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THE MODE OF ACTION OF NEOSTIGMINE AND PHYSOSTIGMINE ON THE GUINEA-PIG TRACHEALIS MUSCLE

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Neostigmine (2.2 μg/ml.) or physostigmine (3.3 μg/ml.) contracted guinea-pig isolated tracheal chains. The anticholinesterase diisopropylphosphodiamidic fluoride (mipafox) itself caused no contractile response even in concentrations of 100 μg/ml., yet neostigmine or physostigmine still caused contractions after treatment with mipafox. The responses were abolished by hyoscine or atropine. Local anaesthetics, cooling, ionic changes and hemicholinium, all known to inhibit the release of acetylcholine from nerve endings, abolished or much reduced the responses. It seems that the contractile response to physostigmine or neostigmine does not depend on their anticholinesterase activity, but on their ability to release acetylcholine from postganglionic parasympathetic nerve endings.

Douglas (1951) showed that the anticholinesterases, ethyl pyrophosphate, dyflos and physostigmine, caused slowly developing contractions of the guinea-pig isolated tracheal muscle. This activity and also the potentiated responses to acetylcholine after treatment with these anticholinesterases were abolished by atropine. The delay in onset suggested to him that the responses followed the accumulation of endogenously released acetylcholine resulting from inhibition of cholinesterase. De Candole, Douglas, Evans, Holmes, Spencer, Torrance & Wilson (1953) observed similar contractions in the guinea-pig tracheal muscle in response to the anticholinesterases sarin, dyflos or ethyl pyrophosphate. Daly (1957) showed that the bronchoconstrictor action of sarin or dyflos in the dog was antagonized by atropine but not by hexamethonium, and suggested that the action was distal to the parasympathetic ganglia and arose by accumulation of endogenous acetylcholine or by a direct action. It has been shown for the guinea-pig tracheal muscle by Foster (1963) that the organophosphorous inhibitor of cholinesterase, diisopropylphosphodiamidic fluoride (mipafox), in a concentration of 10 μg/ml., caused no change in inherent tone while markedly potentiating the actions of added acetylcholine. Carlyle reported to the British Pharmacological Society in 1962 that in the intact guinea-pig trachea mipafox markedly potentiated the action of acetylcholine added to the bath or the response to stimulation of the intrinsic parasympathetic nerves. After treatment of the intact trachea with mipafox a substance resembling acetylcholine was released in the absence of stimulation but no change in tone occurred during 20 min periods, although good contractions were obtained with either

neostigmine or physostigmine in 5 min. These observations suggested that the contractions in response to neostigmine or physostigmine were not solely a result of the ability of the drugs to inhibit cholinesterase.

The experiments described in this paper suggest that neostigmine and physostigmine contract the guinea-pig tracheal muscle by releasing acetylcholine from parasympathetic nerve endings probably by a direct stimulant action on these structures.

METHODS

Pairs of guinea-pigs of 0.5 to 1 kg body weight were killed by stunning and bleeding through the axilla. The tracheae were removed and a pair of matched tracheal chains, each containing six links, was prepared by the method described by Foster (1960). Each chain was suspended in a 12 ml. organ-bath containing Krebs solution at 37° C bubbled with 95% oxygen and 5% carbon dioxide. Contractions or relaxations were recorded with light balsa side-writing levers giving a magnification of 25-times with a load of 300 mg. Krebs solution of the following composition (g/l.) was used: NaCl 6.92, KCl 0.354, $CaCl_2$ 0.282, $NaHCO_3$ 2.1, KH_2PO_4 0.162, $MgSO_4.7H_2O$ 0.294 and glucose 2.0.

Antagonists, except hemicholinium, changes in ionic environment, or cooling were allowed to reach equilibrium with the test chain during 60 min before challenge with drugs; the control chain was untreated for the same length of time. The paired tracheal chain preparation thus allowed effective control of the experimental procedures. The time of contact between agonist and tissue was 5 min, with a total time of 15 min between doses for acetylcholine or histamine; for nicotine 40 min was necessary and 60 min for physostigmine or neostigmine.

Drugs used were diisopropylphosphodiamidic fluoride (mipafox), dyflos, acetylcholine chloride, histamine acid phosphate, physostigmine sulphate, neostigmine methyl sulphate, hyoscine hydrobromide, procaine hydrochloride, nicotine acid tartrate, mecamylamine hydrochloride, atropine sulphate, hexamethonium bromide and hemicholinium dibromide. All drug concentrations are expressed as final bath concentrations in μg/ml. of base.

RESULTS

The responses of the guinea-pig tracheal muscle to neostigmine and physostigmine. Concentrations of 0.1 μg/ml. and upwards of neostigmine or physostigmine gave slowly developing contractions. The latency in onset was longer than with acetylcholine or histamine. The contractions were maintained for up to 90 min and were reversed after washing. In these experiments concentrations of 2.2 μg/ml. of neostigmine and 3.3 μg/ml. of physostigmine were adopted since they give good responses in 5 min.

The effect of mipafox on the guinea-pig tracheal muscle and on the responses to neostigmine or physostigmine. Mipafox, in the high concentration of 100 μg/ml., did not cause the spastic response characteristic with physostigmine or neostigmine, as can be seen by comparison with the control chain (Fig. 1). But the responses to acetylcholine were greatly potentiated, the threshold dose after mipafox being 0.004 μg/ml. compared with the control value of 0.08 μg/ml. The sensitivity to histamine was unaffected, as also were the responses to neostigmine or physostigmine.

The effect of dyflos on responses to neostigmine or physostigmine. Dyflos (2 μg/ml.) gave a strong slowly developing contraction compared with an absence of response to 100 μg/ml. of mipafox, both drugs being left in contact for 90 min (Fig. 2). The response to dyflos was abolished with difficulty by repeated washing. The increased

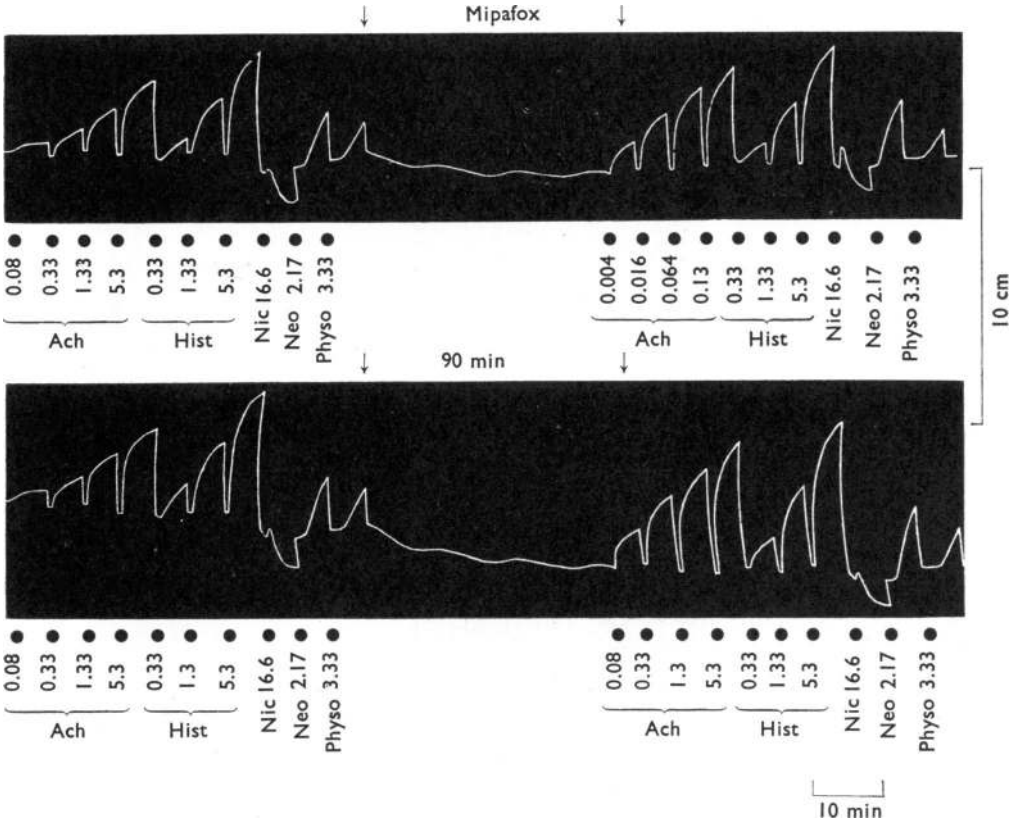


Fig. 1. Paired tracheal chains. The upper record is from the test chain, the lower is from the control chain. Mipafox (100 μg/ml.) in contact with the experimental chain for 90 min failed to cause a contraction. The responses to acetylcholine (Ach) were potentiated after washing out excess mipafox, those to histamine (Hist), neostigmine (Neo) and physostigmine (Physo) were unaffected and the motor response to nicotine (Nic) was increased. A slower drum speed was used between arrows. All drug concentrations are in μg/ml.

sensitivity to acetylcholine was the same after treatment with dyflos or mipafox, as was the stimulatory phase of the response to nicotine, but the responses to neostigmine, physostigmine or histamine were unaffected.

The action of hyoscine. Hyoscine (0.1 μg/ml.) abolished the responses to acetylcholine, neostigmine or physostigmine; it also abolished the stimulatory phase of the response to nicotine. The responses to histamine were not reduced; in fact they were sometimes potentiated, as shown in Fig. 3. Atropine had actions similar to hyoscine. Even in concentrations of 12.7 μg/ml. of neostigmine or 13.3 μg/ml. of physostigmine and with the period of contact extended to 240 min, no response was obtained in the presence of 0.1 μg/ml. of hyoscine.

The action of ganglion-blocking agents. The response to nicotine of the guinea-pig tracheal muscle, although variable, usually consisted of an initial contraction which

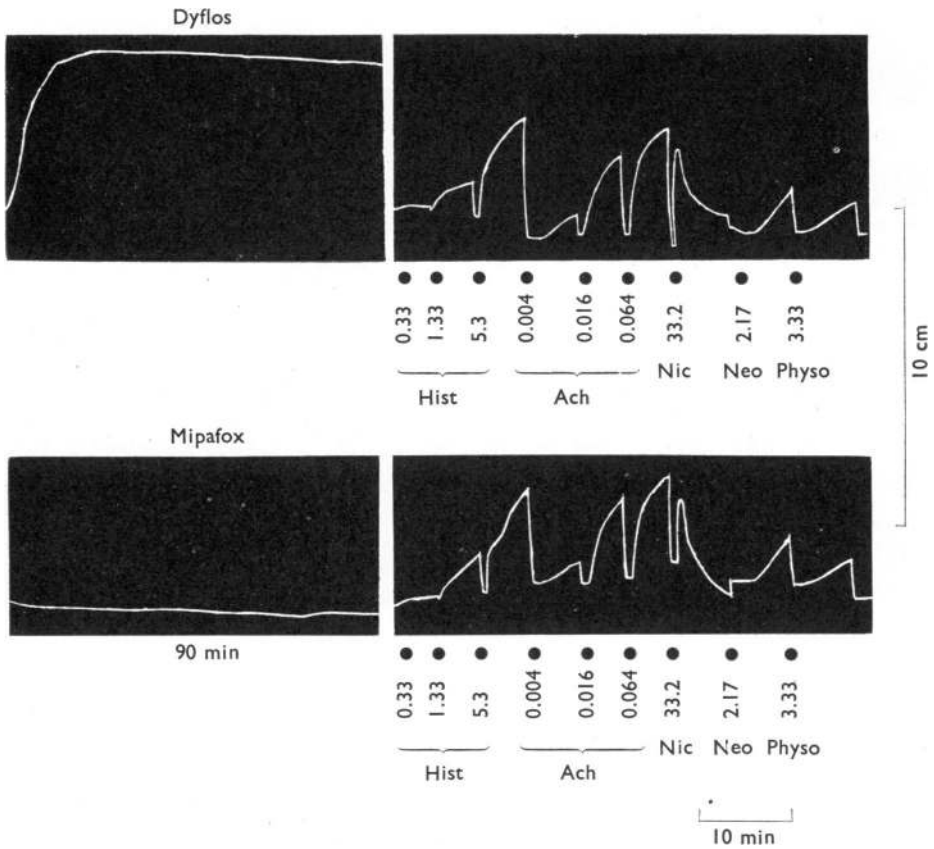
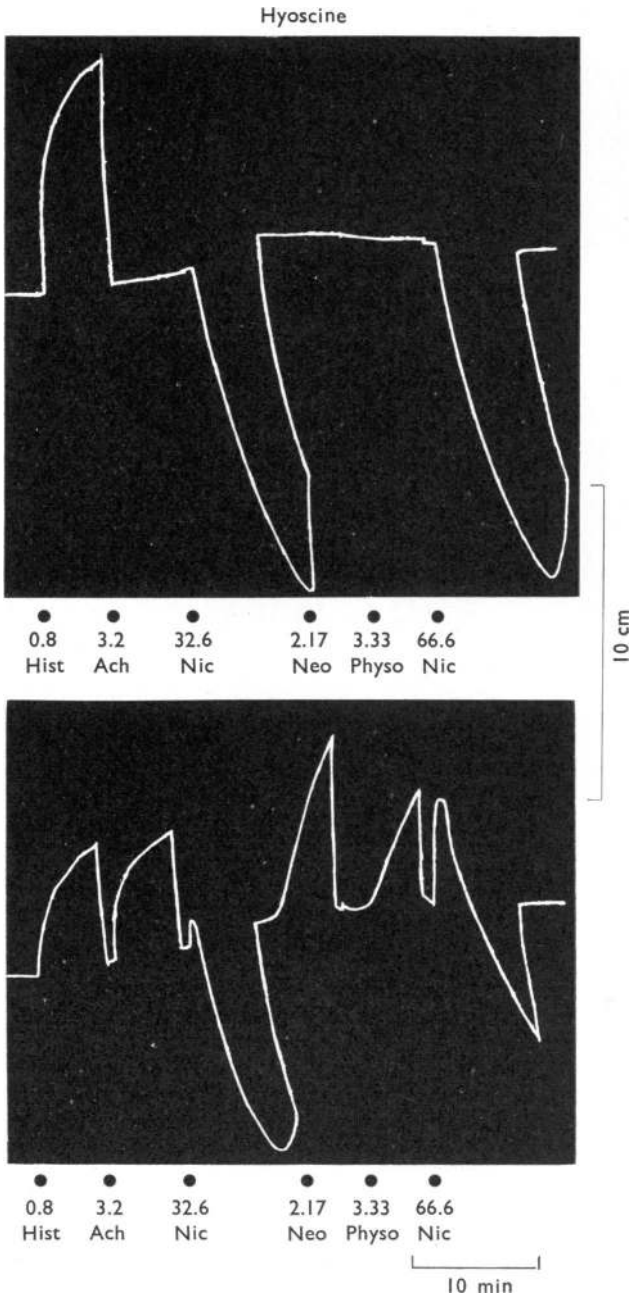


Fig. 2. Paired tracheal chains. The upper chain was exposed to dyflos (2 μg/ml.) and the lower to mipafox (100 μg/ml.) for 90 min (slow drum speed). Treatment with both these irreversible anticholinesterases sensitized the tracheal muscle to acetylcholine (Ach). The responses to histamine (Hist), neostigmine (Neo) and physostigmine (Physo) were unaffected. The motor response to nicotine (Nic) was enhanced.

was followed by relaxation (Fig. 4). Whether any part of the stimulatory action of physostigmine or neostigmine occurred at ganglia was challenged by the use of hexamethonium, mecamylamine or nicotine. Hexamethonium (50 μg/ml.) had no effect on the responses to neostigmine or physostigmine but blocked all the actions of nicotine. Fig. 4 shows the effect of a concentration of 100 μg/ml. of nicotine. Though this concentration competitively blocked the response to a massive dose of nicotine, it did not block the responses to neostigmine or physostigmine. After washing out the nicotine, the motor response to nicotine returned. Mecamylamine, a blocking agent thought to have a different mode of action to nicotine or hexamethonium, in a concentration of 5 μg/ml. also blocked the motor response to nicotine but not that to neostigmine or physostigmine (Fig. 4).



g. 3. Matched tracheal chains. Hyoscine (0.1 μg/ml.) abolished the responses to acetylcholine (Ach), those to histamine (Hist) being slightly potentiated. The motor response to nicotine (Nic) was abolished, as were the responses to neostigmine (Neo) and physostigmine (Physo).

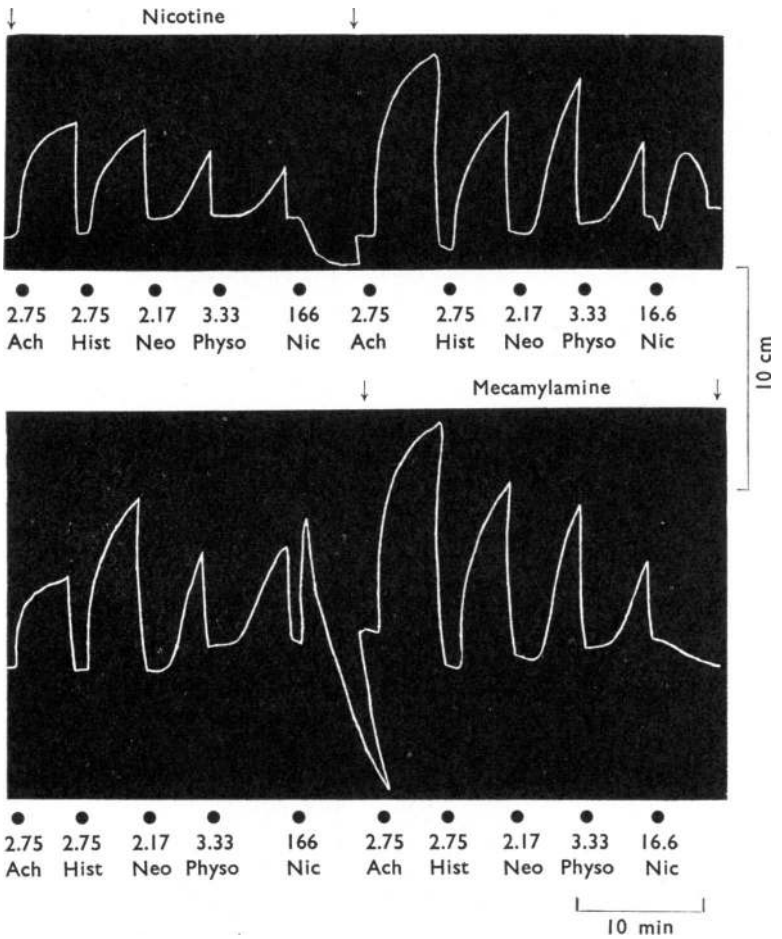


Fig. 4. Matched tracheal chains. A ganglion-blocking concentration of nicotine (100 μg/ml.) failed to abolish the responses to neostigmine (Neo) and physostigmine (Physo) during the period between the arrows in the upper record, the lower record being the control. The response to nicotine (Nic) was competitively blocked. The nicotine was subsequently washed out. A ganglion-blocking concentration of mecamylamine (5 μg/ml.) now failed to block the responses to neostigmine and physostigmine while blocking the motor response to nicotine during the period between the arrows in the lower trace, the upper trace now being the control. Hist= histamine; Ach=acetylcholine.

The action of procaine. Procaine (20 μg/ml.) abolished the responses to nicotine, neostigmine or physostigmine but left the sensitivity to acetylcholine or histamine unaffected (Fig. 5).

Cooling. Cooling the tracheal chains to 19° C depressed the responses to acetylcholine or histamine but abolished the responses to physostigmine and almost eliminated those to nicotine or neostigmine (Fig. 6).

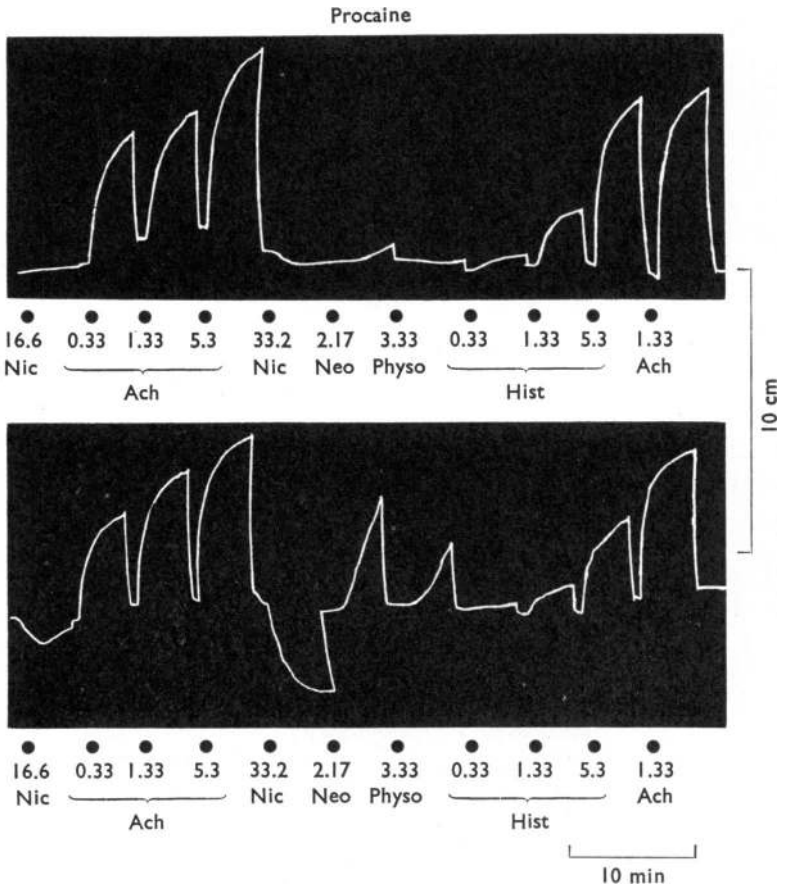


Fig. 5. Matched tracheal chains. Procaine (20 μg/ml.) abolished the responses to nicotine (Nic) and physostigmine (Physo), and almost abolished the response to neostigmine (Neo). The responses to histamine (Hist) and acetylcholine (Ach) were unaffected.

Low calcium and high magnesium concentrations in Krebs solution. Reducing the calcium ion concentration of Krebs solution to one-twentieth of normal greatly reduced the inherent tone of the tracheal muscle and almost abolished the responses to nicotine, neostigmine or physostigmine. In contrast the responses to acetylcholine or histamine were much enhanced (Fig. 7).

Raising the magnesium ion concentration to four-times the normal had no effect on motor responses induced by neostigmine, physostigmine or nicotine. Responses to acetylcholine or histamine were unaffected (Fig. 7).

Hemicholinium. Hemicholinium (123 μg/ml.) was left in contact with the tracheal chain for 2 hr, during which no contraction was seen. The preparation was then washed free from hemicholinium. Subsequently the responses to acetylcholine were unaffected but the responses to neostigmine or physostigmine were greatly reduced and the response to nicotine was abolished (Fig. 8).

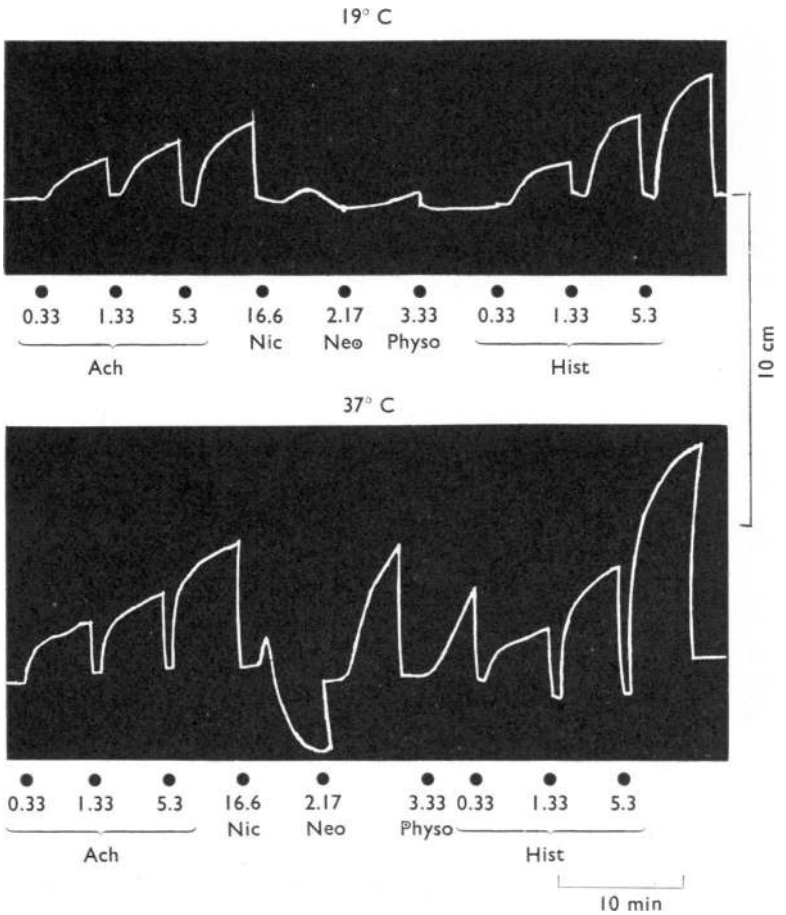


Fig. 6. Matched tracheal chains. Cooling to 19° C almost abolished the responses to nicotine (Nic), neostigmine (Neo) and physostigmine (Physo) while only reducing the responses to acetylcholine (Ach) and histamine (Hist).

DISCUSSION

When physostigmine or neostigmine was added to the fluid bathing the guinea-pig tracheal chain, a slowly developing contraction took place. If this effect is attributed solely to an anticholinesterase action of the added drugs, then this suggests a high level of acetylcholine release and destruction in the untreated preparation. But this is unlikely since, after cholinesterase inhibition by the organophosphorous compound mipafox, acetylcholine is released from the intact trachea in minute amounts, but causes no change in inherent tone. Also hyoscine or atropine do not relax the tracheal muscle in concentrations which abolish the action of acetylcholine added to the bath or which abolish the slow contractions produced by transmural stimulation of parasympathetic intrinsic nerves (Carlyle, unpublished). In contrast,

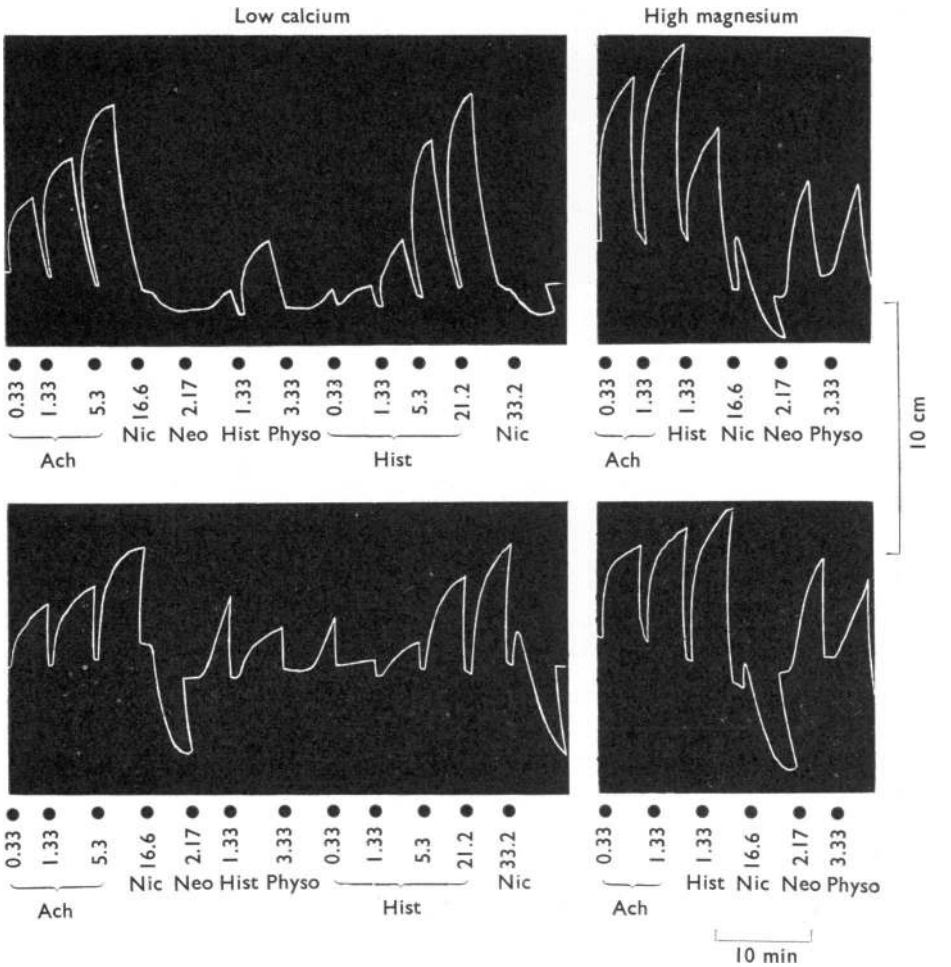


Fig. 7. Matched tracheal chains. One-twentieth normal concentration of calcium greatly reduced the response to neostigmine (Neo) and physostigmine (Physo) while potentiating the responses to acetylcholine (Ach) and histamine (Hist). Raising the magnesium ion concentration to four-times normal had no action on the responses to neostigmine or physostigmine. Nic= nicotine.

the responses to high concentrations of physostigmine or neostigmine left in contact for as long as 4 hr are completely blocked by atropine or hyoscine.

These experiments suggest that neostigmine and physostigmine act by a cholinergic mechanism and not by a direct action on the smooth muscle. In addition the stimulatory responses to physostigmine or neostigmine are not abolished by high concentrations of mipafox (100 μg/ml.) or dyflos (2 μg/ml.) which potentiate the responses to acetylcholine. Thus, the contraction produced by neostigmine or physostigmine is not solely due to their ability to inhibit cholinesterase.

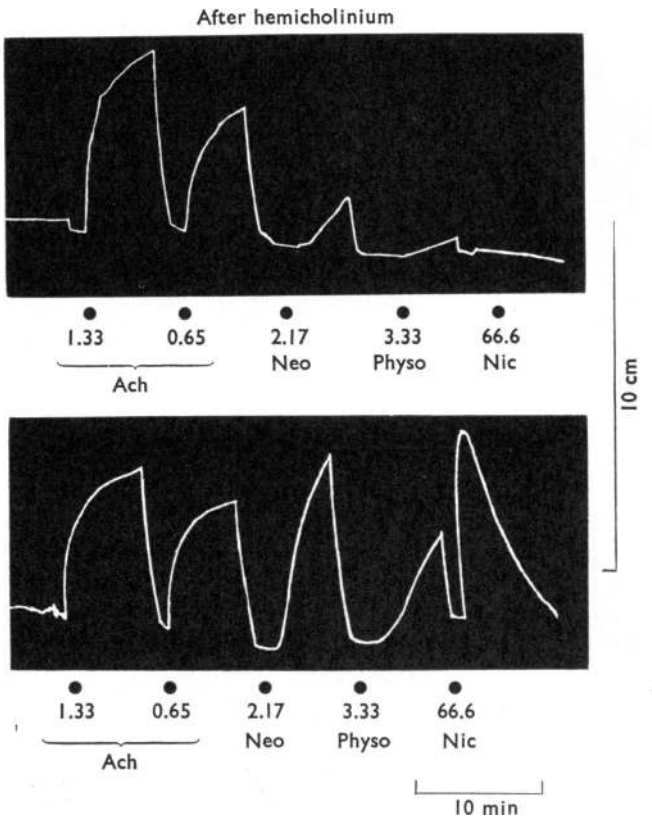


Fig. 8. Matched tracheal chains. Treatment of one tracheal chain (upper record) with 123 μg/ml. of hemicholinium for 120 min, after which it was washed, greatly reduced the responses to neostigmine (Neo) and physostigmine (Physo), blocked the actions of nicotine (Nic) but left the responses to acetylcholine (Ach) unaffected.

Is this stimulatory action on the trachea of ganglionic origin ? Paton & Hawkins (1958) showed that the response of the guinea-pig tracheal muscle to nicotine was both motor and inhibitory, demonstrating the presence of parasympathetic and sympathetic ganglia. But Akcasu (1959) could not obtain a response to nicotine. In the present experiments all preparations responded to nicotine, but the responses varied widely. Only stimulatory responses were seen with neostigmine and physostigmine; thus if a ganglionic action is involved it would have to be at parasympathetic ganglia only. This is unlikely. Moreover, the actions of neostigmine, physostigmine or dyflos at sympathetic ganglia have been demonstrated (Hilton, 1961; Long & Eckstein, 1961; Volle, 1962; Mason, 1962a, b). The failure to block the stimulatory response to physostigmine or neostigmine with hexamethonium, nicotine or mecamylamine supports the argument that ganglionic stimulation is not involved.

Consider next the potential sites of action distal to the parasympathetic ganglion. Feldberg & Lin (1949b) applied local anaesthetic drugs in experiments on the intestine

to block the actions of nicotine without depressing the smooth muscle or muscarinic receptors. In the present experiments procaine blocked the effects of nicotine, neostigmine or physostigmine, the responses to acetylcholine or histamine being unaffected. Ambache (1946) obtained a similar effect by cooling the guinea-pig ileum. Cooling the tracheal chain to 19° C abolished the responses to nicotine, neostigmine or physostigmine but only reduced the responses to acetylcholine or histamine. The selective blocking action of procaine, or of cooling, on the responses to neostigmine or physostigmine suggests an action on nervous structures and strengthens the evidence against a muscarinic or other direct action on the smooth muscle.

Reducing the calcium content of Krebs solution to one-twentieth of its normal value depressed the motor responses of the tracheal muscle to nicotine, neostigmine or physostigmine but potentiated the actions of acetylcholine or histamine. This evidence supports the hypothesis that the anticholinesterases act by releasing acetylcholine from a nervous structure. Increasing the concentration of magnesium ions depressed acetylcholine output at the neuromuscular junction (Straughan, 1959) and at a sympathetic ganglion (Hutter & Kostial, 1954). But a four-fold increase of the magnesium ion concentration in Krebs solution failed to abolish the motor response to nicotine, neostigmine or physostigmine. The smooth muscle cholinergic nerve endings are insensitive to this change of magnesium ion concentration and must differ from endings at nicotinic sites. MacIntosh, Birks & Sastry (1956), Gardiner (1957), Rietzel & Long (1959a, b), Wilson & Long (1959) and MacIntosh (1961) have shown that hemicholinium inhibits the synthesis and release of acetylcholine from nervous structures. In the present experiments hemicholinium did not stimulate the guinea-pig tracheal muscle, and lacked any muscarinic blocking action. Prior treatment with hemicholinium greatly reduced the response to neostigmine or physostigmine, and the response to nicotine was abolished. This supports the view that neostigmine and physostigmine act by the release of acetylcholine from cholinergic nerve endings of the tracheal muscle.

Some anticholinesterases may act on cholinergic nerve endings. Masland & Wigton (1940) showed that neostigmine stimulated antidromic impulses in motor nerves to skeletal muscles by an action on the nerve endings. Riker, Roberts, Standaert & Fujimori (1957), Riker, Roberts, Werner & Kuperman (1959), Werner (1960) and Hubbard & Schmidt (1961) have all stressed the importance of the presynaptic action of substances which include anticholinesterases on nerve terminals at the neuromuscular junction. Similarly dyflos, neostigmine and physostigmine have been shown to act on the preganglionic nerve terminals at a sympathetic ganglion (Volle, 1962; Takeshige & Volle, 1962, 1963). On smooth muscle the situation is less clear. Feldberg & Lin (1949a) used contractions of the guinea-pig ileum induced by physostigmine as a measure of endogenous acetylcholine release and assumed the action of physostigmine to be entirely one of cholinesterase inhibition. Harry (1962) noted that neostigmine and physostigmine each induced strong rhythmic contractions and relaxations of the guinea-pig ileum whereas mipafox did not. He considered that neostigmine and physostigmine possessed potent muscarinic activity, absent in mipafox. Salerno & Coon (1949) found that hexaethyl tetraphosphate,

dyflos, ethyl pyrophosphate, neostigmine and physostigmine each converted the pendulum movements of the rabbit gut to strong rhythmical contractions and relaxations resembling peristalsis. Atropine or procaine restored normal activity. These authors suggested that all these anticholinesterases acted by some neurogenic mechanism.

However, the interpretation of results obtained on the above preparations is made difficult by the possibility of large amounts of acetylcholine being released spontaneously by the intrinsic nerve terminals or non-nervous structures. This complication seems to be absent in the guinea-pig tracheal muscle. Beaver & Riker (1962) deduced that the ciliary nerve endings in the constrictor pupillae muscle in the isolated eye were probably not spontaneously releasing acetylcholine and suggested that the powerful miotic actions of dyflos, neostigmine and physostigmine were not due only to inhibition of cholinesterase. They suggest, by analogy with the neuromuscular junction, that these compounds may act by stimulating the parasympathetic nerve endings.

The experiments described here provide evidence that neostigmine and physostigmine contract the guinea-pig tracheal muscle by an action on postganglionic cholinergic nerve endings causing a release of acetylcholine. It follows that the amount of acetylcholine spontaneously released at rest from the guinea-pig tracheal muscle cannot be assessed by the response caused by physostigmine or neostigmine. This limitation may also apply to other isolated smooth muscle.

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