

THE ACTION OF PHYSOSTIGMINE AND THE DISTRIBUTION OF CHOLINESTERASES IN THE CHICKEN OESOPHAGUS

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Physostigmine produces a contraction of the muscularis mucosae but not of the external muscle of the isolated guinea-pig oesophagus. The oesophagus of guinea-pigs contains a striped external muscle innervated by vagal fibres which are not blocked by hexamethonium, and a muscularis mucosae consisting of plain muscle innervated by post-ganglionic nerves. This led to the suggestion that contractions produced by physostigmine in the isolated guinea-pig oesophagus involved only post-ganglionic nerves or the muscularis mucosae (Bartlet, 1968a, b). The chicken oesophagus has a plain external muscle and muscularis mucosae (Calhoun, 1933, 1954), and in the present experiments physostigmine increased the tone of both the external muscle and the muscularis mucosae. The possibility that physostigmine acted through non-neuronal structures has been investigated by testing its effect in the presence of cocaine. In addition the histochemical localization of cholinesterases was investigated to depict the site of action of physostigmine.

METHODS

Isolated organs

Preparations of external muscle, muscularis mucosae, oesophagus and oesophagus with nerve attached were made as described by Bartlet & Hassan (1968). The terms "external muscle" and "muscularis mucosae" in the text, table and figures refer to the separated oesophageal layers, and the term "oesophagus" refers to the whole organ. The isolated tissues had open ends and were set up for the recording of contractions in a 40 ml. organ bath filled with the saline-bicarbonate solution of Krebs & Henseleit (1932) with the Ca^{++} halved, gassed with 5% carbon dioxide in oxygen and maintained at 35° C. The preparations were attached to an isotonic frontal writing lever which exerted a force of 2 g cm, and the nerves were drawn through an electrode similar to that described by Burn & Rand (1960). The nerve was stimulated for 5-30 sec every 5 min or for 1.5 min every 10 min with square pulses of 10 msec duration, a frequency of 20 stimuli/sec and a voltage adjusted to produce a submaximal or a maximal contraction.

The drugs used were acetylcholine chloride, cocaine hydrochloride, physostigmine salicylate and hyoscine hydrobromide. Quantities of drugs in the text, table and figures refer to the salts.

Histochemistry

Pieces of oesophagus were fixed at 4° C for 4 hr in an isotonic solution of sodium sulphate containing 3.6% v/v formaldehyde. After rinsing the tissue with distilled water, it was transferred to 20% v/v ethanol and kept at 4° C for at least 15 hr and sometimes as long as a week. Sections were cut at a thickness of

40 μ on a freezing microtome. The freshly cut sections were stained for acetylcholinesterase or butyrylcholinesterase by the thiocholine method (Krnjević & Silver, 1965), with incubation in the substrate containing solution being carried out at a pH of 5.4 for 5 hr. Some sections were placed in a solution of physostigmine salicylate (4 $\mu\text{g}/\text{ml}$.) or diisopropyl phosphorofluoridate (DFP) (10 $\mu\text{g}/\text{ml}$.) for 30 min before being incubated in a medium which contained in addition to the substrate the inhibitor at the concentration mentioned. Incubation of sections with butyrylthiocholine produced staining at sites of butyrylcholinesterase activity only, whereas when acetylthiocholine was used as substrate staining was produced at sites of acetylcholinesterase and butyrylcholinesterase activities. DFP (10 $\mu\text{g}/\text{ml}$.) inhibited the activity of butyrylcholinesterase only, and physostigmine salicylate (4 $\mu\text{g}/\text{ml}$.) inhibited both acetylcholinesterase and butyrylcholinesterase activities.

RESULTS

Effect of cocaine on the contractions produced by vagal stimulation, acetylcholine and physostigmine

Exposure of the oesophagus to cocaine (50 $\mu\text{g}/\text{ml}$.) for 20–30 min abolished the contractions produced by submaximal or supramaximal vagal stimulation (seven experiments), and potentiated the contraction of the oesophagus to acetylcholine added to the organ bath.

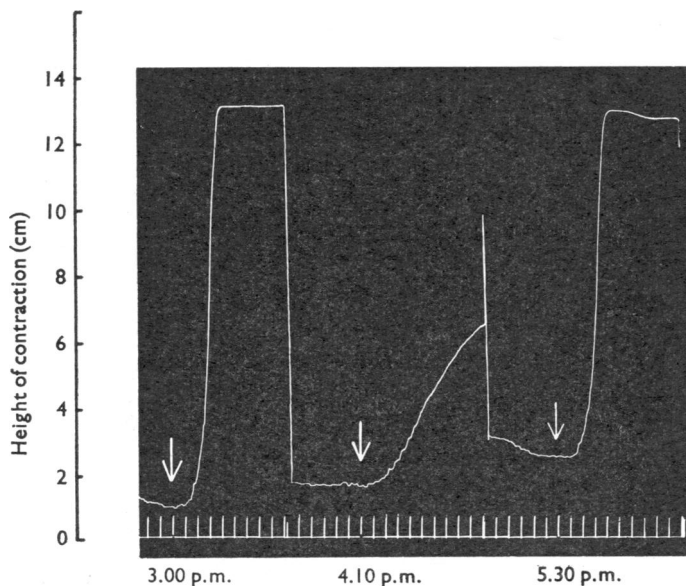


Fig. 1. Contractions of the isolated muscularis mucosae. The arrows mark the addition of physostigmine salicylate (5 $\mu\text{g}/\text{ml}$.) to the organ bath at the times shown. After exposure of the preparation to physostigmine for 5 min the drum was stopped and the drug washed from the organ bath. Cocaine hydrochloride (50 $\mu\text{g}/\text{ml}$.) was present in the organ bath from 3.15 p.m. to 4.15 p.m. Time, 30 sec.

Physostigmine (5 $\mu\text{g}/\text{ml}$.) always produced contractions of the whole oesophagus and external muscle and muscularis mucosae preparations. The preparations contracted after addition of physostigmine to the organ bath after a delay of 0.5–3.5 min, and relaxed only slowly after washing the drug out of the organ bath. Preparations were exposed to

physostigmine long enough to produce a suitable height of contraction—1–3 min in the case of the oesophagus and 5 min in the case of the external muscle or muscularis mucosae preparations. The interval between tests with physostigmine was about one hour and in these conditions a constant response to a given concentration of physostigmine was obtained. Hyoscine (100 ng/ml.) abolished the responses to physostigmine, and exposure to cocaine (50 µg/ml.) for 30–60 min also antagonized it (Fig. 1 and Table 1). The antagonism of physostigmine by cocaine was greater on the oesophagus than on the external muscle or muscularis mucosae preparations ($P < 0.01$).

TABLE 1
EFFECT OF COCAINE ON CONTRACTIONS PRODUCED BY PHYSOSTIGMINE

	Residual contractions to physostigmine salicylate (5 µg/ml.) in the presence of cocaine hydrochloride (50 µg/ml.). The results are expressed as % controls. Mean values ± s.e. (No. expts.)	<i>P</i> (antagonism by cocaine)
Oesophagus	8.8 ± 2.8 (4)	< 0.001
External muscle	38.3 ± 2.8 (7)	< 0.001
Muscularis mucosae	44.0 ± 5.7 (5)	< 0.001



Fig. 2. Transverse section of post-crop oesophagus (×75). Black staining depicts butyrylcholinesterase activity. Dense staining was present in the muscularis mucosae (M) and mucosal glands (Gl).

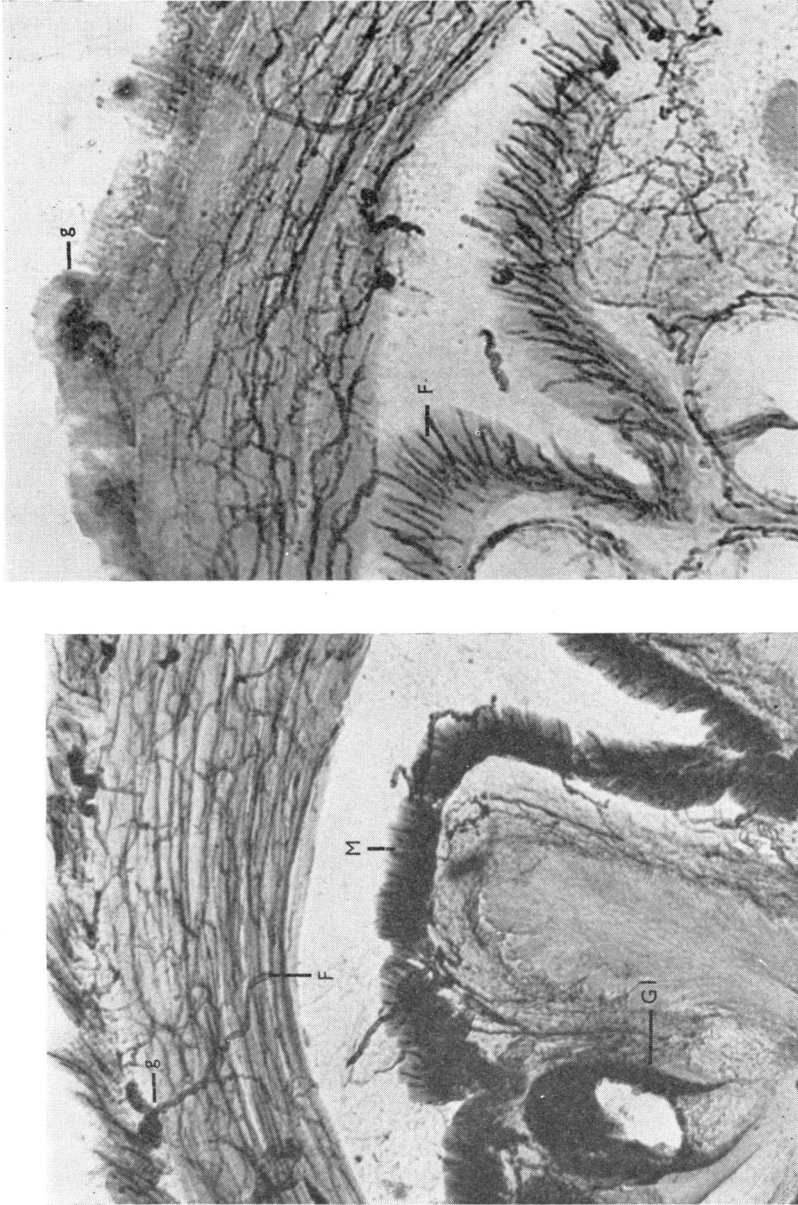


Fig. 3. Transverse sections of post-crop oesophagus incubated with (a) acetylthiocholine and (b) acetylthiocholine and DFP (10 µg/ml.) ($\times 100$). Staining depicts cholinesterase activity in ganglia (g), nerve fibres (F), muscularis mucosae (M) and mucosal glands (Gl). In (a), acetylcholinesterase and butyrylcholinesterase contribute to the stain, and in (b) acetylcholinesterase only is responsible for it.

Cholinesterase staining

Sections made from different parts of the oesophagus exhibited a similar pattern of staining for cholinesterases, and in sections incubated in the presence of physostigmine staining was absent.

In sections incubated with butyrylthiocholine, butyrylcholinesterase activity was demonstrated in the muscularis mucosae and mucosal glands. The stain was usually black (Fig. 2), but in some sections the staining was lighter and brown in appearance. The external longitudinal muscle was usually stained faintly but the circular muscle, ganglia and nerve fibres were not stained. Butyrylcholinesterase activity, and thus staining, was inhibited by pre-treatment of the sections with DFP (10 $\mu\text{g/ml}$).

In sections incubated with acetylthiocholine and DFP (10 $\mu\text{g/ml}$) acetylcholinesterase activity was demonstrated in ganglia and nerve fibres (Fig. 3b). In sections incubated with acetylthiocholine alone, however, some additional staining attributable to butyrylcholinesterase was found in the muscularis mucosae and mucosal glands, and in these sections the staining attributable to mucosal acetylcholinesterase was masked by butyrylcholinesterase being present at the same site (Fig. 3a). Acetylcholinesterase activity was found in ganglia between the external longitudinal and circular muscle layers, in a few ganglia in the muscularis mucosae and in nervous networks associated with the external muscle, the muscularis mucosae and the mucosal glands.

DISCUSSION

The contraction of the isolated chicken oesophagus produced by physostigmine was blocked by hyoscine, and cocaine, in a concentration sufficient to block vagal stimulation, also antagonized it. This suggests that when physostigmine inhibits cholinesterases, an acetylcholine-like substance accumulates and stimulates neural structures. Cocaine reduced the height of the physostigmine-induced contraction of the whole oesophagus to a greater extent than the contractions of the external muscle and muscularis mucosae preparations. This suggests that separation of the external muscle from the mucosa damaged neural structures stimulated by acetylcholine when cholinesterases are inhibited.

The small residual contraction obtained with physostigmine in the presence of cocaine was blocked by hyoscine, which suggests that an acetylcholine-like substance was produced. Such an acetylcholine-like substance could have originated at nerve terminals or at a non-neural structure. According to Cuthbert (1963), an acetylcholine-like substance is present in the nerve-free plain muscle of the chick amnion.

A high level of butyrylcholinesterase activity was found in the muscularis mucosae and mucosal glands and not in the external muscle. Because physostigmine produced similar contractions of anaesthetized preparations of the external muscle and muscularis mucosae, butyrylcholinesterase does not seem to play any part in the metabolism of endogenous acetylcholine. Staining in sections incubated with acetylthiocholine depicted the location of acetylcholinesterase and butyrylcholinesterase, but the activity of the latter enzyme can be selectively inhibited by DFP (10 $\mu\text{g/ml}$). In sections incubated with acetylthiocholine and DFP (10 $\mu\text{g/ml}$) acetylcholinesterase activity was confined to neural structures. Thus the distribution of acetylcholinesterase does not support the

hypothesis that physostigmine causes an accumulation of acetylcholine at a non-neural structure, and we assume that in the chick oesophageal preparations small amounts of acetylcholine were released from anaesthetized nerves.

SUMMARY

1. Physostigmine salicylate (5 μ g/ml.) produced contractions of the isolated whole oesophagus, and external muscle and muscularis mucosae preparations. These contractions were blocked by hyoscine.

2. In the presence of cocaine, at a concentration sufficient to block the effect of vagal stimulation, the contractions produced by physostigmine became smaller whereas those produced by acetylcholine were potentiated.

3. Acetylcholinesterase activity was demonstrated histochemically in ganglia and nerve fibres. Butyrylcholinesterase activity was found only in the muscle and glands of the mucosa.

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