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The effects of eserine and neostigmine on the guinea-pig ileum and on ilial longitudinal muscle strips

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Eserine and neostigmine produced spasms of the guinea-pig isolated ileum that were concentration-related (4 ng–16 μg/ml). Hexamethonium (100 μg/ml) and tetrodotoxin (100 ng/ml) reduced the spasmogenic activity of both drugs. Neostigmine, but not eserine, overcame the hexamethonium antagonism. Pretreatment of the ileum with dyflos (1 μg/ml for 10 min) did not affect the action of either eserine or neostigmine, therefore the spasm was not due to inhibition of cholinesterase. The residual response to eserine or neostigmine after tetrodotoxin was abolished by hyoscine (4 ng/ml). Isolated longitudinal muscle strips were so prepared to be innervated or denervated. Both types of strip gave similar bradykinin maxima but the response of the denervated strip to eserine and neostigmine was reduced to 40% of the innervated strip and the remaining response was blocked by hyoscine (2 ng/ml). It is concluded that eserine and neostigmine have an indirect action on the smooth muscle of the ileum that is the result of acetylcholine release from cholinergic nerves. Inhibition of cholinesterase would not seem to be important for this action. A second component of the action of these two drugs appears to be a direct effect on the muscarinic receptors of the smooth muscle.

The recovery of acetylcholine released from the guinea-pig isolated ileum has been shown to be dependent on the cholinesterase inhibitor used to protect the released acetylcholine (Cox, Hecker & Weston, 1970). Eserine gave higher release rates, mipafox (*NN'*-di-isopropylphosphorodiamidic fluoride) gave lower apparent release rates. It was suggested that organophosphorus inhibitors may give little protection to endogenously released acetylcholine. It was also thought possible that the higher release rates with eserine could be related to some factor other than efficient acetylcholinesterase inhibition. It was noted that eserine at 10^{-5} g/ml caused spasm of the guinea-pig isolated ileum preparation and rendered it unresponsive to either added acetylcholine or to electrical stimulation. This concentration of eserine, has been routinely used in acetylcholine collection experiments (Schaumann, 1957; Ogura, Mori & Watanabe, 1966; Paton & Zar, 1968; Cox & others, 1970). It was possible therefore that the eserine was causing spasm by the release of acetylcholine from cholinergic nerves in the ileum. This is one possible explanation for the high release rates noted previously. We have investigated the mechanisms by which eserine causes spasm of the guinea-pig ileum. Neostigmine was also used.

MATERIALS AND METHODS

Guinea-pig isolated ileum preparation

Segments of non-terminal ileum, 2 cm in length, were suspended in Krebs solution maintained at 37 ± 1° and aerated with 5% carbon dioxide in oxygen. Tension

changes were recorded isometrically using an Ether strain gauge and a potentiometric recorder. The preparation was kept at a resting tension of 1 g throughout.

Isolated longitudinal muscle strip prepared from the guinea-pig ileum

Strips of longitudinal muscle were prepared according to the method of Ambache (1954) as modified by Paton & Zar (1965). A 20 cm length of ileum was removed from the guinea-pig, care being taken to preserve the mesentery. A 1 ml glass pipette of length 35 cm and diameter 0·7 cm was passed down the lumen of the ileum and fixed so that it could be rotated in a shallow bath containing Krebs solution. Incisions were made along the length of the ileum, one on each side of the mesentric attachment, and circumferentially near to one end of the ileum avoiding penetration of the inner circular muscle layer. A long strip of longitudinal muscle was then separated from the circular muscle by freeing it from the circular muscle. Two pieces of this strip were set up as described for the ileum. One piece was taken from the end nearest the horizontal incision. The second piece was taken from the other end. The end nearest the horizontal incision is reported to be innervated by the myenteric plexus and the end furthest from the incision is reported to be denervated (Paton & Zar, 1965). These strips will be referred to as "richly" and "poorly" innervated.

Drugs. Acetylcholine chloride, bradykinin, dyflos, eserine sulphate, hexamethonium bromide, hyoscine hydrobromide, neostigmine methylsulphate, nicotine hydrogen tartrate, tetrodotoxin. Drugs were freshly prepared in Krebs solution, all concentrations refer to final bath concentration of the salts.

RESULTS

Guinea-pig isolated ileum preparation

Effects of eserine and neostigmine. Both eserine and neostigmine produced dose-related increases in the tension of the ileum. The drug contact time was standardized at 60 s, a maximum tension response to any dose used was achieved within this time. With drug concentrations of 32 ng/ml or less, three changes of the bath fluid were sufficient to restore the tissue to its initial resting tension and a 3 min dose cycle was possible. Above 32 ng/ml more frequent washing and a longer dose cycle was needed to restore the resting tension. Log concentration-effect lines for eserine and neostigmine are shown in Fig. 1. None of the tissues tested responded to a drug concentration of 1 ng/ml, most responded at 4 ng/ml and the maximum response was usually at 1 μg/ml. Doses of 64 μg/ml or more of either drug produced smaller tension changes than those recorded with concentrations of 1 and 16 μg/ml. Concentration-effect lines on the ileum could be repeated without any significant change in the position of the line, provided 64 μg/ml was not exceeded.

The effect of hexamethonium, 100 μg/ml, on the concentration-effect lines to eserine and neostigmine (Fig. 1, IA and B). After a 15 min equilibration period hexamethonium produced a shift of the eserine line to the right. It was not possible to elicit a maximum contraction under these conditions as concentrations in excess of 64 μg/ml gave decreased responses in all tissues. The neostigmine line was also shifted to the right, but it was possible to produce a maximum tissue response in the presence of hexamethonium. Hexamethonium (100 μg/ml) reduced the effect of 4 μg/ml nicotine by 75%.

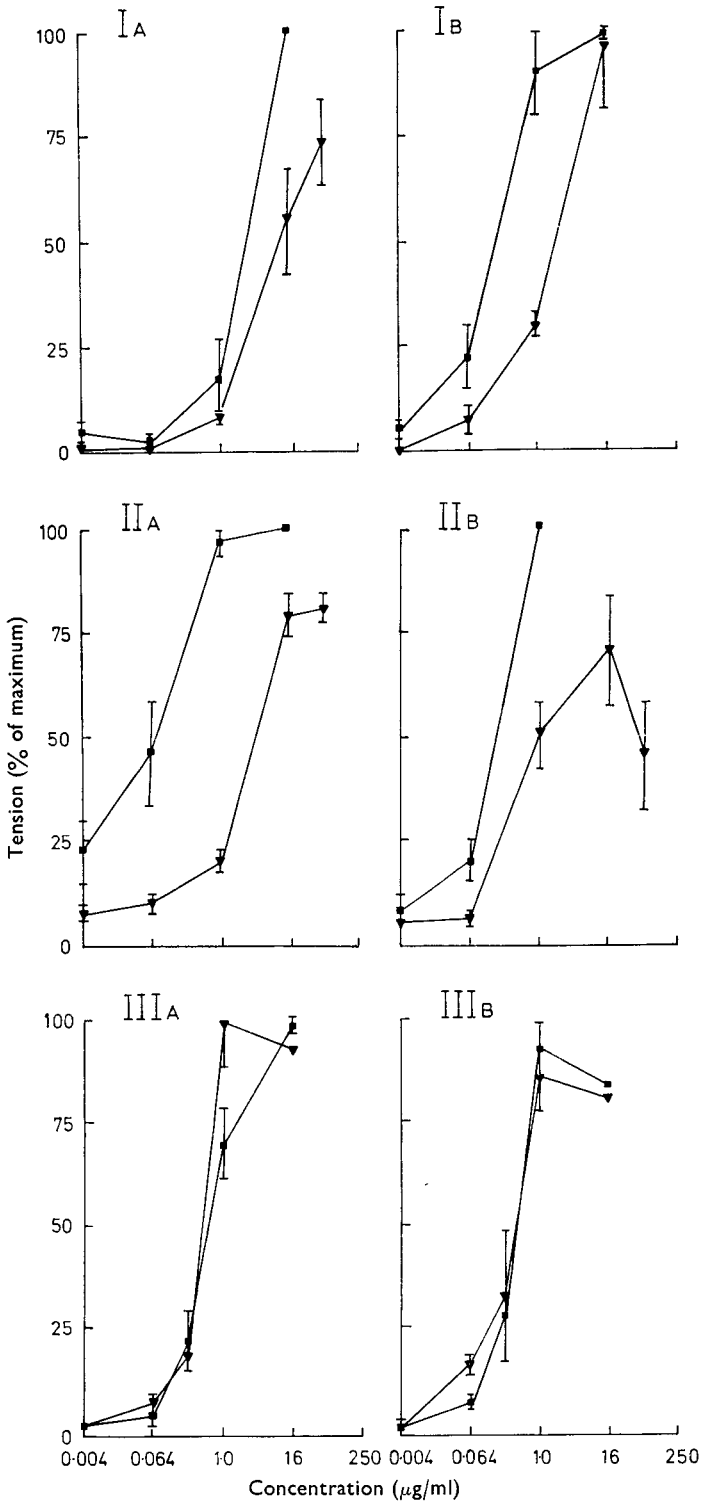

FIG. 1. Log-concentration effect curves for eserine (A) and neostigmine (B) on guinea-pig ileum before (■) and after (▼) I, hexamethonium (100 μg/ml); II, tetrodotoxin (100 ng/ml); III, dyflos (1·0 μg/ml). Each line represents the results from at least 4 separate experiments. Vertical bars are standard errors.

The effect of tetrodotoxin, 100 ng/ml, on concentration effect lines to eserine and neostigmine (Fig. 1, IIA and B). After a 5 min equilibration period tetrodotoxin produced a shift to the right of both the eserine and neostigmine lines. Eserine was shifted further at the ED50 point than was neostigmine. It was not possible to elicit a maximum contraction of the ileum with neostigmine in the presence of tetrodotoxin. The concentration of tetrodotoxin used completely abolished the response of the ileum to transmural stimulation (20–60 V, 0·3 ms pulse width), but did not alter the response to acetylcholine.

The effect of dyflos, 1 μg/ml, on the concentration effect lines to eserine and neostigmine (Fig. 1, IIIA and B). After 10 min equilibration there was no change in the position of these lines. Dyflos, 1 μg/ml, did not alter the response of the ileum to bradykinin, but higher concentrations (10 or 100 μg/ml) depressed the response. The effect of 1 μg/ml dyflos on the cholinesterase of guinea-pig ileum was tested by a titrimetric method (Alles & Hawes, 1940). The rate of hydrolysis of acetylcholine was determined for an untreated homogenate of guinea-pig ileum and for a homogenate preincubated with dyflos for varying times. A 100% inhibition of the hydrolysis occurred with preincubation times of 10 min or longer.

Effect of tetrodotoxin and dyflos on the response to eserine and neostigmine. In this experiment the ileum was preincubated with both dyflos (1 μg/ml) and tetrodotoxin (100 ng/ml). Shifts in the concentration effect lines were similar to those obtained when tetrodotoxin was used on its own. Hyoscine, 4 ng/ml, completely inhibited the effects of eserine and neostigmine over the range 4 ng/ml to 64 μg/ml.

Isolated longitudinal muscle strip preparation

In all experiments "richly" and "poorly" innervated strips were set up side by side, under the same experimental conditions. Before a pair of strips were accepted for use, it was established that both showed similar tension changes when exposed to a standard concentration (200 ng/ml) of bradykinin, that the "richly" innervated strip responded strongly to nicotine (4 μg/ml) but that the "poorly" innervated strip did not.

Eserine and neostigmine produced dose related increases in tension of both "richly" and "poorly" innervated isolated longitudinal muscle strip preparations. An example of log concentration effect lines for these two drugs are shown in Fig. 2A and B. The maximum response of the "poorly" innervated strip was only 40%

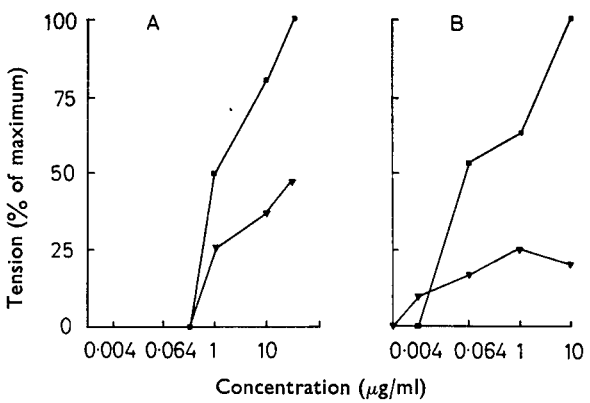

FIG. 2. Log-concentration effect curves for eserine (A) and neostigmine (B) on the longitudinal muscle strip preparation prepared so as to be either "richly" (■) or "poorly" innervated (▼).

of that obtained with the "richly" innervated strip. Two pairs of strips were pre-incubated with dyflos 1 μg/ml for 10 min and then the effects of eserine and neostigmine tested. The responses to these drugs were not affected by this pre-treatment. On the "richly" innervated strip, hyoscine (2 ng/ml) reduced the maximum response to both eserine and neostigmine by 90%, while on the "poorly" innervated strip the responses were completely inhibited.

DISCUSSION

There have been previous reports that eserine has actions other than those of inhibition of the cholinesterase enzymes. Bell (1966), found that eserine (10 and 100 μg/ml) produced an increase in tone of the innervated toad bladder by releasing acetylcholine from cholinergic nerve endings. A similar mechanism of action for eserine-induced spasm of guinea-pig trachealis muscle has been proposed by Carlyle (1963). In spite of these reports eserine has been routinely employed to protect the acetylcholine released from innervated tissues in concentrations known to have other actions as well as causing acetylcholinesterase inhibition (see introduction). The non-specificity of neostigmine seems to have been more widely appreciated and this compound has not usually been used in acetylcholine collection experiments. Neostigmine stimulates autonomic sympathetic ganglia (Mason, 1962a, b) and acts in a similar way to eserine on the guinea-pig trachealis muscle (Carlyle, 1963).

In most of the reported studies with eserine and neostigmine, only one or two doses of the drugs have been used. Therefore we decided to investigate more fully the pharmacological actions of these two drugs by studying concentration-effect relations on the guinea-pig isolated ileum since this has been often used for acetylcholine collection experiments.

We found that the ileum did not respond to transmural electrical stimulation in the presence of eserine 10 μg/ml. A similar observation has been made for the guinea-pig colon (Beani, Bianchi & Crema, 1969). These workers also noted that whilst hexamethonium (50–200 μg/ml), did not modify eserine-induced spasm of the colon, it did significantly reduce its acetylcholine output measured in the presence of eserine. These results could imply that eserine acts on autonomic ganglia or pre-ganglionic nerve endings causing acetylcholine release. Supportive evidence for an action of eserine at ganglia comes from our own findings that hexamethonium antagonizes an eserine-induced spasm of the ileum. Tetrodotoxin antagonized the action of eserine more than did hexamethonium. This suggests that a part of the eserine spasm might be a nerve-action potential mediated effect, not necessarily involving stimulation of nicotinic receptors. The response to neostigmine, on the other hand, was blocked to the same extent by hexamethonium and by tetrodotoxin, suggesting that the indirect component of this spasm is primarily due to stimulation of nicotinic sites. That neostigmine can overcome the hexamethonium blockade points to it being more potent than eserine at these sites.

The experiments with hexamethonium and tetrodotoxin do not rule out that an indirect component in the action of eserine and neostigmine is cholinesterase inhibition. Thus an action at nicotinic sites could be due to potentiation of pre-synaptically-released acetylcholine. However, the experiments with dyflos suggest that this is not so as it was used in a concentration that completely inhibited the ability of ileum homogenate to hydrolyse acetylcholine, but it did not modify either the eserine or the neostigmine dose-response lines. Further, the experiments in

which the ileum was pretreated with both dyflos and tetrodotoxin show that when nerve action potentials are inhibited the residual spasm is not due to inhibition of acetylcholinesterase at the neuroeffector junctions. It would seem therefore that part of the eserine and neostigmine spasm is neuronally mediated, by a mechanism not involving inhibition of cholinesterase.

The experiments on the ileum with hyoscine indicate that the spasm remaining after loss of the nerve action potential-mediated component is due to an action of eserine and neostigmine on muscarinic receptors. This could be either a direct action on muscarinic receptors or release of acetylcholine from nerves by a mechanism not involving a nerve action potential. Therefore, longitudinal muscle strips were prepared which were either "richly" or "poorly" innervated. The lack of effective innervation in the poorly innervated strips was demonstrated by the absence of a response to nicotine. Such strips gave a contraction only 40% of that of the "richly" innervated strips—further evidence of the neuronal component. The residual contraction of the denervated strip produced by either eserine or neostigmine was antagonized by hyoscine indicating their direct action on muscarinic receptors.

In conclusion therefore, both eserine and neostigmine can cause spasm of the guinea-pig ileum by an indirect action involving acetylcholine release, and this could explain the high values obtained for the so called "resting" rate of acetylcholine release measured in the presence of eserine. Experiments which purport to measure the effect of drugs or other agents on acetylcholine release rates in this situation, may in fact be actually measuring a drug interaction, one of the variables being an eserine-induced acetylcholine release. It is obvious that caution must be exercised in interpretation of this type of result. However a satisfactory alternative experimental design is not easy to find. In some experiments (Bolton, 1969), low concentrations of eserine (10 ng/ml) have been used in an attempt to ensure specificity for the cholinesterase system. Our results show that this concentration has little effect on innervated smooth muscle preparations, but it is by no means certain that efficient protection of released acetylcholine has been obtained. The use of organophosphrus inhibitors would not appear to be a satisfactory alternative as it is also unlikely that they give adequate protection to neuronally released acetylcholine (Cox & others, 1970).

REFERENCES

ALLES, G. A. & HOWES, R. C. (1940). *J. biol. Chem.*, **133**, 375–390.
AMBACHE, N. (1954). *J. Physiol., Lond.*, **125**, 53–55P.
BEANI, L., BIANCHI, C. & CREMA, A. (1969). *Br. J. Pharmac.*, **36**, 1–17.
BELL, C. (1966). *Biochem. Pharmac.*, **15**, 1085–1092.
BOLTON, T. B. (1969). *Br. J. Pharmac.*, **35**, 112–120.
CARLYLE, R. F. (1963). *Ibid.*, **21**, 137–149.
COX, B., HECKER, S. E. & WESTON, A. H (1970). *J. Pharm. Pharmac.*, **22**, 231–232.
MASON, D. F. J. (1962a). *Br. J. Pharmac. Chemother.*, **18**, 76–86.
MASON, D. F. J. (1962b). *Ibid.*, **18**, 572–587.
OGURA, Y., MORI, Y. & WATANABE, Y. (1966). *J. Pharmac. exp. Ther.*, **154**, 456–462.
PATON, W. D. M. & ZAR, M. A. (1965). *J. Physiol., Lond.*, **179**, 85–86P.
PATON, W. D. M. & ZAR, M. A. (1968). *Ibid.*, **194**, 13–33.
SCHAUMANN, W. (1957). *Br. J. Pharmac. Chemother.*, **12**, 115–118.