Br. J. Pharmac. (1979), **65**, 261–269

EVIDENCE THAT ADRENALINE IS RELEASED FROM ADRENERGIC NEURONES IN THE RECTUM OF THE FOWL

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1 The rectum isolated from the fowl was perfused with Tyrode solution via the caudal mesenteric artery. Noradrenaline and adrenaline were biologically or fluorimetrically assayed in perfusates collected before and during stimulation of Remak's nerve or of the periarterial nerves.

2 Perfusates collected during nerve stimulation relaxed the chick rectum and rat stomach strips which served as assay tissues. This effect was attributed to the action of noradrenaline or adrenaline released from adrenergic nerve endings which appeared in the perfusates.

3 Perfusates obtained during stimulation (30 Hz for 60 s) of Remak's nerve contained both noradrenaline and adrenaline when measured fluorimetrically. The mean output per stimulus train was 0.8 ± 0.2 ng/g wet wt. tissue for noradrenaline and 1.7 ± 0.2 ng/g wet wt. tissue for adrenaline ($n = 7$). Perfusates obtained during stimulation (30 Hz for 60 s) of the periarterial nerves contained noradrenaline in a concentration of 1.6 ± 0.3 ng/g wet wt. tissue per stimulus train, but almost no adrenaline ($n = 7$).

4 Neither stimulation of Remak's nerve nor the periarterial nerves liberated catecholamines when the rectum was perfused with Tyrode solution containing low Ca^{2+} (0.1 mM) and high Mg^{2+} (10 mM).

5 Infusion of high potassium solution (50 mM) increased markedly the output of noradrenaline and adrenaline.

6 Adrenaline as well as noradrenaline may function as the adrenergic neurotransmitter in the rectum of the fowl.

Introduction

In general, the main catecholamine found in tissues receiving a sympathetic nerve supply is noradrenaline, although variable concentrations of adrenaline may be present (von Euler, 1946; Anton & Sayre, 1962). A high proportion of adrenaline has been found in the heart, spleen, vas deferens and brain of the fowl (review by Holzbauer & Sharman, 1972). Recently, DeSantis, Längsfeld, Lindmar & Löffelholz (1975) showed that both noradrenaline and adrenaline function as neurotransmitters in the cardiac and splenic adrenergic fibres of the fowl.

In the preceding paper, the concentrations of noradrenaline and adrenaline in the chick intestine were found to vary with the region examined and with age. High concentrations of adrenaline were detected in the rectum, which is richly supplied by adrenergic fibres from Remak's nerve, and from periarterial nerves running along the mesenteric arteries (Bennett & Malmfors, 1970; Takewaki, Ohashi & Okada, 1977a).

The present experiments were undertaken to examine whether adrenaline found in the rectum was derived from the adrenergic nerve fibres.

A brief report of the present results has appeared elsewhere (Takewaki, Ohashi & Okada, 1977b).

Methods

White Leghorn cocks, more than 150 days old were stunned and bled and the whole rectum removed together with Remak's nerve, and the caudal mesenteric artery and vein. The rectal contents were removed by washing with Tyrode solution.

Remak's nerve was tied off and a 1 cm-length separated from the rectal wall (measured from the cut end of the intestine aborally) (Figure 1). The oral cut end of the rectum was tied together with the vessels and Remak's nerve. An L-shaped glass tube 7 mm in diameter was inserted about 1 cm into the

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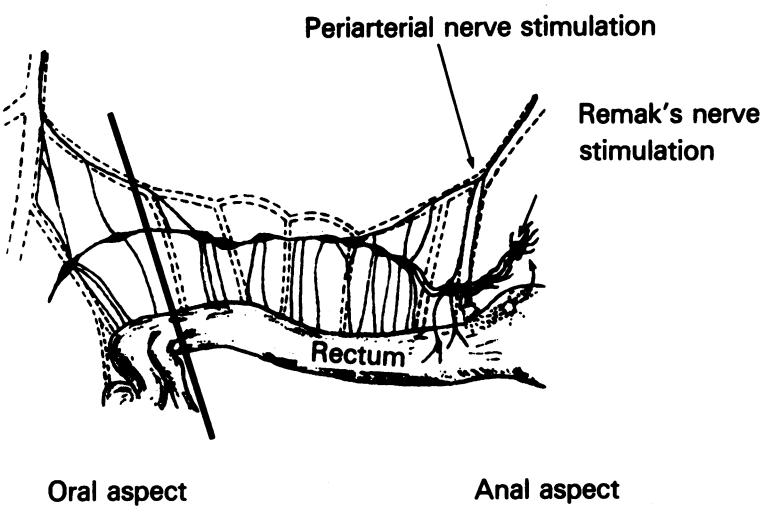

Figure 1 Diagram of the fowl rectum. Broken lines indicate arteries; the bold diagonal line shows where the rectum was cut. Sites of nerve stimulation are indicated by the arrows.

rectal lumen from the anal cut end, and tied to the rectal wall. The tube served both as a cannula through which mucus-like liquid could be removed from the lumen, if necessary, and as support when clamped to a rigid upright. The caudal mesenteric artery and vein were each cannulated with glass tubes of 1.0 and 2.0 mm i.d., respectively. All vessels other than those described above were ligated.

Perfusion of the fowl rectum

The isolated rectum with its nerve supply was mounted in a 200 ml organ bath filled with Tyrode solution and perfused at a constant rate (2.0 to 2.5 ml/min) by means of a roller pump via the arterial cannula with Tyrode solution containing citrated bovine fibrinogen (0.5 mg/ml) (Figure 2). The bathing and perfusing solutions were aerated and maintained at 37°C.

After completion of the experiment, the rectum was quickly blotted and weighed on a torsion balance.

Nerve stimulation

Remak's nerve and the nerve which runs along the caudal mesenteric artery (periarterial nerve, Figure 1) were stimulated at the frequencies indicated in the text with rectangular pulses (0.5 ms), by means of electrodes of the type described by Burn & Rand (1960).

Biological assay of catecholamines in perfusates

The rectum of 1- to 7-day-old chicks and strips of rat stomach were used to detect catecholamines in the perfusates. The fundi of the stomach from Wistar rats, of either sex (200 to 250 g), were prepared as described by Vane (1957). The stomach strip was preincubated (60 min) in Tyrode solution containing atropine (0.1 μg/ml) to prevent contractions produced by acetylcholine.

Each preparation was mounted in a separate superfusion jacket (see Figure 2). Each drop of perfusate leaving the perfused rectum passed over the surface of each assay preparation. Isotonic mechanical changes in tension of each assay preparation were recorded on smoked paper. The resting tone of the assay preparations was adjusted by applying a load of 1 or 2 g to the lever (magnification ×12). Errors resulting from friction between the writing lever and the smoked paper were minimized by a vibrator attached to the vertical rod to which the lever was clamped. All the preparations were allowed to equilibrate for at least 30 min before starting the experiments.

Chemical assay of catecholamines in perfusates

Collection of perfusate In experiments where catecholamines in the perfusate were chemically determined, Tyrode solution (without bovine fibrinogen) was used for perfusion. Two isolated rectums were perfused in parallel in an experiment. The perfusate from each rectum collected during a 60 s period of nerve stimulation, and during the subsequent period of 60 s (total 120 s) was placed in a test tube in ice-cold water and protected from light. This procedure was repeated four times every 20 min. Finally the contents of 8 test tubes were pooled to give a total

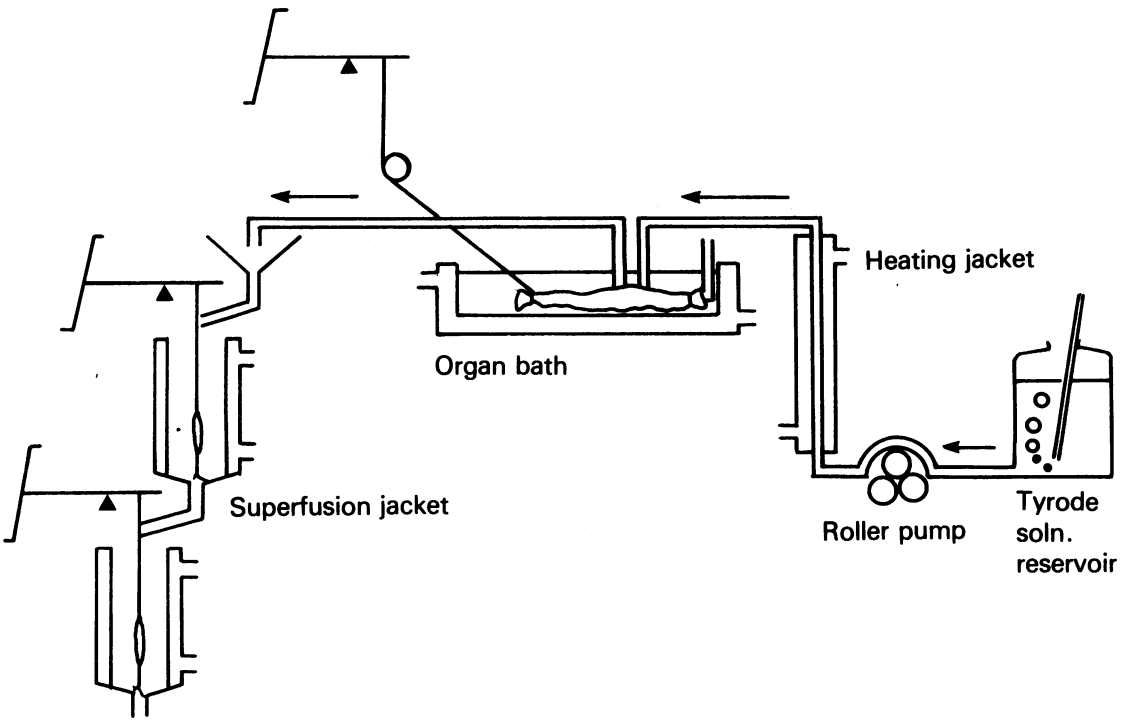

Figure 2 Diagrammatic sketch of the apparatus used to perfuse the isolated rectum and to collect the perfusate for superfusion assay.

volume of about 40 ml. Perfusates (total volume 70 to 100 ml) were collected in the absence of nerve stimulation and assayed for the resting output of catecholamines. In some experiments, after the initial series of samples of perfusate had been collected during nerve stimulation, the rectum was perfused without nerve stimulation for 40 min and a second series of samples collected and assayed.

Stimulation by high potassium was carried out by replacement of the perfusion solution with a Tyrode solution in which K_2SO_4 was substituted for 50 mM of the NaCl. During exposure to the high potassium solution the perfusate was collected for 5 min (about 25 ml).

Estimation of catecholamine concentrations in perfusates The noradrenaline and adrenaline content of the perfusates was adsorbed onto and then eluted from aluminium oxide and fluorimetrically determined as described in the preceding paper (Konaka, Ohashi, Okada & Takewaki, 1979).

Amounts of 5 to 10 ng of noradrenaline and adrenaline in each perfusate sample were required to give a fluorescence reading which was 1.5 times as large as that of the corresponding blank.

Mean recoveries of noradrenaline and adrenaline, when added separately or together to perfusates in amounts ranging from 10 ng to 100 ng, varied from 70 to 85%. The values given here are uncorrected for the percentage recovery. The amount of catecholamines released per minute was expressed as ng/g wet wt. tissue.

Drugs

The drugs used were (−)-noradrenaline bitartrate, (−)-adrenaline, hexamethonium bromide, propranolol hydrochloride, guanethidine sulphate, reserpine, phenoxybenzamine hydrochloride and citrated bovine fibrinogen (Sigma). Concentrations of drugs, except for noradrenaline and adrenaline, are expressed in terms of their salts in g/ml, whereas those of noradrenaline and adrenaline are in terms of their bases.

A certain amount of concentrated drug solution was added to the bathing solution or the perfusion solution to give the final desired concentration. Noradrenaline or adrenaline were applied directly to the assay preparations; an appropriate dilution of respective stock solution was made with Tyrode solution (immediately before use) and added, with a fine syringe to the perfusate leaving the perfused rectum, in a volume of 0.1 ml over a period of about 5 s.

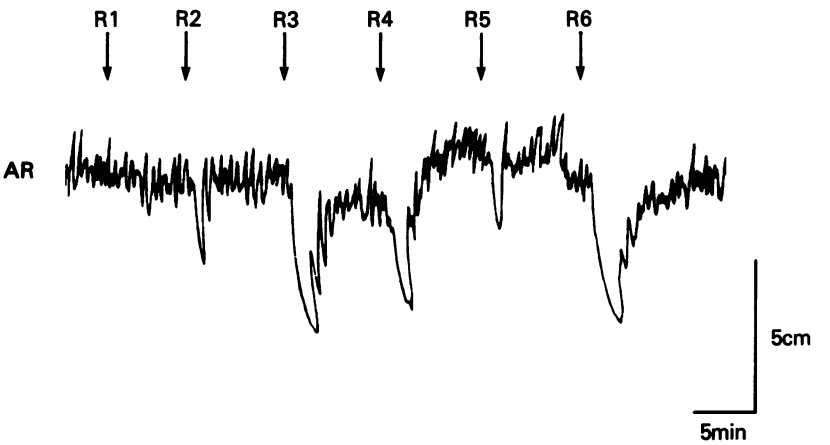

Figure 3 Responses of the chick rectum (assay tissue), AR, to perfusate obtained from the perfused fowl rectum during stimulation (for 30 s) of Remak's nerve at 10 Hz (R_1), 20 Hz (R_2), 30 Hz (R_3 & R_6), 50 Hz (R_4) and 100 Hz (R_5).

Results

Stimulation of Remak's nerve caused a biphasic response of the perfused fowl rectum, consisting of an initial contraction and a delayed relaxation, as previously observed (Takewaki *et al.*, 1977a). The amplitude and duration of both components varied with the frequency of stimulation. With a train of pulses delivered at a frequency of 30 Hz for 30 s, the first response, contraction, started immediately and decayed during the first 10 s of the period of stimulation. Relaxation, following the contraction, continued to develop over the stimulation period, attained a maximum level within 60 s after stimulation had stopped, and subsided so slowly that complete recovery of the tone to the control level took a few minutes.

Perfusate collected during stimulation of Remak's nerve usually relaxed the chick rectum and rat stomach strip, assay preparations. Stimulation of Remak's nerve for example at 10, 20, 30, 50 and 100 Hz for 30 s relaxed the chick rectum (Figure 3). The relaxation was maximum at 30 Hz and again declined at higher frequencies. At each frequency, relaxation of the chick rectum was much larger than that of the rat stomach strip.

Perfusate collected during stimulation of the periarterial nerve also produced relaxations for both assay tissues; the optimum frequency for the rat stomach strip was again 30 Hz and this was used for subsequent experiments. The perfusate, unlike that collected during stimulation of Remak's nerve was less effective on the chick rectum than on the rat stomach strip.

Responses of the assay tissues changed little over a period of a few hours when intervals between trains of stimuli were not less than 15 min. This demonstrated both the reproducibility of responses and the maintained ability of the nerves to release transmitters in the perfused rectum over a prolonged period.

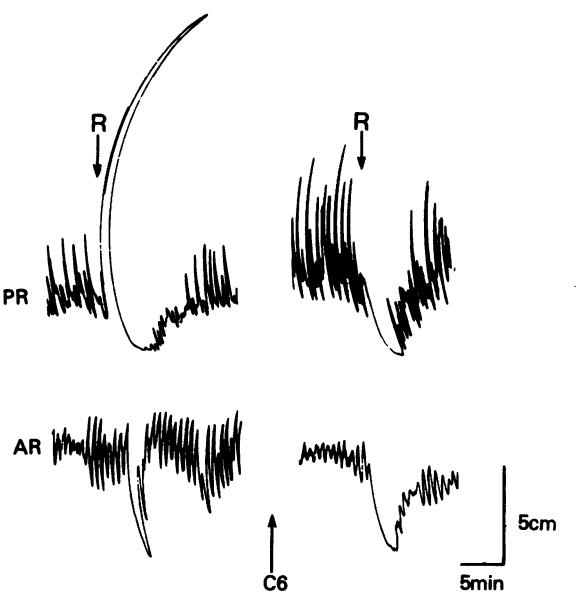

Figure 4 Effects of hexamethonium (C_6) on (upper trace) the responses of the perfused fowl rectum (PR) and (lower trace) of the assay tissue (chick rectum, AR) to the perfusate from the perfused fowl rectum produced by Remak's nerve stimulation (R). Left hand panels, controls; right hand panels, 20 min after application of C_6, 0.5 mg/ml.

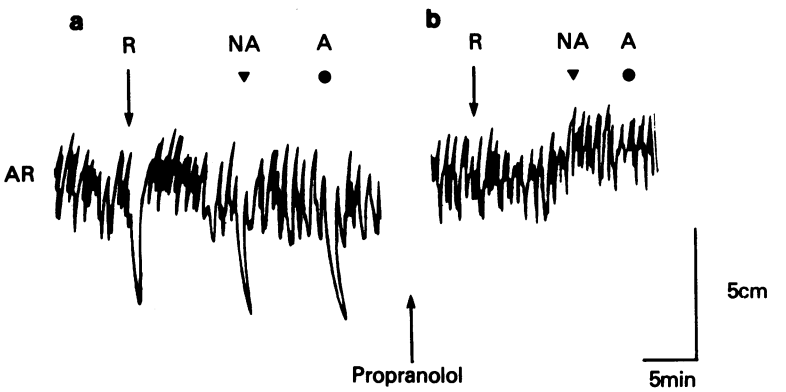

Figure 5 Effects of propranolol on the responses of the assay tissue (chick rectum, AR) to the perfusate from the fowl rectum during Remak's nerve stimulation (↓R, 30 Hz for 30 s) and to added noradrenaline (▼NA, 0.5 μg/ml) and adrenaline (●A, 5 ng/ml). (a) Control; (b) 20 min after beginning perfusion with propranolol, approx 5 μg/ml.

Effects of hexamethonium on responses of the perfused preparation

Hexamethonium (0.5 mg/ml in the bathing solution) inhibited or abolished the contraction but not the relaxation in response to stimulation of Remak's nerve. Relaxation of the assay tissue (chick rectum) was more marked after abolition of contraction of the perfused preparation (Figure 4). This indicated that the active material which appeared in the perfusate was released from nerves, and not squeezed out following contraction of the perfused rectum.

Effects of drugs on responses of the assay preparations

The β-adrenoceptor blocker, propranolol was added directly, at a rate of 0.5 ml/min to the venous effluent from the perfused rectum to give a final concentration of from 1 to 5 μg/ml and passed over the assay tissue (chick rectum). Propranolol caused a slight rise in tone in some cases. The chick rectum treated with propranolol (Figure 5, 5 μg/ml) responded neither to the perfusate obtained during stimulation of Remak's nerve nor to added noradrenaline (0.5 μg/ml) or adrenaline (5 ng/ml). Relaxations of the rat stomach strip to the perfusate during stimulation of the periarterial nerve and to noradrenaline and adrenaline were antagonized completely only after combined treatment with propranolol and phentolamine each at a concentration of 5 μg/ml.

Guanethidine (5 μg/ml) was applied to the perfused rectum by adding to both perfusing and bathing media for about 30 min. This drug decreased markedly the duration of relaxation of the perfused preparation and abolished relaxations of the assay tissues following nerve stimulation. Noradrenaline- and adrenaline-induced relaxations were usually unaffected or slightly potentiated in some preparations (Figure 6).

Three cocks were pretreated with reserpine (3.5 mg/kg i.p. for two days) and the rectums perfused. Perfusates from each reserpine-treated rectum collected during nerve stimulation did not relax the assay preparations although the response to added catecholamines appeared to be unaffected (Figure 7). The catecholamine content of the rectum from reserpine-treated cocks was less than 10% of control. Phenoxybenzamine (50 μg/ml) added to the solution perfusing the rectum, increased markedly the amplitude and duration of relaxation of the assay preparation (rat stomach strip) produced either by stimulation of Remak's nerve or following periarterial nerve stimulation (Figure 8).

This evidence indicates that the active material in the perfusate is a catecholamine which may be released from adrenergic nerves.

Differentiation of noradrenaline from adrenaline in the perfusate

Noradrenaline and adrenaline relaxed both assay preparations. The ED_{50} values for adrenaline and noradrenaline were respectively 5 ng/ml and 0.5 μg/ml (chick rectum) and 0.1 μg/ml for both amines (rat stomach strip) (see Figure 9). The sensitivity of the rat stomach strip to adrenaline and to noradrenaline is very similar whereas the sensitivity of the chick rectum to adrenaline is about 100 times more than that to noradrenaline. Therefore, the predominance of either adrenaline or noradrenaline in perfusates could be seen directly from simultaneous observations on the responses of the two different tissues. The rela-

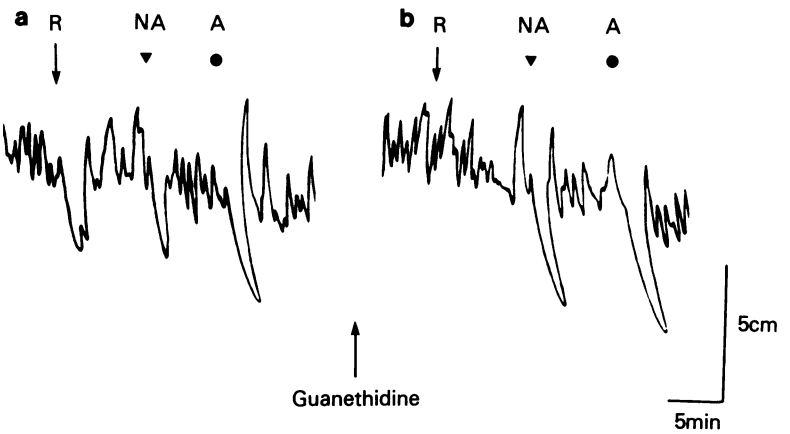

Figure 6 Effects of guanethidine on the responses of the assay tissue (chick rectum, AR) to the perfusate from the fowl rectum during Remak's nerve stimulation (↓R, 30 Hz for 30 s) and to exogenously-applied noradrenaline (▼NA, 0.1 μg/ml) and adrenaline (●A, 10 ng/ml). (a) Control; (b) 30 min after beginning perfusion with guanethidine, 5 μg/ml.

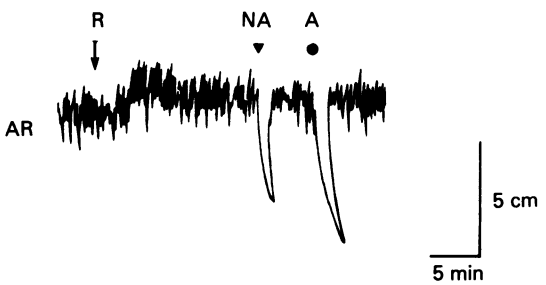

Figure 7 Responses of the assay tissue (chick rectum, AR) to the perfusate from the rectum of cocks pretreated with reserpine (3.5 mg/kg daily i.p. for 2 days) during Remak's nerve stimulation (↓R, 30 Hz for 30 s) and to added noradrenaline (▼NA, 0.1 μg/ml) and adrenaline (●A, 10 ng/ml).

tive amplitudes of the relaxation of the chick rectum and of the rat stomach following stimulation of Remak's nerve was roughly equal to the relative amplitude of the relaxation of both tissues induced by adrenaline in concentrations ranging from 5 ng to 10 ng/ml (Figure 10). This indicated that adrenaline predominated in the perfusate. However, since the minimum effective dose of noradrenaline was 5 to 10 ng/ml for the assay tissues (see above), the possibility could not be excluded that there was a concurrent overflow of a small amount of noradrenaline into the perfusion solution. In 5 out of 14 experiments, the response of the rat stomach to the perfusate was inconsistent with an adrenaline-induced relaxation; the perfusate caused relaxation followed by a contraction in two preparations and a small tone rise in three others. Atropine, added to the perfusion fluid (0.5 μg/ml) did not affect the excitatory effects of the perfusate.

A similar comparison of the relaxation of the assay tissues during stimulation of the periarterial nerve and by noradrenaline and adrenaline was made. The neurally-mediated responses resembled the noradrenaline-induced relaxations in doses from 50 ng to 0.1 μg/ml in 5 out of 7 experiments. In the remaining experiments, the perfusate caused a larger relaxation of the chick rectum than noradrenaline if this drug was applied in a dose required to produce the same amplitude of relaxation of the rat stomach strip as the relaxation elicited by the perfusate. These results suggest that the perfusate during stimulation of the periarterial nerve contained mainly noradrenaline.

Chemical assay of noradrenaline and adrenaline in perfusates

To confirm and extend the results described above, noradrenaline and adrenaline in perfusates were assayed fluorimetrically. Neither noradrenaline nor adrenaline was detected in perfusates (70 to 100 ml) before nerve stimulation. Thus, the catecholamine concentration was less than 0.1 ng/ml (see Methods). The mean weight of the recta was 2.0 g and the resting output per min less than 0.125 ng/g wet wt. tissue. The amounts of both noradrenaline and adrenaline in the perfusates increased markedly during Remak's nerve stimulation. The mean values (at 30 Hz, 60 s) were 0.8 $\pm$ 0.2 ng/g wet wt. tissue per min for noradrenaline and 1.7 $\pm$ 0.2 ng/g wet wt. tissue per min for adrenaline (n = 7). Stimulation of the periarterial nerve also markedly increased the noradrenaline concentration in the perfusate and the output was estimated to be 1.6 $\pm$ 0.3 ng/g wet wt. tissue per min,

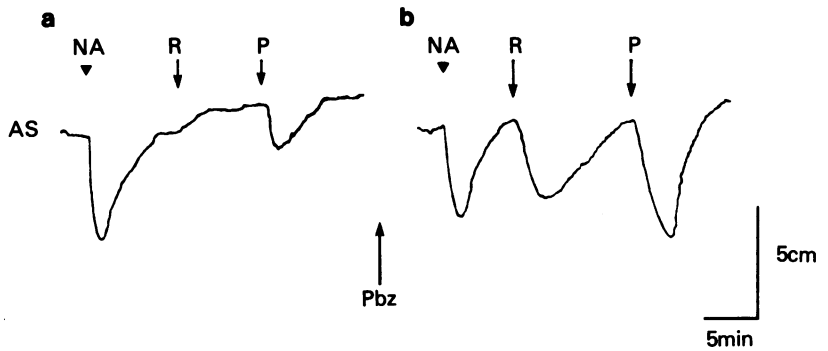

Figure 8 Effects of phenoxybenzamine (Pbz) on the responses of the assay tissue (rat stomach strip, AS) to the perfusate from the fowl rectum during stimulation (30 Hz for 30 s) of Remak's nerve (↓R) and of the periarterial nerves (↓P), and to added noradrenaline (▼NA), 0.5 μg/ml). (a) Control; (b) 20 min after beginning perfusion with Pbz, 50 μg/ml.

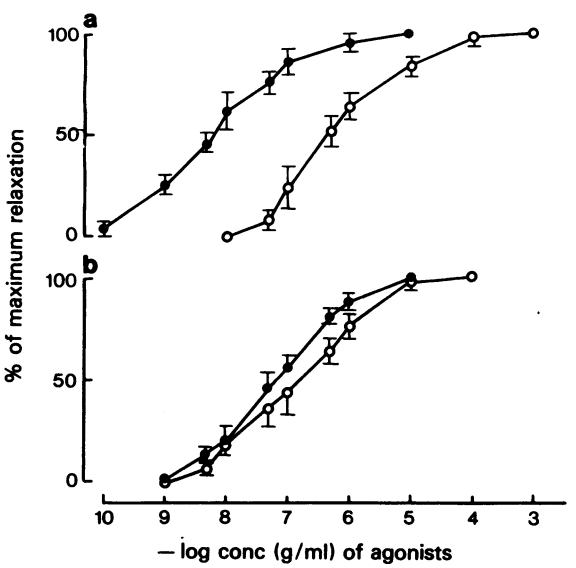

Figure 9 Dose–response curves, showing the effects of added noradrenaline (○) and adrenaline (●) on the assay tissues (chick rectum in a; rat stomach strip in b). Each point presents the mean of five to eight separate determinations; vertical lines show s.e. means.

but in contrast to stimulation of Remak's nerve, periarterial nerve stimulation was much less effective in increasing adrenaline. A very small amount of adrenaline was detected in only two out of seven samples. The output of noradrenaline and adrenaline per min in response to a second train of stimulation of Remak's nerve (30 Hz, 60 s) was reduced to about 60% of the control, but the ratio between noradrenaline and adrenaline remained almost unaltered.

Treatment of the perfused rectum with phenoxybenzamine (50 μg/ml) for 30 min increased the mean output of noradrenaline by about seven fold and that of adrenaline by about three fold in response to the Remak's nerve stimulation (Table 1).

The output of both amines was also increased when the isolated rectum was perfused with a potassium-rich (50 mM) Tyrode solution. The mean values per minute (ng/g wet wt. tissue) were 3.3 ± 0.4 for noradrenaline and 1.0 ± 0.1 for adrenaline.

During perfusion with a solution containing low calcium (0.1 mM) and high magnesium (10 mM), no release of noradrenaline and adrenaline was detected during stimulation of Remak's nerve in three experiments.

Discussion

The findings that adrenaline is present in a higher concentration in the fowl rectum suggest that this catecholamine may act as a neurotransmitter in the adrenergic fibres in the rectum of this species. This agrees with the recent observation that both noradrenaline and adrenaline act as sympathetic neurotransmitters in the fowl heart and spleen (DeSantis *et al.*, 1975).

There was a considerable difference in the concentration ratio of noradrenaline to adrenaline in perfusates following stimulation of the two different nerve pathways. Perfusate from the isolated rectum when Remak's nerve was stimulated contained about 70% of the total catecholamines (noradrenaline plus adrenaline) as adrenaline. On the other hand, noradrenaline represented almost all of the catecholamines in the perfusate during stimulation of the periarterial nerves. The concentration ratio of adrenaline to noradrenaline was maintained throughout repetitive stimulation provided an appropriate interval (40 min) was used although the adrenaline and noradrenaline

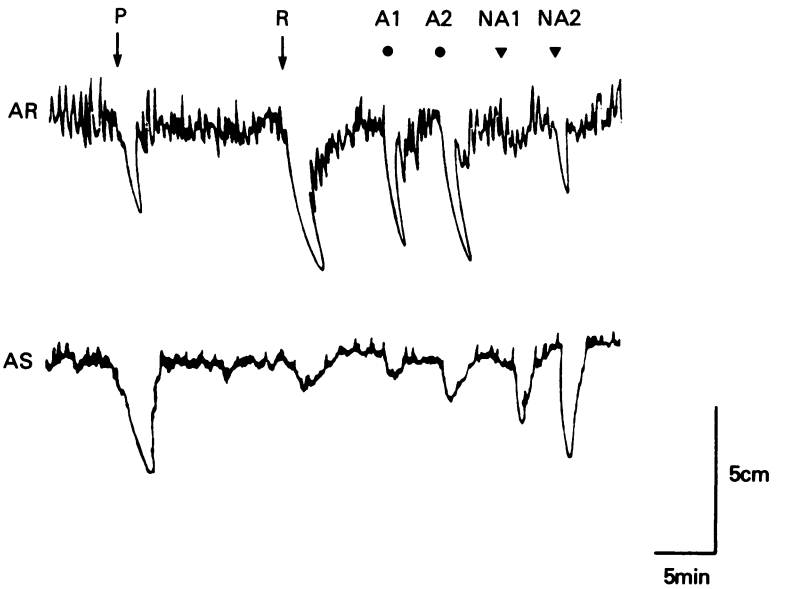

Figure 10 Responses of assay tissues (chick rectum, AR; rat stomach strip, AS) to the perfusate from the fowl rectum during stimulation (30 Hz for 30 s) of Remak's nerve (↓R), of periarterial nerves (↓P), and to added noradrenaline (▼NA1, 50 ng/ml; NA2, 0.1 μg/ml) and adrenaline (●A1, 10 ng/ml; A2, 20 ng/ml). Both assay tissues were superfused in series. Note similar pattern of responses between P and NA2, and between R and A2.

concentrations were reduced. On the other hand, infusion of a high potassium solution released adrenaline and noradrenaline in a concentration ratio of 1:3 which is approximately the same as that of endogenous adrenaline and noradrenaline (see preceding paper). This may mean that stimulation of either the periarterial nerves or of Remak's nerve causes a selective excitation of some of the adrenergic fibres distributed in the wall of the rectum, whereas potassium infusion stimulates the rectal adrenergic nerve plexus indiscriminately.

On the basis of the view that a neurone releases one kind of transmitter substance (Dale, see Eccles, 1957), adrenergic fibres derived from Remak's nerve may consist of adrenaline-releasing and noradrenaline-releasing fibres with a predominance of the former, but those from the periarterial nerves may contain noradrenaline-releasing fibres only. Alternatively, individual adrenergic nerve fibres may contain both amines in varied concentration ratios, the amount released in response to stimulation being directly related to their ratio (Bacq, 1934).

The total output of adrenaline and noradrenaline released into the perfusate was larger during stimulation of Remak's nerve than during stimulation of the periarterial nerves. However, the perfusate col-

Table 1 Effects of phenoxybenzamine on the output of noradrenaline and adrenaline from the perfused fowl rectum during Remak's nerve stimulation

	n	*Noradrenaline (ng/g wet wt. tissue per min)*	*Adrenaline (ng/g wet wt. tissue per min)*
Control	3	0.7 ± 0.3	1.6 ± 0.4
After treatment with phenoxybenzamine (50 μg/ml)	3	5.1 ± 1.0	5.2 ± 0.9

Values are mean ± s.e. mean of noradrenaline and adrenaline outputs per stimulus train (60 s at 30 Hz) determined fluorimetrically.

lected during Remak's nerve stimulation invariably caused a smaller relaxation of the rat stomach strip, the sensitivity of which is similar to adrenaline and noradrenaline. This discrepancy could be due to the simultaneous release of a stimulating substance(s) other than acetylcholine, to which the rat stomach muscle is more sensitive. Stimulation of Remak's nerve induces non-cholinergic contractions (Bartlet & Hassan, 1971; Takewaki *et al.*, 1977a) and excitatory junction potentials (Takewaki & Ohashi, 1977) in the fowl rectum. Furthermore, the release of a prostaglandin-like substance occurred following mechanical stimulation of this nerve trunk (unpublished observation).

The origin and physiological function of Remak's nerve have not been well understood (Kuntz, 1910; Nolf, 1934; Yntema & Hammond, 1952; Yasukawa, 1959; Watanabe, 1972; Hukuhara, Naitoh & Kameyama, 1974). The present findings suggest that the nerve may play a different role in regulating the rectal motility from that of the periarterial nerves.

We wish to thank Dr T.B. Bolton for his helpful criticism of the manuscript.

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(Received May 31, 1978. Revised August 1, 1978.)