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A pharmacological study of oesophageal muscularis mucosae from the cat, dog and American opossum (*Didelphis virginiana*)

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1 Strips of muscularis mucosae from the oesophagi of cat, dog and opossum have been studied to determine their responses to drugs and to electrical field stimulation.

2 All tissues were contracted by acetylcholine, histamine and, with the exception of strips of muscularis mucosae from the opossum proximal oesophagus, noradrenaline. The effects of acetylcholine and histamine were competitively antagonized by atropine (50 nM) and mepyramine (50 nM) and were abolished by atropine (1 μM) and mepyramine (1 μM) respectively. Contractile responses to noradrenaline were competitively antagonized by phentolamine (50 nM) but were converted to propranolol (50 nM)-sensitive relaxations by phentolamine (1 μM). Relaxations were abolished by propranolol (1 μM).

3 Cholecystokinin octapeptide, gastrin 1 and vasoactive intestinal polypeptide were ineffective on any of the tissues examined. Substance P caused contractions in tissue from all three species. These effects were atropine and tetrodotoxin insensitive.

4 All tissues gave atropine (50 nM)- and tetrodotoxin (100 nM)-sensitive contractions in response to electrical field stimulation. Contractions were not followed by relaxations and spontaneous mechanical activity was not suppressed between periods of stimulation. No evidence was obtained for the presence of non-adrenergic, non-cholinergic inhibitory innervation of the oesophageal muscularis mucosae in any species.

5 During electrical field stimulation noradrenaline always reduced the amplitude of evoked contractions and, with the exception of tissue from proximal opossum oesophagus, increased resting tension.

6 In opossum distal oesophageal muscularis mucosae, the effects of noradrenaline during electrical field stimulation were abolished by a 30 min pretreatment of the tissue with phentolamine (1 μM) and propranolol (1 μM). To achieve this in all other tissues, it was also necessary to use yohimbine (1 μM).

7 In all tissues where noradrenaline caused a phentolamine (1 μM)-and propranolol (1 μM)-resistant depression of electrically evoked responses, clonidine produced a yohimbine (1 μM)-sensitive depression.

8 Evidence was obtained for the presence of excitatory α_1-and inhibitory α_2- and β-adrenoceptors. Inter-species differences in their distribution are discussed.

Introduction

The role of the muscularis mucosae throughout the gastrointestinal tract is essentially unknown. If its function is to be understood it is first necessary to determine the nature and regulation of its movements. For this reason we set out to examine by pharmacological methods the controls of spontaneous and evoked contractions in one example of this tissue.

Although the muscularis mucosae of the stomach, small and large intestine have, over the years, been the subject of pharmacological investigation (Magnus, 1904: Gunn & Underhill, 1914: King & Church,

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1923: King & Robinson, 1945: King *et al.*, 1947: Walder, 1953: Onori *et al.*, 1971: Gallacher *et al.*, 1973: Angel *et al.*, 1982) little is known of the pharmacology of oesophageal muscularis mucosae.

Early studies by Hughes (1955; 1957) showed that oesophageal muscularis mucosae from the cat, rabbit, rat and human foetus were contracted by acetylcholine and pilocarpine and, with the exception of human foetal tissue were, depending on resting tone, relaxed by adrenaline. More recently, Kamikawa & Shimo (1979), Okhawa (1980) and Kamikawa *et al.* (1982) have examined the responses of the guinea-pig isolated oesophageal muscularis mucosae to electrical stimulation and to drugs. From this work it appears that the muscularis mucosae of the guinea-pig oesophagus is pharmacologically very similar to the longitudinal muscle of the ileum in that species. These similarities include the presence of an excitatory cholinergic innervation which responds to low frequency electrical stimulation (0.1 Hz) the function of which is depressed by atropine, tetrodotoxin and catecholamines.

The innervation, physiological function and pharmacological features of the muscularis mucosae of the oesophagus in non-rodent species are not known and it seemed appropriate, therefore, to study this tissue in animals that are neither rodents nor herbivores. Furthermore, anatomical differences exist in the arrangement (Oppel, 1896) and pharmacological characteristics (Hughes, 1955; 1957) of the oesophageal muscularis mucosae between species. For these reasons, the cat, dog (both carnivores) and North American opossum (*Didelphis virginiana*) (an omnivorous marsupial) were chosen. The present study was designed to investigate the types of receptors present in the oesophageal muscularis mucosae, the transmitters released from the intrinsic innervation and the interplay of these during electrically evoked responses.

Methods

Cats (31) and opossums (43) (2–4 kg) of either sex were anaesthetized by intraperitoneal injection of pentobarbitone (30 mg kg^{-1}). The abdomen and thorax were opened and an 8 cm length of oesophagus including the lower oesophageal sphincter (L.E.S.) was removed. Dogs (17) (18–20 kg) of either sex were anaesthetized by slow intravenous administration of pentobarbitone (25 mg kg^{-1}); 10 cm of oesophagus and the L.E.S were then removed.

Once removed, segments of oesophagus were placed in Krebs solution at 37 ± 1°C and adherent connective tissue was removed from the serosal surface. In each case the oesophagus was opened along the line of the lesser curvature of the stomach and pinned out flat (mucosal surface uppermost) on a Sylgard (Dow Corning Co.)-covered platform in Krebs solution at 37 ± 1°C.

For dog and opossum, strips of mucosa, muscularis mucosae and submucosa 4 to 5 cm long and 4 mm wide were cut by pulling on the mucosal surface and cutting the submucosal connection to the underlying circular muscle. Strips were cut in an oral direction starting either a few millimetres or 3.5 cm above the L.E.S. These strips are designated as distal and proximal, respectively. In the case of the cat, it was necessary to cut longitudinal sections of the desired size from the mucosal to the serosal surface, invert them and then remove the longitudinal and circular muscle from the submucosa. If this procedure was not observed the submucosal plexus tended to be damaged during removal. Since the presence of mucosal epithelium did not seem to interfere with the ability of the tissue to respond to electrical stimulation and to drugs, it was not removed.

Strips were tied in the middle with 4–0 gauge surgical thread and folded (mucosal surface inwards) to be half their original length. The oral and aboral ends, now side by side, were tied together and the entire preparation was mounted at 37 ± 0.5°C in a 10 ml organ bath under either the equivalent of 1 to 2 g tension (isometric recording) or a 2 g load (isotonic recording). Isometric responses were measured with Grass FTO3C transducers, isotonic responses with Harvard isotonic transducers. In each case, responses were displayed on a Beckman RM dynograph recorder.

Electrical stimuli from a Grass S88 stimulator were delivered through two Ag/AgCl electrodes mounted on the inside wall of the organ bath. The stimulus parameters used were, for the cat and the opossum, 5s trains of pulses at 20 Hz, 1 to 2 ms duration, 115 to 125 mA given once every 2 min; in the case of the dog, optimum responses were obtained at a stimulus frequency of 50 Hz.

A Krebs solution of the following composition (mM) was used throughout: NaCl 118.5, KCl 4.75, $CaCl_2$ 2.54, NaH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 25, glucose 11, choline chloride 0.02, gassed with a mixture of 95% O_2 and 5% CO_2.

Drugs

The following drugs were used, acetylcholine chloride, atropine sulphate, cholecystokinin octapeptide, clonidine hydrochloride, gastrin 1 (fragments 1–13), (all Sigma), histamine acid phosphate (BDH), mepyramine maleate (Sigma) noradrenaline bitartrate (Sigma), phentolamine hydrochloride (Ciba-Geigy), propranolol hydrochloride, substance P, tetrodotoxin, vasoactive intestinal polypeptide,

yohimbine hydrochloride (all Sigma).

Acetylcholine chloride was dissolved in a 5% NaH_2PO_4 solution and diluted with Krebs solution lowered to pH 4 with 0.1N HCl. Noradrenaline bitartrate, histamine acid phosphate and yohimbine hydrochloride were dissolved in, and diluted with, a modified Krebs solution of the following composition (mM): NaCl 143, KCl 4.75, $CaCl_2$ 2.54, ascorbic acid 0.15. Tetrodotoxin was dissolved in a citrate buffer containing citric acid (50 mM) and NaH_2PO_4 (48 mM). Dilutions were made with double-distilled H_2O. All other drugs were dissolved in and diluted with double-distilled H_2O. As clonidine has been shown to adhere to glass (Muir & Smart, 1983) doses were added cumulatively and baths were washed repeatedly with acid between experiments.

Results

Effects of acetylcholine, histamine and noradrenaline

With the exception of proximal strips of opossum oesophageal muscularis mucosae, all the tissues examined developed spontaneous mechanical contractions during the course of an experiment. Proximal and distal strips of oesophageal muscularis mucosae from the cat, dog and opossum gave concentration-dependent concentrations in response to acetylcholine and histamine over the range 10nM to 1mM (Figure 1a,b). Contractions developed slowly and, at the higher agonist concentrations, took several minutes to disappear, despite repeated washing of the tissue. Contractions to acetylcholine and histamine were competitively antagonized by atropine (50nM) and mepyramine (50nM), 15 min exposure, and abolished by a 30 min exposure to atropine (1μM) and mepyramine (1μM).

In the case of noradrenaline, 40% of all strips of cat oesophageal muscularis mucosae failed to respond with either a contraction or a relaxation. This was independent of the area from which the strips were taken. The remaining 60% always responded with a contraction (Figure 1c). Contractions to noradrenaline were competitively antagonized by phentolamine (50nM) but were converted to relaxations after a 30 min exposure to phentolamine (1μM); these relaxations were competitively antagonized by a 15 min exposure to propranolol (50nM) and abolished following a 30 min exposure to propranolol (1μM).

All strips of dog oesophageal muscularis mucosae responded to noradrenaline with concentration-dependent contractions (Figure 1c) which were competitively antagonized by phentolamine (50nM, 15 min exposure), but were converted to propranolol (1μM, 30 min exposure)-sensitive relaxations by phentolamine (1μM, 30 min exposure). These relaxations were completely antagonized by propranolol (50nM, 15 min exposure). No differences between areas with respect to noradrenaline sensitivity were observed.

No proximal strips of opossum oesophageal muscularis mucosae responded to noradrenaline by either contracting or relaxing. All distal strips, however, gave contractions (Figure 1c) which could be converted by a 30 min exposure to phentolamine (1μM) to relaxations; these relaxations themselves were abolished by a 30 min exposure to propranolol (1 μM). The contractions were competitively antagonized by a 15 min exposure to phentolamine (50 nM); the relaxations were antagonized competitively by a 15 min exposure to propranolol (50 nM).

These data show that the predominant response to exogenous noradrenaline is an α_1-adrenoceptor mediated contraction, but that if this response is abolished, a β-adrenoceptor mediated relaxation is revealed.

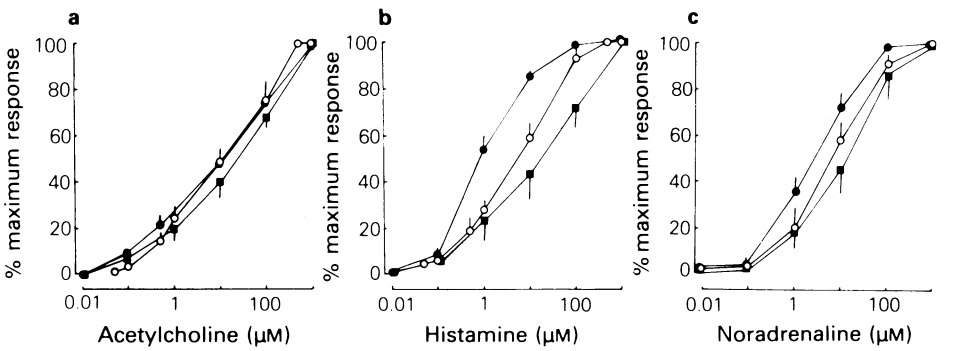

Figure 1 Log concentration-response curves for the effects of (a) acetylcholine, (b) histamine and (c) noradrenaline on strips of oesophageal muscularis mucosae from the cat (■), dog (●) and opossum (○). Results are expressed as a percentage of the maximum response of each tissue to the individual agonist and are the mean of 5 experiments with vertical lines representing s.e. mean.

Effects of the peptides

In each species cholecystokinin octapeptide (0.4 nM to 4 μM) occasionally caused small contractions of the muscularis mucosae which were most notable if isotonic contractions were recorded. This effect appeared to be independent of the concentration of the peptide and the area from which the strip was chosen.

Gastrin (5 nM to 5 μM) and vasoactive intestinal polypeptide (1.5 nM–1.5 μM) were without effect on strips of muscularis mucosae from any area of the cat, dog or opossum oesophagus.

The effects of substance P (3.3 nM to 3.3 μM) were quite different in each species. In the cat, substance P caused concentration-dependent increases in resting tension and either an increase in the amplitude of spontaneous mechanical activity, or the appearance of spontaneous activity where none was originally present (Figure 2).

Dog oesophageal muscularis mucosae responded to substance P with small contractions, the threshold for which was never less than 300 nM.

Strips from the opossum oesophagus, on the other hand, were more sensitive to the effects of substance P than to those of any other agonist (Figure 3). As was found with higher concentrations of acetylcholine and histamine, concentrations of substance P causing near-maximal contractions had a persistent effect.

In each species there was no difference between proximal and distal strips with regard to their sensitivity to substance P. Pretreatment of the tissues with atropine (1 μM, 30 min exposure) or tetrodotoxin (100 nM, 4 min exposure) failed to modify their responses to substance P when compared to control values.

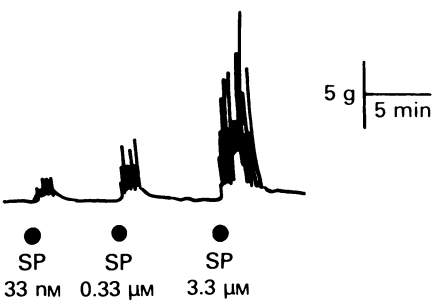

Figure 2 The effects of increasing concentrations of substance P (SP) on a strip of cat oesophageal muscularis mucosae. Substance P was washed from the bath after a 2 min exposure period and 5 min were allowed to elapse before a further addition was made.

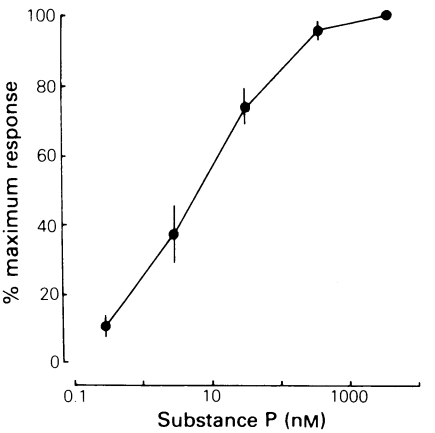

Figure 3 Log concentration-response curve for the effect of substance P on oesophageal muscularis mucosae from the opossum. Results are expressed as a percentage of the maximum response of the tissue to substance P and are the mean of 5 experiments with vertical lines representing s.e. mean.

Electrical field stimulation

Muscularis mucosae from all species responded to electrical field stimulation (e.f.s.) with atropine (50 nM)-and tetrodotoxin (100 nM)-sensitive contractions when isometric but not isotonic transducers were used. This resulted from the inability of the tissue to relax to its original length in the periods between trains of stimuli. Under the latter recording conditions, this phenomenon was also observed as an increase in the time taken for tissues to recover from agonist-induced contractions.

Contractions, which always occurred during rather than after trains of electrical stimuli, were not followed by periods of relaxation. Spontaneous mechanical contractions were not suppressed between trains of stimuli. When electrically-evoked responses were abolished by atropine (50 nM), tissues maintained a resting tension equivalent to 2–3 g. Continued e.f.s. during this time at frequencies of 5–20 Hz did not reveal any inhibitory responses. Similarly, if the resting tension was increased to the equivalent of 5–6 g by the addition of histamine (1–10 μM) in the presence of atropine, e.f.s. (5–20 Hz) again failed to produce an inhibition. There were no species differences in these observations.

With some notable exceptions, noradrenergic innervation of the intestine is usually associated with an inhibition. Since noradrenaline was found to have an excitatory action on the muscularis itself, its potential

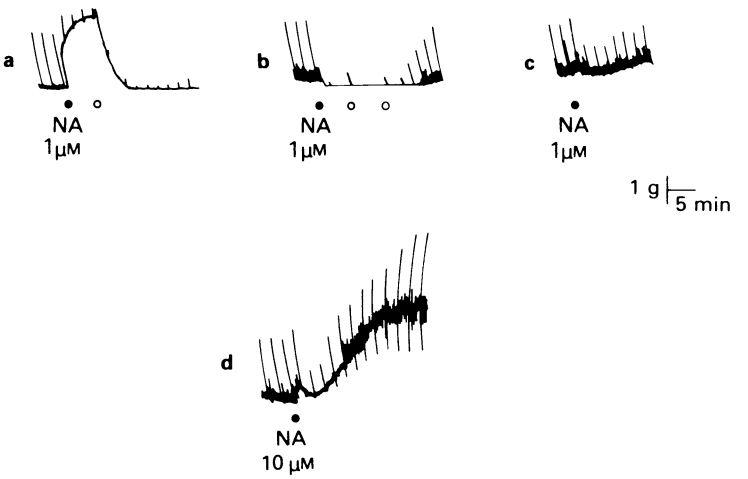

Figure 4 (a) The response to noradrenaline (NA) 1 μM (●) during electrical field stimulation (50 Hz, 1 ms, 120 mA, 5 s duration) of dog oesophageal muscularis mucosae. (b) The response to noradrenaline 1 μM (●) in the same tissue following a 30 min exposure to, and in the continuous presence of, phentolamine (1 μM). (c) The response to noradrenaline 1 μM (●) of the same tissue following a 30 min exposure to, and in the continuous presence of, phentolamine (1 μM) and propranolol (1 μM). (d) On a separate piece of dog oesophageal muscularis mucosae the effect of noradrenaline (10 μM) following a 30 min exposure to, and in the continuous presence of, propranolol (1 μM) and yohimbine (1 μM). (○) Wash out.

to act as a modulator of neuroeffector transmission was studied by observing its effects on electrically-evoked responses.

Strips of oesophageal muscularis mucosae from the cat were contracted by the addition of noradrenaline (1 to 10 μM) during e.f.s. This was accompanied by a small reduction (< 20%) in the amplitude of evoked contractions. Following a 30 min exposure to (and in the continued presence of) both phentolamine (1 μM) and propranolol (1 μM) subsequent administration of noradrenaline was without effect in 20% of these strips. In the remaining strips, there was a residual inhibition which was not seen if noradrenaline was again added after the tissue had been exposed to phentolamine (1 μM), propranolol (1 μM) and yohimbine (1 μM) for 30 min. The differences between strips were independent of the area from which they were taken.

During e.f.s. of dog oesophageal muscularis mucosae, noradrenaline (1 to 10 μM) increased resting tension and decreased the size of evoked contractions (Figure 4a). The latter phenomenon was quite marked even when the increase in resting tension was relatively small. Furthermore, in the continued presence of phentolamine (1 μM, 30 min exposure) a depression of evoked contraction was again seen, although there was no increase in resting tone (Figure 4b). Following a 30 min exposure to, and in the continued presence of phentolamine (1μM) and propranolol (1 μM), noradrenaline still caused a reduction in the amplitude of evoked contractions (Figure 4c). When this was repeated in the continued presence of phentolamine (1 μM), propranolol (1 μM) and yohimbine (1 μM), noradrenaline was without effect. In the presence of only propranolol (1 μM) (to exclude any β-adrenoceptor mediated effect) and yohimbine (1 μM), noradrenaline caused an increase in resting tension but failed to decrease the amplitude of evoked contractions (Figure 4d). The responses of proximal and distal strips were identical. It appears, therefore, that the yohimbine-sensitive depressions of evoked contractions by noradrenaline are not related to stimulation of α_1- or β-adrenoceptors.

Proximal and distal strips of opossum oesophageal muscularis mucosae responded in different ways to the addition of noradrenaline during e.f.s.

On proximal strips noradrenaline depressed evoked contractions without affecting resting tension (Figure 5a). This effect was not abolished by a 30 min exposure of the tissue to propranolol (1 μM) (Figure 5b) but was effectively reduced by a combination of propranolol (1 μM) and yohimbine (1 μM) (Figure 5c).

By way of contrast, noradrenaline increased the resting tension of distal strips while causing a minimal depression of evoked contractions (Figure 5d). This effect was abolished by a 30 min exposure to a combination of phentolamine (1 μM) and propranolol (1 μM) (Figure 5e).

These latter observations suggest that the actions of noradrenaline on opossum distal oesophageal muscularis are mediated only by α_1- and β-receptors.

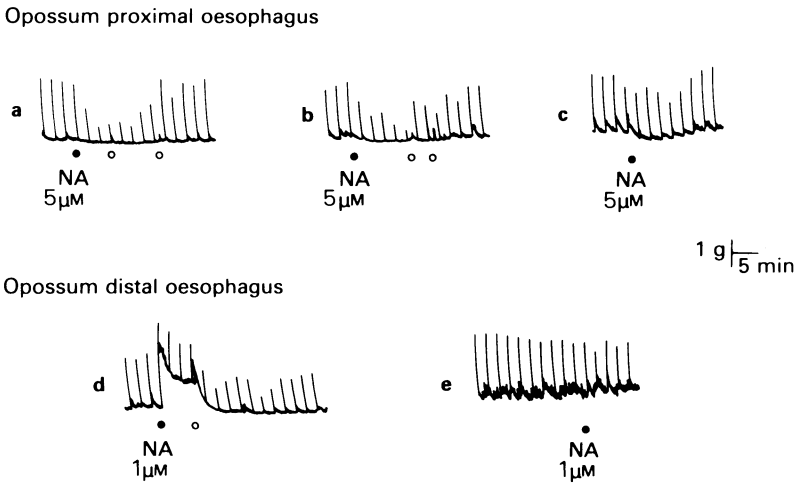

Figure 5 (a) The response to noradrenaline (NA) 5 μM (●) during electrical field stimulation of opossum proximal oesophageal muscularis mucosae. (b) The response to noradrenaline 5 μM (●) in same tissue following a 30 min exposure to, and in the continuous presence of, propranolol (1 μM). (c) The response to noradrenaline 5 μM (●) in the same tissue following a 30 min exposure to, and in the continuous presence of, propranolol (1 μM) and yohimbine (1 μM). (d) The response to noradrenaline 1 μM (●) in muscularis mucosae from opossum distal oesophagus during electrical field stimulation. (e) The response to noradrenaline 1 μM (●) in the same tissue following a 30 min exposure to, and in the continuous presence of phentolamine (1 μM) and propranolol (1 μM). (○) Wash out.

On proximal muscularis mucosae, noradrenaline appears to have a yohimbine-sensitive inhibitory action not associated with α_1- or β-adrenoceptors.

To investigate further the noradrenaline-induced, phentolamine- and propranolol-insensitive depression of electrically evoked responses, the relatively specific α_2-adrenoceptor agonist clonidine was employed.

On muscularis mucosae from the cat oesophagus, clonidine (10nM–100 μM) caused small depressions (< 20%) of evoked responses and often greatly increased the amplitude and frequency of spontaneous contractions. These increases were absent if the tissue was first treated for 15 min with phentolamine (1 μM). Depressions of evoked responses were not seen following a 15 min pretreatment of the tissue with yohimbine (1 μM).

Clonidine (10nM–100μM) both raised the resting tone and depressed, by a maximum of 30%, evoked contractions of dog oesophageal muscularis mucosae. Changes in resting tone were abolished by phentolamine (1 μM, 15 min) but not yohimbine (1 μM, 15 min). Yohimbine alone (1 μM, 15 min) abolished the effect of clonidine on evoked contractions.

On proximal opossum oesophageal muscularis mucosae, clonidine (10 nM–1 μM) depressed evoked contractions up to a maximum of 30% with no associated change in resting tension. Following a 15 min pretreatment with yohimbine(1 μM) clonidine was without effect. Distal oesophageal muscularis mucosae was not affected by clonidine in this concentration range.

It was not possible to determine the nature of the antagonism between yohimbine and clonidine on any of these tissues because a maximum inhibition was never achieved, and because, at concentrations exceeding 100 μM, clonidine caused a near-maximal, yohimbine-resistant depression of evoked responses.

Discussion

The results of the present study extend the observations of Hughes (1955; 1957) regarding inter-species differences in oesophageal muscularis mucosal pharmacology and have underlined a number of similarities.

In all species where the muscularis mucosae of the oesophagus has been subjected to electrical stimulation, a cholinergic excitatory innervation predominates. Furthermore, non-cholinergic, non-adrenergic responses are apparently absent. This contrasts with results obtained using muscularis mucosae from the large intestine of rabbits and dogs where electrical stimulation produces a relaxation (Gallacher *et al.*, 1973: Angel *et al.*, 1982) and from cat colonic muscularis mucosae where cholinergic nerves are reportedly absent (Onori *et al.*, 1971).

Although cat and opossum oesophageal mus-

cularis mucosae responded to low concentrations of substance P, dog tissue was singularly unresponsive. The other peptides used were ineffective on tissues from all three species. In this lack of peptide sensitivity, the muscularis mucosae of the oesophagus contrasts with that of human stomach (Walder, 1953), dog stomach and colon (Angel *et al.*, 1980; 1983), which have been found to be sensitive to the actions of a variety of peptides. This implies that peptides have little place in regulation of the gross movements of the oesophageal muscularis mucosae, although they might be involved in interneuronal transmission or act as neuromodulators.

In terms of catecholamines, oesophageal muscularis mucosae may be considered in two groups; those where the predominant response of the unstimulated tissue is a relaxation – guinea-pig and rat (Hughes, 1955; Kamikawa *et al.*, 1982) and those where contraction is seen – cat, rabbit (Hughes, 1955), dog and opossum.

The actions of noradrenaline on the electrically-evoked responses of oesophageal muscularis mucosae were usually inhibitory. In the guinea-pig (Kamikawa *et al.*, 1982) and opossum proximal oesophageal muscularis mucosae, inhibition was not accompanied by an increase in resting tension, whereas in the cat, dog and opossum (distal) tissue it was.

Thus, in rodents the sympathetic innervation of the oesophageal muscularis mucosae may act in a manner analogous to that found elsewhere in the gastrointestinal tract. In the non-rodents studied this would require that the sympathetic nerves impinge only on the intrinsic innervation and not on the muscularis mucosae itself.

The results obtained using yohimbine to block the phentolamine- and propranolol-insensitive noradrenaline-induced inhibition of electrically-evoked contractions are not, by themselves, sufficient to establish the presence of α_2-adrenoceptors. The actions of clonidine, however, provide further evidence. Most notable is that in all tissues where phentolamine and propranolol in combination failed to block completely the effects of noradrenaline, clonidine elicited a yohimbine-sensitive inhibition. Furthermore, in opossum distal oesophageal muscularis mucosae where no phentolamine- and propranolol-resistant inhibition was seen, clonidine was without effect.

Alterations in resting tone and spontaneous contractions produced by clonidine seem related to its ability to act as an α_1-adrenoceptor agonist, since both were abolished by phentolamine.

As has been noted for the circular muscle of the opossum oesophageal body (Christensen, 1970) oesophageal muscularis mucosae exhibits a gradient in noradrenaline sensitivity along its length. In the muscularis mucosae this is also manifest as a gradient of adrenoceptor types. The most distal region has easily demonstrated excitatory α_1-and inhibitory β-adrenoceptors which do not extend more than approximately 3 cm proximal to the L.E.S. On the other hand, inhibitory α_2-adrenoceptors are a feature of the proximal rather than the distal oesophageal muscularis mucosae. This suggests that inhibitory noradrenergic innervation is unlikely to be found in the most distal 3 cm of the opossum oesophageal muscularis mucosae.

In the cat and the dog the lack of regional variation in the response of the oesophageal muscularis mucosae to noradrenaline implies that, if a gradient exists at all, it extends over an area greater than that used in the present study.

On the basis of the results of the present series of experiments, cat and dog tissue appear to have excitatory α_1-, inhibitory α_2- and inhibitory β- adrenoceptors. In tissue from all three species it would seem reasonable to deduce that both the α_1- and β-adrenoceptors are located postjunctionally, since the actions of noradrenaline on the muscle alone are sensitive to phentolamine and propranolol. The α_2-adrenoceptors appear to be located prejunctionally, since they are not evident unless either clonidine is employed or noradrenaline is added (in the continuous presence of both phentolamine and propranolol), while the tissue is being electrically stimulated.

The functional significance of noradrenergic excitation rather than inhibition of oesophageal muscularis mucosae has not been explained by the present study. It may only be concluded, therefore, that there are fundamental differences between rodent and non-rodent oesophageal muscularis mucosae. The significance of these differences with respect to oesophageal function, however, remains unclear.

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