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A NON-ADRENERGIC INHIBITORY NERVOUS PATHWAY IN GUINEA-PIG TRACHEA

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1 Electrical stimulation of the guinea-pig isolated tracheal tube causes a biphasic response, initially excitatory and then inhibitory. The excitatory response was abolished by atropine leaving the inhibitory response unaffected.

2 The inhibitory response was greatly reduced but not abolished by propranolol or guanethidine. A residual inhibitory response was still present in tracheas in which sympathetic nerve function had been abolished by pretreatment with syrosingopine or 6-hydroxydopamine. These results show that the inhibitory response is predominantly adrenergic but that a small non-adrenergic component is also present.

3 The non-adrenergic inhibitory response was abolished by lignocaine and tetrodotoxin suggesting that it is nervous in origin.

4 Optimal stimulation parameters for the predominantly adrenergic inhibitory response were a pulse width of 0.7-2 ms, a stimulation period of 7 s and a frequency of 20 Hz. For the non-adrenergic inhibitory response, optimal stimulation parameters were a pulse width of 2 ms, a stimulation period of 12 s and a frequency of 20 Hz.

5 Evidence obtained with pharmacological antagonists, enzyme inhibitors and activators suggested that the transmitter mediating the non-adrenergic inhibitory nervous response is unlikely to be: acetylcholine, histamine, 5-hydroxytryptamine, cyclic 3',5'-adenosine monophosphate or a prostaglandin.

6 The adenosine uptake blocking drugs dipyridamole, hexobendine and Dilazep potentiated the non-adrenergic inhibitory nervous response and unmasked inhibitory responses to adenosine and adenosine 5'-triphosphate.

7 It is concluded that electrical stimulation of the guinea-pig trachea, in addition to activating cholinergic and adrenergic nervous pathways, may activate a separate and distinct inhibitory nervous pathway. This pathway has some features in common with the non-adrenergic non-cholinergic inhibitory pathways in gastro-intestinal muscle.

Introduction

A preparation of isolated tracheal tube of the guinea-pig has been described by Farmer & Coleman (1970). Electrical stimulation of the trachea was shown to cause a biphasic response, initially excitatory and then inhibitory. The excitatory response was abolished by atropine and the inhibitory response greatly reduced by propranolol.

Further experiments have shown that the inhibitory response of the trachea includes a small component that is resistant to the blocking effect of propranolol. A more detailed investigation of the inhibitory response to electrical stimulation was therefore undertaken, the results of which are described in this paper.

A preliminary account of this work was presented to a meeting of the British Pharmacological Society in March 1973 (Coleman, 1973).

Methods

Electrically stimulated trachea

Guinea-pigs weighing 300-400 g were killed by a blow on the head and the tracheas excised. Two tracheal tube preparations were made from each animal by dividing the trachea halfway along its length. Each portion was mounted on a tracheal electrode as described by Farmer & Coleman (1970). The preparations were stimulated through intraluminal and extraluminal platinum wire electrodes with square wave alternating pulses. The

intraluminal pressure (1 mmHg = 1.333 mbar) was monitored continuously. Physiological salt solution maintained at 37° C was in contact with both inner and outer surfaces of the trachea. It had the following composition (g/l): NaCl, 6.9; KCl, 0.35; KH_2PO_4, 0.16; $MgSO_4 . 7H_2O$, 0.29; glucose, 2.0; $NaHCO_3$, 2.1; $CaCl_2 . 6H_2O$, 0.28. In those experiments in which barium chloride was used to maintain intraluminal pressure, $MgCl_2 6H_2O$ replaced $MgSO_4 . 7H_2O$ in the physiological salt solution.

Unstimulated trachea

Preparations were set up as described above. A high level of resting tone was induced in the trachea by the method of Coleman & Farmer (1971). The preparation was used to detect any direct inhibitory effects of drugs.

Abolition of sympathetic nerve function

Guinea-pigs were depleted of neuronal catecholamine stores by intraperitoneal injection of syrosingopine, 5 mg/kg, 16-20 h before use (Orlans, Finger & Brodie, 1959). The treatment was judged to have been effective as no formaldehyde-induced fluorescence could be detected by histochemical examination.

Guinea-pigs were pretreated with 6-hydroxydopamine to produce 'chemical sympathectomy' (Tranzer & Thoenen, 1967; Malmfors & Sachs, 1968). 6-Hydroxydopamine was injected intravenously, 2 x 25 mg/kg on day 1 and 2 x 50 mg/kg on day 7, and experiments were carried out on days 8-10. Destruction of sympathetic nerve endings in the trachea was judged to have been complete as no formaldehyde-induced fluorescence could be detected by histochemical examination and responses to tyramine were abolished (Coleman & Levy, 1972).

Drugs

Aspirin (Ralph N. Emanuel); adenosine (Koch-Light); N^6, 0^2 dibutyryl adenosine 3',5'-cyclic monophosphoric acid (dibutyryl cyclic AMP, Sigma); adenosine 5'-triphosphate (ATP, BDH); atropine sulphate (BDH); burimamide (SKF); tetrahydro-1H-1,4-diazepine-1,4(5H)-dipropanol,3,4,5-rimethoxybenzoate (diester) (Dilazep, Asta Werke); dipyridamole (Boehringer Ingelheim); guanethidine sulphate (Ciba); hexamethonium bromide (Koch-Light); hexobendine (Oesterreichische Stickstoffwerke); histamine acid phosphate (BDH); 5-hydroxytryptamine creatinine sulphate (Koch-Light); 6-hydroxydopamine hydrochloride (Hassle); imidazole (BDH); indomethacin (MSD); lignocaine hydrochloride (Macfarlan Smith), 2-amino-7-methyl-5-propyl-syn-triazolo [2,3-c]-pyrimidine (M30966,I.C.I.); mepyramine maleate (M&B); methysergide bimaleate (Sandoz); (−)-noradrenaline bitartrate (Winthrop); (±)-propranolol hydrochloride (I.C.I.); prostaglandin E_2 (Upjohn); syrosingopine (CIBA); tetrodotoxin (Calbiochem); theophylline (choline theophyllinate, A&H). Concentrations in the text refer to the appropriate free acid or base.

Indomethacin and aspirin were dissolved in 0.2% w/v sodium carbonate solution. A solution of syrosingopine (10 mg/ml) was made by dissolving 40 mg of the drug in 0.1 ml glacial acetic acid and adding 0.8 ml propylene glycol, 0.8 ml ethanol and distilled water up to 4 ml. Dipyridamole was dissolved in distilled water. All other drugs were dissolved in the physiological salt solution. Ascorbic acid (200 μg/ml) was present in solutions of noradrenaline.

Quantitative analysis

Values quoted in the text are geometric means with 95% confidence intervals in parentheses.

Results

Responses to electrical stimulation

Alternating square wave pulses were delivered at a frequency of 20 Hz, a pulse width of 2 ms and supramaximal voltage for 7 s in every 2 minutes. The tissue responded with an initial rapid increase in pressure (excitatory response) followed by a decrease and a slow return to normal (inhibitory response) (see control responses in Figure 1a). Some preparations exhibited a small degree of spontaneous activity; this was superimposed on the responses to electrical stimulation (see for example Figures 1c and 2a).

Effect of antagonists on the biphasic response to electrical stimulation

The excitatory response was markedly reduced by atropine, 0.01 μg/ml (Fig. 1a) and was abolished by 0.1 μg/ml. The inhibitory response was unaltered by these concentrations of atropine.

The β-adrenoceptor antagonist propranolol produced a graded reduction of the inhibitory response in the concentration range 0.01-0.1 μg/ml ($n = 4$). The inhibitory response was reduced by a maximum of 71% (60.5-81.5%) at 0.1 μg/ml (Figure 1b). Higher concentrations of propranolol (up to 1 μg/ml) had no greater effect.

The adrenergic neurone blocking drug

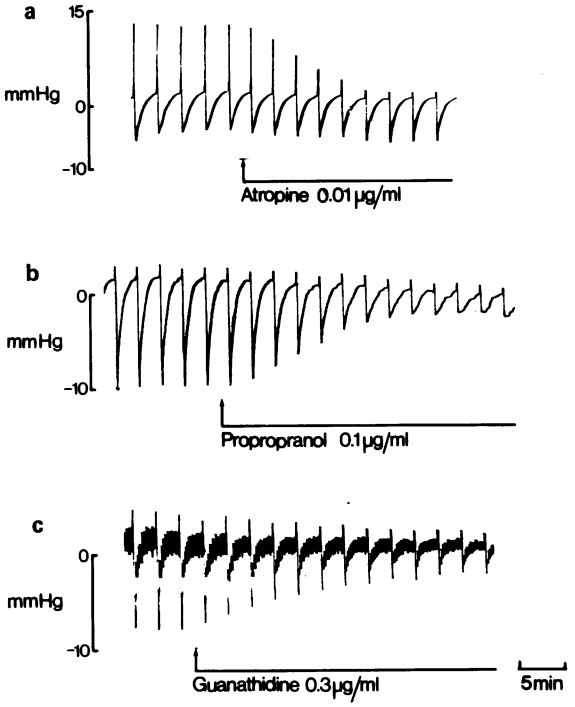

Fig. 1 Guinea-pig isolated tracheal tube. Electrical stimulation (20 Hz, 2 ms for 7 s in every 2 minutes). (a) Effect of atropine on biphasic response. (b) Effect of propranolol and (c) guanethidine on inhibitory response. In (b) and (c), atropine (0.1 μg/ml) in bathing solution. Note residual inhibitory response after maximally effective blocking doses of propranolol and guanethidine.

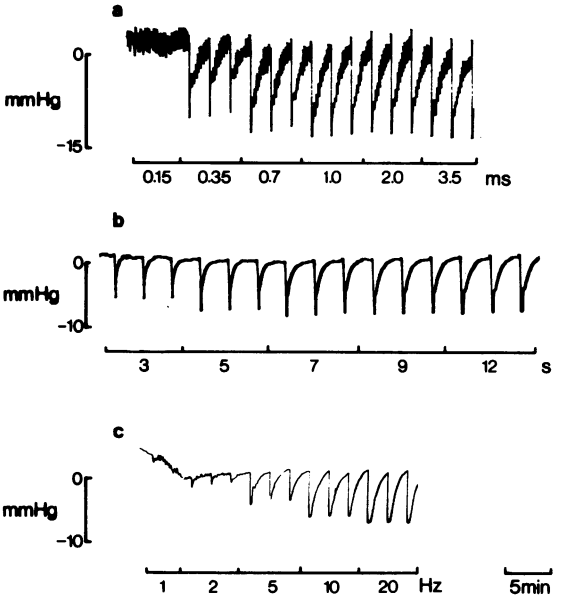

Fig. 2 Guinea-pig isolated tracheal tube. Atropine (0.1 μg/ml) in bathing solution. (a) Effect of variation in (a) pulse width (frequency 20 Hz, stimulation period 7 s); (b) stimulation period (frequency 20 Hz, pulse width 2 ms); (c) frequency (pulse width 2 ms, stimulation period 7 seconds).

guanethidine produced a graded reduction of the inhibitory response in the concentration range 0.1-0.3 μg/ml ($n = 4$). The inhibitory response was reduced by a maximum of 74% (62-87%) at 0.3 μg/ml (Figure 1c). As with propranolol, higher concentrations of guanethidine (up to 5 μg/ml) had no greater blocking effect.

In all subsequent experiments on the response of the trachea to electrical stimulation the excitatory component was abolished by the addition of atropine, 0.1 μg/ml, to the bathing fluid.

Effect of changes in pulse width, stimulation period and frequency on the inhibitory response to electrical stimulation

The optimal pulse width was 0.7-2 ms and stimulation period 7 seconds. The inhibitory response increased in magnitude with increasing stimulation frequencies up to 20 Hz, the highest frequency examined (Figure 2). The preparation responded to these stimulation parameters with a mean fall in intraluminal pressure of 10.5 (9.3-11.5) mmHg ($n = 43$).

Effect of pretreatment with syrosingopine or 6-hydroxydopamine on the inhibitory response to electrical stimulation

Tracheas from guinea-pigs pretreated with syrosingopine ($n = 64$) or 6-hydroxydopamine ($n = 6$) still gave inhibitory responses to electrical stimulation. However, these responses differed from responses in normal tracheas in a number of ways. Firstly, they were smaller, with a decrease in intraluminal pressure of 4.1 (3.5-4.7) mmHg. (This value is significantly different from that in the normal trachea–see above, $P < 0.05$.) Secondly, they were unaffected by high concentrations of either propranolol (1 μg/ml, $n = 6$) or guanethidine (5 μg/ml, $n = 8$) (Figure 3a and b). Thirdly, the optimal stimulation parameters differed slightly in that the optimal pulse width was 2 ms and optimal stimulation period 12 seconds. The relationship between response and stimulation frequency was unchanged (Figure 3c).

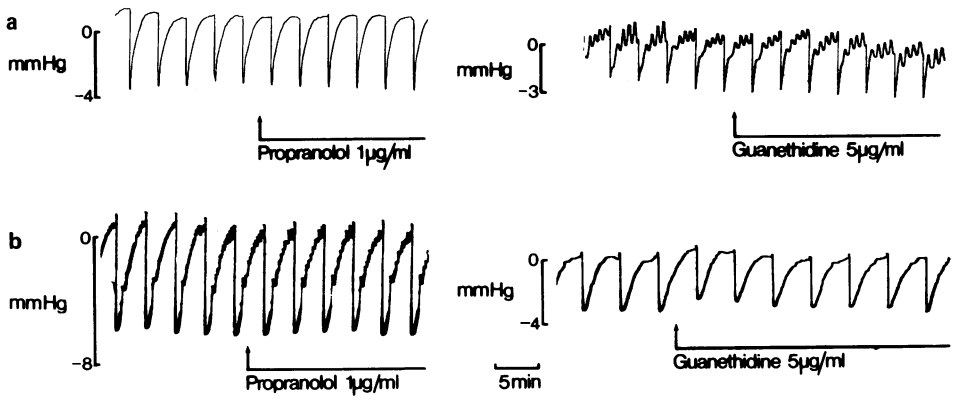

Fig. 3 Guinea-pig isolated tracheal tube. Atropine (0.1 μg/ml) in bathing solution. Preparations from animals pretreated with (a) syrosingopine and (b) 6-hydroxydopamine. Effect of propranolol and guanethidine on the non-adrenergic inhibitory response to electrical stimulation (20 Hz, 2 ms for 12 seconds).

These results show that there are usually two distinct components to the inhibitory response to electrical stimulation. The predominant component is adrenergic and the other is non-adrenergic. In all subsequent experiments on the non-adrenergic inhibitory response, preparations from animals pretreated with syrosingopine were used. As before, atropine 0.1 μg/ml, was added to the bathing solution to eliminate the excitatory response.

Effect of tetrodotoxin, lignocaine and hexamethonium on the non-adrenergic inhibitory response to electrical stimulation

The non-adrenergic inhibitory response was abolished by lignocaine (100-300 μg/ml, $n \mp 5$) and by tetrodotoxin (0.03-0.1 μg/ml, $n = 5$) (Figure 4a and b). Neither drug, in the concentrations used, had any effect on inhibitory responses to noradrenaline (0.01-0.3 μg/ml) in the unstimulated trachea.

Hexamethonium (10 μg/ml, $n = 4$) had no effect on the non-adrenergic inhibitory response.

The results with tetrodotoxin and lignocaine indicate that the non-adrenergic inhibitory response is nervous in origin.

Histamine, 5-hydroxytryptamine, cyclic 3',5'-AMP and prostaglandins as possible transmitter substances for non-adrenergic inhibitory nerves

Histamine

Unstimulated trachea. Histamine causes relaxation of tracheobronchial muscle in the sheep by activating H_2-receptors, and in the cat by activating both H_1 and H_2-receptors (Eyre, 1973). However, in the present experiments in guinea-pig trachea only an excitatory response to histamine was obtained even in preparations with a high intraluminal pressure ($n = 8$). The contractile response to histamine (0.1-10 μg/ml) was abolished by the H_1-antagonist mepyramine (0.1 μg/ml, $n = 4$), but no inhibitory response was unmasked.

Electrically stimulated trachea. Neither mepyramine (3 μg/ml, $n = 4$) nor the H_2-antagonist burimamide (10 μg/ml, $n = 5$) had any effect on the non-adrenergic inhibitory response of the trachea to electrical stimulation.

5-Hydroxytryptamine

Unstimulated trachea. 5-Hydroxytryptamine (0.01-1.0 μg/ml) caused excitatory responses which were abolished by methysergide (0.1 μg/ml, $n = 4$). In the presence of methysergide, 5-hydroxytryptamine produced a small inhibitory response which was blocked by propranolol (1 μg/ml).

Electrically stimulated trachea. The non-adrenergic inhibitory response to electrical stimulation was unaltered by concentrations of methysergide as high as 10 μg/ml ($n = 4$).

Cyclic AMP

Unstimulated trachea. The dibutyryl ester of cyclic AMP was used since it is more resistant to

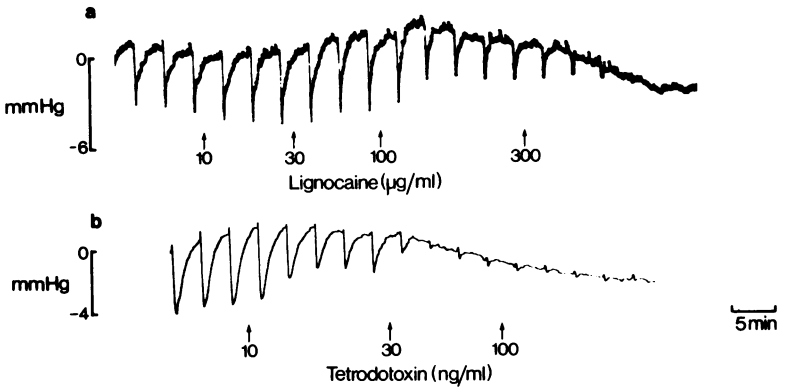

Fig. 4 Guinea-pig isolated tracheal tube. Atropine (0.1 μg/ml) in bathing solution. Preparations from animals pretreated with syrosingopine. Effect of lignocaine (a) and tetrodotoxin (b) on the non-adrenergic inhibitory response to electrical stimulation.

breakdown by phosphodiesterase and may penetrate cells more readily than cyclic AMP (Posternak, Sutherland & Henion, 1962). Dibutyryl cyclic AMP caused an inhibitory response at 500 μg/ml ($n = 5$). The response was slow in onset but similar in magnitude to the maximum inhibitory response to isoprenaline. Little or no recovery of resting tone was obtained, even after repeated washing of the preparation.

Electrically stimulated trachea. Cyclic AMP is inactivated by the enzyme phosphodiesterase. Theophylline which inhibits phosphodiesterase (Sutherland & Rall, 1958; Butcher & Sutherland, 1959) had no effect on the non-adrenergic inhibitory response to electrical stimulation in concentrations below those producing a direct spasmolytic effect (1.0-10 μg/ml, $n = 4$). M30966, another compound known to possess phosphodiesterase inhibitory activity (Martin & Vardy, unpublished observations), also had no effect in concentrations below those producing a direct spasmolytic effect (0.03-0.1 μg/ml, $n = 2$).

The phosphodiesterase activator imidazole (Butcher & Sutherland, 1962) at a concentration of 1 mg/ml had no effect on the non-adrenergic inhibitory response ($n = 4$).

Prostaglandins

Unstimulated trachea. Prostaglandin E_2 (0.01-1.0 μg/ml, $n = 10$) caused inhibitory responses, thus confirming previous findings in guinea-pig tracheal muscle (Main, 1964; Lynn-James, 1969).

Electrically stimulated trachea. Indomethacin and aspirin inhibit prostaglandin synthesis in guinea-pig lung (Vane, 1971). The effects of these two drugs on the non-adrenergic inhibitory response to electrical stimulation were therefore investigated. Analysis of the effect of indomethacin on the inhibitory response was complicated by the fact that the drug itself abolished resting tone, in agreement with the findings of Farmer, Farrar & Wilson (1972). The study was therefore carried out in preparations in which a high resting tone was maintained by the addition of barium chloride (100 μg/ml) to the bathing solution. Under these conditions, indomethacin (1-10 μg/ml, $n = 5$) had no blocking effect on the non-adrenergic inhibitory response, and in three experiments the response was enhanced. Aspirin (10-30 μg/ml, $n = 6$) had very little direct effect on resting tone. Like indomethacin, aspirin (10-30 μg/ml, $n = 6$) had no blocking effect on the non-adrenergic inhibitory response and in five experiments the response was enhanced.

Effects of adenosine uptake blocking drugs on the non-adrenergic inhibitory response to electrical stimulation and on responses to adenosine and ATP

Unstimulated trachea. Adenosine ($n = 4$) and ATP ($n = 4$) produced little or no inhibitory response in the trachea in the concentration range 1-30 μg/ml. In three preparations a small excitatory response to ATP was observed (Figure 5).

Dipyridamole has been shown to block the

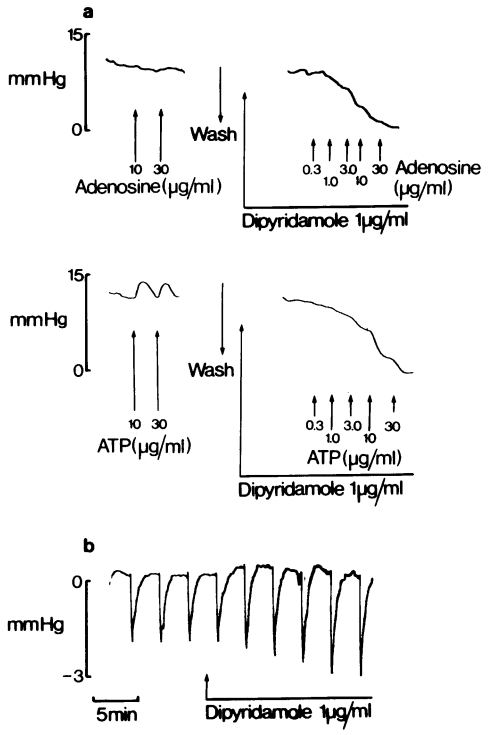

Fig. 5 Guinea-pig isolated tracheal tube. Atropine (0.1 μg/ml) in bathing solution. Preparations from animals pretreated with syrosingopine. (a) Unstimulated preparation. Responses to adenosine and ATP in the absence and presence of dipyridamole. (b) Electrically stimulated preparation. Effect of dipyridamole on the non-adrenergic inhibitory response.

uptake of adenosine in the heart (Kolassa, Pfleger & Rummel, 1970) and to potentiate responses to adenyl compounds in several tissues (Hockerts & Bögelmann, 1959; Stafford, 1966; Bowman & Stafford, 1968; Buyniski & Rapela, 1969; Nott, 1970; Raberger & Kraupp, 1971).

In the presence of dipyridamole, 1 μg/ml, a concentration which itself had no effect on the tone of the tracheal preparation, both adenosine ($n = 25$) and ATP ($n = 12$) produced inhibitory responses in the concentration range 0.3-30 μg/ml (Figure 5). This potentiating action was specific for the adenyl compounds since inhibitory responses to noradrenaline (0.01-0.3 μg/ml) were unaltered in the presence of dipyridamole (1 μg/ml, $n = 4$).

Electrically stimulated trachea. In 9 out of 11 experiments dipyridamole, 1 μg/ml, potentiated the non-adrenergic inhibitory response to electrical stimulation by 9-55% (Figure 5). In contrast, the predominantly adrenergic response of the normal trachea was unaffected by dipyridamole, 1 μg/ml ($n = 9$).

Similar results were obtained with two other drugs reported to block adenosine uptake, hexobendine (Kraupp, Wolner, Adler-Kastner, Chirikdjian, Ploszczanski & Tuisl, 1966) and Dilazep (Sano, Katsuki & Kawada, 1972; Buyniski, Losada, Bierwagen & Gardier, 1972). Thus both agents unmasked inhibitory responses to adenosine and ATP and potentiated the non-adrenergic inhibitory response to electrical stimulation. A difference in the time course of the potentiating action of these agents was observed. With dipyridamole and hexobendine the potentiating effect on the non-adrenergic inhibitory response and on responses to the adenyl compounds was maximum within 20 min; with Dilazep the maximum effect took over 30 min to develop.

Discussion

Previous reports have shown that electrical stimulation of the isolated trachea of the guinea-pig produces a biphasic response, the excitatory component resulting from activation of cholinergic nerves and the inhibitory component from activation of adrenergic nerves (Foster, 1964; Farmer & Coleman, 1970; Rikimaru & Sudoh, 1971). The present results confirm the cholinergic nature of the excitatory response but show that the cause of the inhibitory response is more complicated than activation of adrenergic nerves alone. Thus, an additional inhibitory factor has been demonstrated, distinguishable from the major adrenergic factor by its resistance to measures used to abolish adrenergic function and by its different optimal stimulation parameters.

The susceptibility of the non-adrenergic inhibitory response to lignocaine and tetrodotoxin shows that it is nervous in origin, and its resistance to ganglion-blocking concentrations of hexamethonium (Mason, 1962) suggests that the response results from stimulation of post-ganglionic nerves. Further experiments were directed towards determining the nature of the transmitter involved. Several criteria must be satisfied before a substance can be established as a neurotransmitter (Eccles, 1964). Of these, two were selected for use in the present work. First, the effect of the neurotransmitter should be mimicked by the exogenous administration of the substance to the effector; second, drugs which modify the response to the transmitter after its release by nerve stimulation should modify the

response to the exogenously applied substance in a similar way.

Histamine, 5-hydroxytryptamine and cyclic AMP were rejected as possible neurotransmitters because they failed to meet either one or both of these criteria. An inhibitory prostaglandin is also an unlikely candidate because indomethacin and aspirin, known prostaglandin synthetase inhibitors, did not reduce the response.

Burnstock and his co-workers have demonstrated the presence, in gastro-intestinal smooth muscle, of intramural inhibitory nerves which are neither cholinergic nor adrenergic. As the principal active substance released from these nerves appeared to be a purine nucleotide, most probably ATP, they were called 'purinergic' (see Burnstock, 1972 for detailed references). Although the purinergic nature of these nerves has not been unequivocally established, for example Weston (1973) has recently concluded that ATP is not the transmitter involved, we did consider the possibility that the non-adrenergic inhibitory nerves in the trachea were purinergic.

Burnstock's model for purinergic nerve transmission involves the release of ATP from the nerve endings, its action on post-synaptic receptors, its metabolic breakdown to adenosine and other metabolites, and re-uptake of adenosine into the purinergic nerves. The adenosine uptake blocking drug dipyridamole has been shown to potentiate responses to purinergic nerve stimulation and to exogenously administered adenosine and ATP. Burnstock (1972) postulated that dipyridamole produces these effects by blocking uptake of adenosine into purinergic nerves. This effect is analogous to that of cocaine, which potentiates responses to adrenergic nerve stimulation and exogenously administered noradrenaline by blocking neuronal uptake (Trendelenburg, 1966).

In the present experiments dipyridamole produced a specific potentiation of the non-adrenergic inhibitory nervous response and unmasked inhibitory responses to exogenously administered adenosine and ATP. Hexobendine and Dilazep, which also block adenosine uptake, had similar potentiating effects. In these respects the non-adrenergic inhibitory nerves in the trachea resemble the intramural inhibitory nerves in the gut, but more evidence would be required to establish that they are unequivocally purinergic in nature.

Shortly after the initial communication from this laboratory (Coleman, 1973), Coburn & Tomita (1973) also reported the presence of non-adrenergic, non-cholinergic inhibitory nerves in guinea-pig tracheal muscle. These workers consider that the relative importance of the adrenergic and non-adrenergic components of the inhibitory response to electrical stimulation varies depending upon the particular part of the trachea under study. No such distinction has been observed by us. However, apart from this the results of the two studies agree closely.

Non-adrenergic, non-cholinergic nerves, possibly purinergic in nature may be present in other tissues, for example in the urinary bladder of fish, amphibians, reptiles and mammals and in the lungs of amphibians and reptiles (Campbell, 1970; Burnstock, 1972). The occurrence of such inhibitory nerves in the lungs of amphibians and reptiles is of particular interest in relation to the present work. In these animals the inhibitory fibres are derived from the vagus nerve (see Burnstock, 1972). Quite possibly the non-adrenergic inhibitory nerves reported in this study are also vagal in origin and may be responsible for several reports of bronchodilatation in mammals after vagal stimulation (see Widdicombe, 1963).

An important physiological role has been ascribed to the non-adrenergic non-cholinergic nerves supplying the alimentary tract (Burnstock, 1972). The presence of such nerves in airways smooth muscle raises a question about their possible physiological function, but this remains to be determined.

We wish to thank Mr G.F. Ainge for carrying out fluorescence histochemical studies.

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