Brit. J. Pharmacol. (1964), **22**, 126–136.

THE RESPONSES OF THE GUINEA-PIG ISOLATED INTACT TRACHEA TO TRANSMURAL STIMULATION AND THE RELEASE OF AN ACETYLCHOLINE-LIKE SUBSTANCE UNDER CONDITIONS OF REST AND STIMULATION

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(*Received September* 18, 1963)

A preparation of the guinea-pig isolated intact trachea is described which allows transmural stimulation of the intrinsic nerves of the tracheal muscle with simultaneous measurement of the volume changes of the trachea caused by contraction or relaxation of the tracheal muscle. The apparatus allows fluid to be removed from the lumen and outside of the trachea for assay of acetylcholine. The response to transmural stimulation over a range of 1 to 50 shocks/sec is first a contraction followed by a relaxation. The contraction is best seen at high frequencies of stimulation and is abolished by atropine. The contraction is enhanced by treatment of the trachea with the anticholinesterase *NN*-diisopropylphosphodiamidic fluoride (mipafox), and is thus probably due to stimulation of intrinsic cholinergic nerves. The relaxation is maximal at low frequencies of stimulation. After treatment with mipafox a substance resembling acetylcholine is released in the absence of stimulation. The acetylcholine output rises following transmural stimulation, probably due to release of acetylcholine from cholinergic motor nerves. No relationship between frequency of transmural stimulation and release of acetylcholine has been seen in nine of ten experiments. The resting and stimulated release of acetylcholine is not greatly reduced by attempted separation of the tracheal muscle from the bulk of the trachea. This result suggests that acetylcholine outputs from the intact trachea are a measure of release from the smaller tracheal muscle.

Though the guinea-pig trachea has been widely used in the form of tracheal chains in the study of its pharmacology (Castillo & De Beer, 1947; Douglas, 1951; Akcasu, 1952, 1959; McDougal & West, 1953; Hawkins, 1955; Hawkins & Paton, 1958; Foster, 1960, 1963) there has been little attention paid to the reactions of the intact isolated trachea, and in particular the responses to, and release of possible transmitters by transmural stimulation of its intrinsic nerves.

Thornton (1934) showed that vagal stimulation caused a substance resembling acetylcholine to appear in the fluid (containing physostigmine) perfusing the guinea-pig isolated lungs; he found no release in the absence of stimulation. Foster (communication to the British Pharmacological Society, July, 1960; 1963) described a capillary manometer method for measuring the minute changes in volume of the intact guinea-pig trachea produced by transmural stimulation. Electrical stimuli,

which were assumed to be activating nervous elements, at first caused a contraction and then a relaxation. The contraction could be abolished by atropine and was presumably cholinergically mediated. Recently Jamieson (1962) used a capillary manometer method similar to that of Foster (1963) to study the action of broncho-constrictor and bronchodilator drugs on guinea-pig and rat isolated intact tracheas.

In the work described here the motor responses to transmural stimulation of the intrinsic nerves of the guinea-pig tracheal muscle have been further studied by an extension of Foster's (1963) method. After treatment of the trachea with an organo-phosphorous anticholinesterase an acetylcholine-like substance was released both in the absence of, and during transmural stimulation. This paper describes the tentative identification of this substance as acetylcholine and the demonstration that the tracheal muscle is the principal site of release.

METHODS

The apparatus used is shown in Fig. 1. The organ-bath (B) was made of Perspex. At the lower end a three-way tap (T1), with side-tubes of 1.5 mm internal diameter, was fitted.

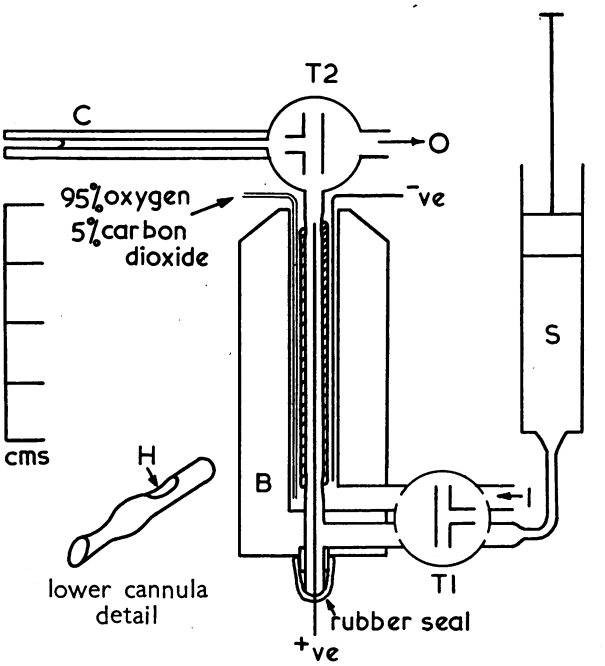

Fig. 1. Diagram of apparatus used to record volume changes induced in the guinea-pig trachea by transmural stimulation and to allow removal of organ-bath and tracheal lumen fluid for assay. B, Perspex organ-bath; C, recording capillary, 1 mm internal bore; H, hole in lower cannula allowing removal of tracheal lumen contents; I, inlet for oxygenated Krebs solution at 37° C; S, 2 ml. syringe for removing organ-bath and tracheal lumen contents; O, overflow tube; T1, lower three-way tap allowing removal of fluid from tracheal lumen and organ-bath, and washing; T2, upper tap allowing washing of tracheal lumen and recording of tracheal volume changes. Taps T1 and T2 are here shown in positions for recording volume changes of the trachea. The electrodes for transmural stimulation are marked $-_{ve}$ and $+_{ve}$.

A polyethylene cannula, 3 mm outside diameter, was tied into each end of the trachea. The lower cannula was made as shown in Fig. 1, and allowed the simultaneous withdrawal of fluid both from the organ-bath and from the tracheal lumen through the hole (H). A watertight joint between the lower cannula and the lower part of the organ-bath was ensured by a slight bulge in the cannula just above the withdrawal hole. The upper cannula was attached to a small three-way tap (T2). This tap enabled fluid to pass to waste (O) or connected the lumen of the trachea to the capillary (C) by which changes in tracheal volume were monitored. The lower tap enabled Krebs solution warmed to 37° C to be passed through the inlet (I) or the contents of the organ-bath and lumen to be removed for assay by a syringe (S). The whole apparatus was maintained at 37° ±0.5° C in a water-bath. The organ-bath and replacement fluid were both gassed with a mixture of 95% oxygen and 5% carbon dioxide.

Transmural stimulation was applied by two platinum electrodes, one placed in the lumen of the trachea and one outside, and arranged so that the stimulus was applied across the region of the tracheal muscle. Rectangular pulses of 0.3 msec duration and 40 V were applied at frequencies of 1 to 50 shocks/sec, unless otherwise stated. The organ-bath had a total volume of 2 ml. including a dead space of 0.3 ml. Krebs solution of the following composition was used (g/l.): NaCl 6.92, KCl 0.354, $CaCl_2$ 0.282, $NaHCO_3$ 2.10, KH_2PO_4 0.162, $MgSO_4$ $7H_2O$ 0.294 and glucose 2.0.

Female guinea-pigs of 0.5 to 1.0 kg were killed by stunning and bleeding from the axilla. The whole length of the trachea plus the larynx was removed and placed in cold Krebs solution. The trachea was then cut across immediately above the bifurcation and also just below the larynx. The oesophagus and connective tissue adhering to the trachea were next carefully removed, particularly that overlying the tracheal muscle; the lumen was then thoroughly washed out. The trachea was then tied to the two cannulae and placed in 100 ml. of oxygenated Krebs solution at 37° C containing 10 or 100 μg/ml. of *NN*-diisopropyl-phosphodiamidic fluoride (mipafox) and left for 75 to 90 min. The trachea and cannulae were then fixed in place in the organ-bath and the lumen and organ-bath perfused for 30 min with Krebs solution to wash out excess anticholinesterase. The lower tap was then closed and the upper tap set to connect the tracheal lumen with the recording capillary. Readings of the capillary were now taken every minute for 20 min after which the contents of the organ-bath and also of the tracheal lumen were removed for assay. The lumen and organ-bath were then perfused with Krebs solution for 2 min before a second experimental period of 10 min during which transmural stimulation was applied. Samples were taken before every period of stimulation and represented the resting release of acetylcholine. This cycle gave well-maintained responses for many hours. The volumes of samples were measured and they were immediately stored at −20° C until assayed, usually on the same day. If kept till the next day standard solutions of acetylcholine in Krebs solution were stored with them. Despite these precautions, some experiments yielded samples in which there was no detectable activity after storage.

The isolated tracheal muscle preparation. The trachea (Fig. 2, A) was cut along its length about 1 mm on each side of the tracheal muscle (Fig. 2, B). The muscle preparation was then transferred to a Perspex rod (Fig. 2, C) and attached by fine threads, care being taken to ensure that the tracheal muscle was held as near to its normal length and width as possible (Fig. 2, D). The whole assembly was then returned to the organ-bath and arranged for transmural stimulation.

Estimation of acetylcholine. Samples were assayed on pieces of guinea-pig ileum which had been previously incubated with the irreversible anticholinesterase, mipafox (10 μg/ml.), for 60 to 75 min in 100 ml. of Krebs solution at 37° C. The pieces of ileum were suspended in 2 ml. of Krebs solution at 37° C. The use of morphine (Paton, 1957; Blaber & Cuthbert, 1961; Birmingham, 1961) in the organ-bath was found to be unnecessary for the conduct of a successful assay. A contact time of 20 sec and a cycle of 90 sec were used. About three-quarters of all ileal segments were not sensitive enough to acetylcholine for assay purposes. A preparation was considered adequate only if it gave a response to 50 pg of acetylcholine

and no contraction artifact to 0.4 ml. of Krebs solution added to the 2 ml. organ-bath. Standard acetylcholine solutions were made in Krebs solution. Acetylcholine is expressed as base throughout.

Identification of the spasmogenic substance as acetylcholine. The contractile response of the ileum to samples was readily abolished by hyoscine (0.1 μg/ml.) leaving the response to histamine unaffected. A variable but small residual response was occasionally obtained after hyoscine (0.1 μg/ml.), and this residual response was substracted from the assay value.

The activities both of standard acetylcholine and of the samples were abolished by boiling for 5 min with N-sodium hydroxide solution, but were unaffected by boiling with N-hydrochloric acid.

No activity could be detected in samples taken from tracheas not treated with mipafox.

The assay preparation was much less sensitive to other possible contaminating substances. Thus a response to 0.1 ng of acetylcholine was matched by those to 4 ng of histamine, 0.5 μg of 5-hydroxytryptamine, 200 μg of adenosine triphosphate, 75 μg of potassium (as chloride) and 1 μg of choline. On segments of terminal ileum a matching response could be obtained to 1 to 3 μg of adrenaline.

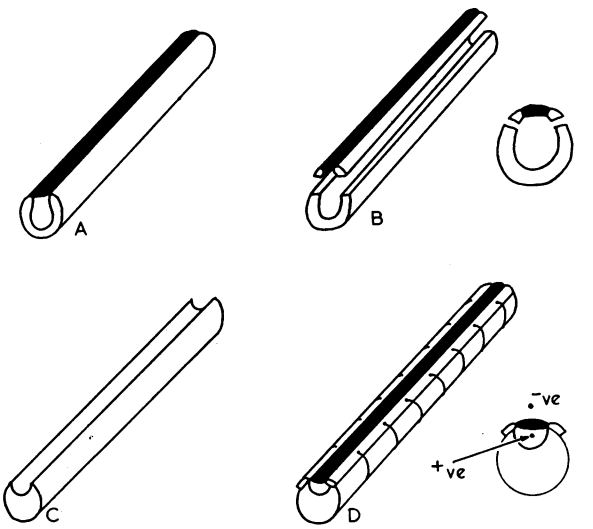

Fig. 2. The construction of the isolated tracheal muscle preparation from the intact trachea (A). The tracheal muscle and a narrow strip of cartilage on each side are removed as in B and transferred to the Perspex rod (C). The tracheal muscle is attached at as near to its normal length as possible (D). The upper diagrammatic cross-section shows the trachea with cuts to separate the tracheal muscle. The lower section shows the isolated muscle preparation positioned for transmural stimulation. The electrodes are marked $-_{ve}$ and $+_{ve}$.

Chang & Gaddum (1933) used five test objects to distinguish acetylcholine from other choline esters. But the samples obtained from the guinea-pig trachea have an acetylcholine content which allows their assay only on the more sensitive test organs. Thus it has only been possible to assay particularly potent samples on an additional test organ. This is the rabbit tracheal chain preparation treated with mipafox (10 μg/ml.) for 60 to 75 min. The preparation consists of eight tracheal rings in a chain, suspended in 2 ml. of Krebs solution at 37° C. A load of 180 mg and twenty-fold magnification were used. With a contact time of

2 min and a cycle of 7 min good responses to 1 ng of acetylcholine in 2 ml. of Krebs solution were obtained. The preparation was insensitive to 5-hydroxytryptamine and to histamine. The samples tested were equipotent on the guinea-pig ileum and rabbit tracheal chain preparations treated with mipafox.

Choice of anticholinesterase. *NN*-diisopropylphosphodiamidic fluoride (mipafox) has been used in all the experiments described here as it lacks the ability to release acetylcholine from parasympathetic nerve endings in the guinea-pig tracheal muscle (Carlyle, 1963).

Drugs. These were (−)-adrenaline acid tartrate, acetylcholine chloride, atropine sulphate, adenosine triphosphate, choline chloride, hexamethonium bromide, histamine acid phosphate, hyoscine hydrobromide, 5-hydroxytryptamine creatine sulphate, potassium chloride and procaine hydrochloride. All concentrations are expressed as μg/ml. of base except for that of mipafox. Outputs of acetylcholine are expressed as ng of base.

RESULTS

Responses of the tracheal muscle to transmural stimulation

In most tracheas the response to transmural stimulation at 0.3 msec duration, 40 V and 1 to 50 shocks/sec was first a variable contraction, which was followed by relaxation (Fig. 3, *a*). After discontinuing stimulation there was usually a further relaxation before recovery of the inherent tone.

After atropine (1 μg/ml.) the response was converted to relaxation only (Fig. 3, *c*). Also after exposure to mipafox (10 μg/ml.) the contractions were augmented (Fig. 3, *b*). The inhibitory response obtained after atropine was nearly maximal at the low rate of stimulation of 3 shocks/sec. In contrast contractions were most easily obtained by the use of high frequencies of stimulation. Hexamethonium (30 μg/ml.) did not reduce the contractile or relaxant responses to transmural stimulation; in many preparations both kinds of response were potentiated. Procaine (100 μg/ml.) abolished the contraction, before and after addition of mipafox. The inhibitory response was resistant to this concentration of procaine which had no effect on contractions induced by acetylcholine and histamine.

After treatment with mipafox (10 or 100 μg/ml.) no significant effect on the inherent tone was observed. The contractile responses to transmural stimulation after this treatment were usually well held for the experimental periods of 10 min.

Even in the presence of atropine (100 μg/ml.), which had no significant effect on muscle tone, it was possible to cause a contraction by transmural stimulation with parameters of 3 msec duration, 120 V and 6 shocks/sec and higher. This response was presumably due to direct stimulation of the smooth muscle cells.

Resting release of acetylcholine

After treatment of the trachea with mipafox (100 μg/ml.) and in the absence of transmural stimulation a substance resembling acetylcholine appeared in the bath. In 108 experimental resting periods of 20 min from twenty-five experiments a release of acetylcholine was found in all but two; in these samples the minimal acetylcholine content that could have been detected by the assay preparation was 125 pg in 2 ml. In these 108 periods the resting release was 0.062 ± 0.007 ng/min (mean and standard error).

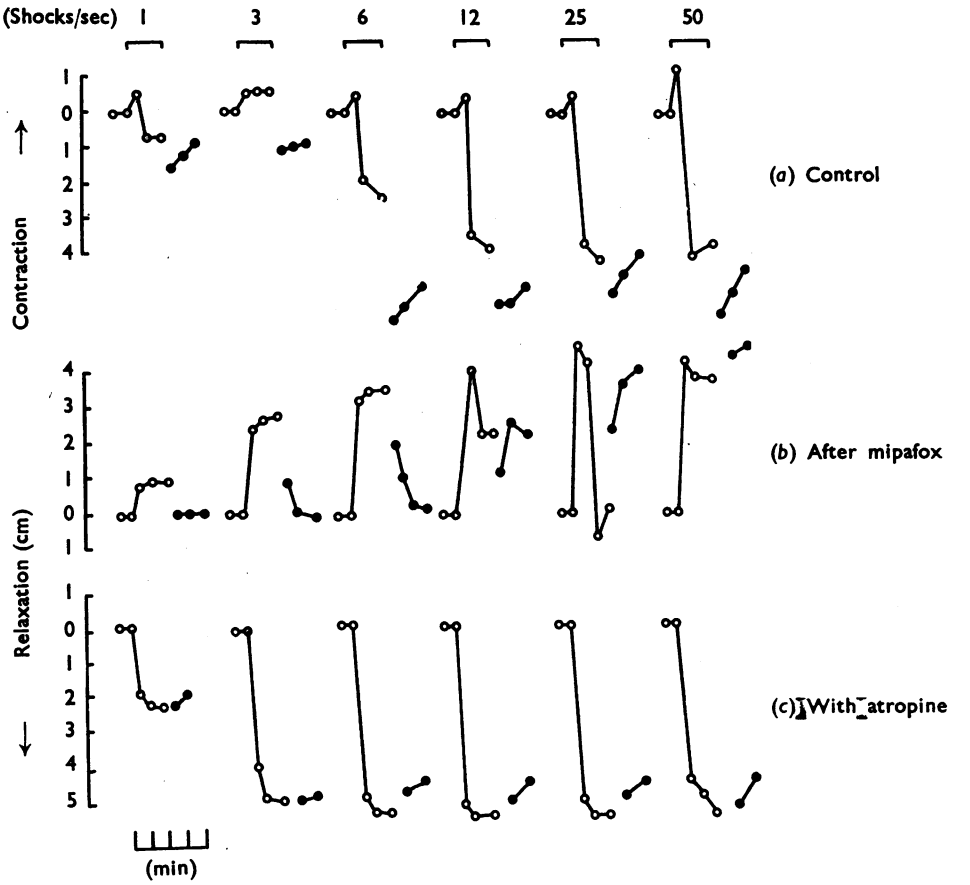

Fig. 3. Typical responses of the intact trachea preparation to transmural stimulation. Records show movement of fluid in the recording capillary. A downward movement from zero represents relaxation of the tracheal muscle and an upward movement contraction. Empty circles give the position of the capillary fluid during the period of stimulation and filled circles after cessation of stimulation. (*a*) represents responses to transmural stimulation at frequencies of 1 to 50 shocks/sec (figures at top) for periods of 2 min; (*b*) similar stimulations after 60 min incubation with mipafox (10 μg/ml.); (*c*) shows the responses to stimulation in the presence of atropine (1 μg/ml.).

Relation of time and amount of acetylcholine released

Samples were taken at 5, 10, 20 and 40 min from unstimulated tracheas. As the absolute values for acetylcholine output varied in ten experiments between 1 and 4 ng of acetylcholine in 40 min, the outputs at 5, 10 and 20 min were expressed as a percentage of the output at 40 min. The results are represented by the graph in Fig. 4 which gives the means and standard errors for ten experiments. The output appears to be simply related to time up to 20 min, but the yield at 40 min is less than would be predicted from the output of acetylcholine during 5, 10 and 20 min collection periods.

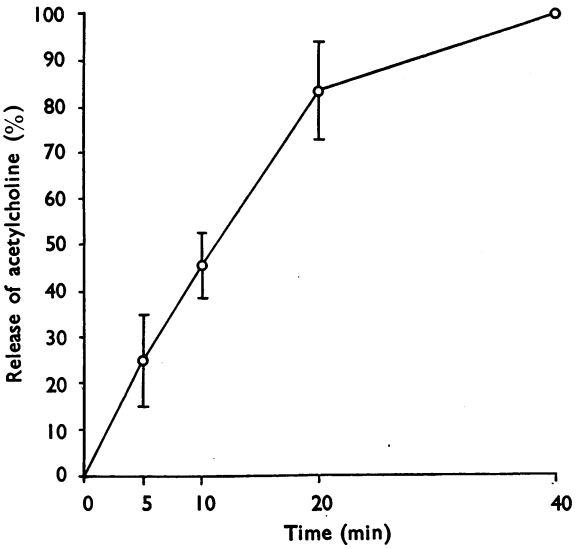

Fig. 4. Graph showing the relationship between the output of acetylcholine from the guinea-pig trachea at rest and the duration of the collection period in min. The output of acetylcholine is expressed as a percentage of the output in a 40-min sample. The means and standard errors of ten experiments are given.

During these experiments no significant contraction was observed during the 5, 10, 20 and 40 min collection periods. In many tracheas a fall of the inherent tone was noted during the 40 min collection period.

The resting release between periods of transmural stimulation often fluctuated or steadily increased with time but was occasionally constant throughout an experiment. The output of acetylcholine during any period of stimulation was therefore compared with the resting release measured in the period immediately preceding.

Release of acetylcholine during transmural stimulation

Transmural stimulation of the trachea after treatment with mipafox (100 μg/ml.) usually caused well-held contractions and there was usually an increase in acetylcholine output in samples obtained after stimulation compared with the previous resting value.

Stimulus frequencies were applied in the ascending order of 1, 3, 6, 12, 25 and 50 shocks/sec in six experiments, and in a random order in four experiments. There was no apparent difference in the results from these two methods. In these ten experiments the output of acetylcholine rose in forty-seven experimental periods of stimulation, was unaffected in four and fell in eight.

A typical experiment is illustrated in Fig. 5. This shows the effect of applying, at random, stimulation at rates of 1 to 50 shocks/sec for 10 min periods, on the contraction of the tracheal muscle and on acetylcholine output. Though an increase in acetylcholine output above the resting level occurred on stimulation, this was in no way related to the stimulus frequency or to the size of the contraction. Also,

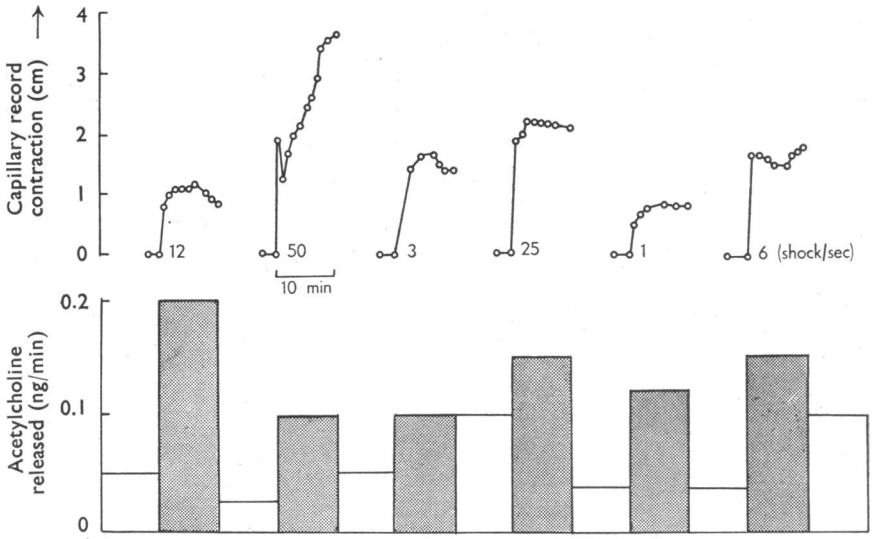

Fig. 5. Diagram showing the contractile responses to transmural stimulation of the intact guinea-pig trachea at frequencies of 1, 3, 6, 12, 25 and 50 shocks/sec applied in a random order for periods of 10 min, and the release of acetylcholine. The upper graphs show the contractile responses elicited by transmural stimulation as recorded by capillary fluid movement. The lower histogram represents the acetylcholine released at rest (empty panels) and during transmural stimulation (shaded panels). Acetylcholine outputs are in ng/min.

the contraction showed no appreciable fatigue, even at stimulation rates as high as 50 shocks/sec. The resting release of acetylcholine also showed variations throughout the course of nine experiments. In the remaining experiment acetylcholine output increased with each increase in stimulus frequency in the range 6 to 50 shocks/sec only.

Release of acetylcholine from the isolated tracheal muscle preparation

Kordik, Bülbring & Burn (1952) showed that acetylcholine was connected with ciliary movement in the mucous membrane of the rabbit trachea. Thus it was important to determine to what extent the cartilage and mucosal lining of the trachea contributed to the total acetylcholine output. The isolated tracheal muscle preparation shown in Fig. 2 was used and the release of acetylcholine at rest and during transmural stimulation was determined both for the intact trachea and for the tracheal muscle preparation following incubation with mipafox (100 μg/ml.).

The results have been expressed as the percentage release from the muscle preparation compared with the release from the intact trachea. In five experiments the resting release of the tracheal muscle preparation was $110 \pm 22\%$ (mean and standard error) of the value for the whole trachea and the release on transmural stimulation was $92 \pm 12\%$ of the value for the whole trachea. The weight of the tracheas was 194 ± 18 mg (mean and standard error) and the weight of the tracheal muscle preparation was 47 ± 5 mg. Thus, although the weight of the tracheal muscle preparation was only about one-quarter of the total tracheal weight, the outputs of acetylcholine at rest and during stimulation were similar.

In one experiment the body of the trachea and its lining did, after removal of the muscle, produce a small but detectable release of acetylcholine; this release was not affected by stimulation and was only 10 to 20% of the usual resting release for the intact trachea.

DISCUSSION

These experiments show that the guinea-pig tracheal muscle is innervated by at least two groups of antagonistic intrinsic nerves; a motor group, presumably cholinergic, and an inhibitory group, presumably adrenergic. Transmural stimulation of the trachea causes an initial contraction followed by a relaxation. This result confirms the original observations of Foster (1963). After treatment with the irreversible anticholinesterase, mipafox, the contractions may become dominant and well-held on transmural stimulation. After atropine the motor responses are abolished. These experiments provide good evidence that these contractions are caused by stimulation of intrinsic cholinergic nerves. An interesting aspect of the contractions is the absence of any obvious fatigue at the high stimulus frequency of 50 shocks/sec. This is in contrast to the responses of the transmurally stimulated guinea-pig isolated ileum (Paton, 1957) which failed to hold a tetanus at stimulus frequencies of over 20 shocks/sec. The failure of hexamethonium to block the contractions or relaxations after transmural stimulation suggested that the stimulation was effectively postganglionic.

A resting release of acetylcholine in the absence of stimulation has been observed in the guinea-pig ileum by Feldberg & Lin (1949), Paton (1957), Schaumann (1957), Harry (1962) and Johnson (1963). A similar release from the guinea-pig stomach was shown by Paton & Vane (1963). In the course of these experiments the trachea appeared to be able to produce acetylcholine continuously for many hours; in fact the resting release may increase during the course of an experiment. The acetylcholine from a 40 min period of collection at rest is less than would be expected from the values at 5, 10 and 20 min. Schaumann (1957) observed that the resting release from the guinea-pig ileum over a 60 min collection period was less than the total of several collection periods of shorter duration. He thought that this was caused by a retardation of acetylcholine release from the tissue by the high organ-bath concentration of acetylcholine. This is only one of a number of possibilities. Under the conditions of these experiments the inherent tone of the tracheal muscle is well held for up to 20 min but after this time often decreases. This observation suggests that conditions within the small organ-bath during periods of longer than 20 min are not optimal. The fall of the predicted acetylcholine output during a 40 min collection period may also arise from these changed conditions. Furthermore it has already been noted that in some samples no acetylcholine could be detected; this may be caused by spontaneous hydrolysis of the low concentrations of acetylcholine at the alkaline reaction of Krebs solution.

It is of interest to note that the irreversible anticholinesterase mipafox induces no significant change in the inherent tone of the guinea-pig tracheal muscle, though after this treatment a release of acetylcholine at rest can be demonstrated. This result is in marked contrast to the contraction induced in the guinea-pig ileum

by its resting release of acetylcholine after cholinesterase inhibition by mipafox (Johnson, 1963), and suggests that in the tracheal muscle the acetylcholine receptors have a low sensitivity to the endogenous acetylcholine released or that the release is remote from the receptor. The site of origin of the acetylcholine released at rest from the trachea cannot at present be stated, though it is likely that the parasympathetic nerve terminals are only feebly spontaneously active at rest (Carlyle, 1963).

The increase in acetylcholine output above the resting level during transmural stimulation follows a familiar pattern. In nine of ten experiments over the range of stimulus frequencies 1 to 50 shocks/sec there was no clear increase in output with increase in frequency. Paton (1957) showed that the release per stimulus in the guinea-pig isolated ileum fell during transmural stimulation at frequencies of 3.5/min to 27/sec. This pattern was confirmed on the guinea-pig ileum by Harry (1962). Similarly, Straughan (1960) showed that in the rat and guinea-pig diaphragms the release of acetylcholine during indirect stimulation in the frequency range 6 to 100 shocks/sec did not rise in the manner predicted from the release at 16 shocks/sec. Perry (1953) showed that in the cat superior cervical ganglion perfused with saline containing physostigmine the acetylcholine output produced by preganglionic nerve stimulation did not alter with increase in frequency from 5 to 100 shocks/sec, the output falling off rapidly after the onset of stimulation. The rise in acetylcholine output from the guinea-pig trachea after transmural stimulation may be tentatively ascribed to a release of acetylcholine from intrinsic cholinergic nervous structures. These may be both post- and preganglionic since Hawkins & Paton (1958) have shown the presence of both parasympathetic and sympathetic ganglia in the guinea-pig tracheal muscle.

Since the acetylcholine output of the isolated tracheal muscle preparation was similar to that of the intact trachea, values obtained from the latter preparation may be taken to represent the output of the smaller tracheal muscle. This assumption is of practical value because, in recording responses of the tracheal muscle during transmural stimulation, the trachea must be intact.

The release of acetylcholine in detectable quantities from the cartilaginous part of the trachea and its mucosal lining accords with the findings of Kordik *et al.* (1952) who found choline acetylase, cholinesterase and acetylcholine in the mucosal lining of the rabbit trachea.

This work was communicated to the British Pharmacological Society in July, 1962. I wish to thank Professor G. Brownlee for his help and guidance throughout this work, which was carried out during the tenure of a grant from the Lederle Laboratories Division of the American Cyanamid Company.

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