoll & Vizi (1970; 1971) with noradrenaline added to the organ bath and by Cowie, Louise & others (1970) with morphine.

Since without a cholinesterase inhibitor it is not possible to measure acetylcholine release, the question arises as to how the presence of eserine influences the effect of noradrenaline or parasympathetic stimulation on acetylcholine release. All the data obtained during the study of the effect of sympathetic nerve stimulation, or of guanethidine, on contraction of intestine evoked by parasympathetic nerve stimulation are in agreement with the data obtained from direct measurement of acetylcholine output. Sympathetic nerve stimulation, guanethidine, by releasing noradrenaline, and noradrenaline added to the bath affected in a similar way both the responses to parasympathetic nerve stimulation and resting and stimulated acetylcholine output.

The single field stimulation was sometimes followed by reduction of pendular movement. It is probably due to the noradrenaline, or some other inhibitory substance, released since the non-adrenergic inhibitory innervation is a general feature of mammalian intestinal smooth-muscle (Burnstock, Campbell & others, 1963, 1964; Bülbring & Tomita, 1967; Furness, 1969). After guanethidine pretreatment, stimulattion of the sympathetic extrinsic nerve caused a contraction in rabbit jejunum, as seen by Day & Rand (1961).

A detailed analysis of the inhibitory effect of noradrenaline or adrenaline on acetylcholine output from nerve elements of intestine at stimulation (Paton & Vizi, 1969; Vizi, 1968; Knoll & Vizi, 1970; 1971), and the similar observations by others (Kosterlitz & others, 1970; Beani & others, 1969; Del Tacca & others, 1970) also in gut, or the reduction of acetylcholine release by noradrenaline or adrenaline from ganglia *in situ* (Paton & Thompson, 1958) or *in vitro* (Dawes & Vizi, unpublished) present evidence that noradrenaline may play a role in transmission by reducing the acetylcholine release. Another interesting coincidence is that adrenaline (McIsaac, 1966) and guanethidine (Maxwell, Plummer & others, 1957) were each able to reduce ganglionic transmission at low frequency of stimulation (0·33 and 0·5 Hz respectively). These data and the fact that noradrenaline released either by sympathetic nerve stimulation or by guanethidine was capable of inhibiting acetylcholine release, support the concept that noradrenaline physiologically controls the release of acetylcholine. Nerve impulses in sympathetic nerve, on reaching the nerve endings liberate noradrenaline which, in turn, blocks the release of acetylcholine. This seems to be a more economic form of counteraction between acetylcholine and noradrenaline than that which takes place at the postsynaptic membrane.

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