

The effect of immunosympathectomy and of 6-hydroxydopamine on the responses of the rat anococcygeus to nerve stimulation and to some drugs

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Summary

1. The rat anococcygeus muscle possesses a dense motor adrenergic innervation and also a powerful inhibitory innervation whose transmitter is unknown. The possibility that the adrenergic nerves released both noradrenaline and the unknown inhibitory transmitter was investigated by destroying the adrenergic nerves either by immunosympathectomy or by 6-hydroxydopamine.
2. Immunosympathectomy was only partially effective in destroying the adrenergic neurones to the anococcygeus. 6-Hydroxydopamine destroyed the terminal adrenergic varicosities abolishing almost completely the motor response. Neither treatment affected the inhibitory response.
3. 6-Hydroxydopamine induced a specific hypersensitivity of the muscle to noradrenaline which was not shared with carbachol.
4. The maximum tension response of the anococcygeus muscle was greater in the male than in the female probably because the muscle is bigger.

Introduction

The responses of the anococcygeus smooth muscle of the rat to nerve stimulation and to drugs have recently been described (Gillespie, 1971, 1972). One particularly interesting feature of this preparation is the possession of a powerful inhibitory innervation whose presence is uncovered by raising the tone of the preparation. The inhibitory chemical transmitter is unknown but is neither a catecholamine, acetylcholine, ATP, prostaglandin E₁, E₂ or F_{2a} nor γ -aminobutyric acid (Gillespie, 1972 and unpublished). The fibres of origin of the inhibitory transmitter could be either a separate set of inhibitory nerves or possibly a second pharmacologically active substance is liberated by the adrenergic nerves. The experiments to be described were intended to distinguish between these two possibilities by destroying selectively the adrenergic neurone or its varicose terminal by immunosympathectomy or 6-hydroxydopamine treatment respectively and studying the effect on the inhibitor response. We have also examined the degree and selectivity of the hypersensitivity induced by these two forms of chemical sympathectomy.

Methods

Daily intraperitoneal injections of nerve growth factor (Burroughs Wellcome) were given to new-born rats with a dose schedule of 0.1:0.1:0.2:0.2 and 0.4 ml from the first to the fifth day after birth. 6-Hydroxydopamine hydrobromide (6-OHDA

Sigma) was given to adult rats by intraperitoneal injection in doses of 2×50 mg/kg of the salt on day one and 2×100 mg/kg on day four (Thoenen & Tranzer, 1968). These animals were killed and the anococcygeus muscles examined on the fifth or sixth day.

Rats were killed by a blow on the head and bleeding. The anococcygeus muscles were isolated as previously described (Gillespie, 1972), suspended in a 30 ml isolated organ bath in Krebs bicarbonate saline at 37°C and gassed with a mixture of 95% O_2 + 5% CO_2 . Tension was recorded isometrically with an Ether strain gauge tension transducer and displayed on a Devices M2 pen recorder. The muscles were mounted under a resting tension of 0.2–0.5 g. When the intramural nerve fibres were to be stimulated the muscle was drawn through a pair of platinum loop electrodes embedded in epoxy resin (Araldite, Ciba) and stimulated by square pulses of 1 ms duration and supramaximal voltage from a Palmer stimulator. Drugs dissolved in 0.9% w/v NaCl (saline) were added to the bath by syringe; the maximum volume added was 0.4 ml.

The success of the procedures used to destroy the adrenergic nerve terminals was checked histochemically by examining in each animal the other, paired muscle by the technique of Falck & Hillarp. The technique of freeze drying and exposure to formaldehyde vapour has already been described (Gillespie & Kirpekar, 1966). The tissue sections were mounted in liquid paraffin and examined with a Leitz Ortholux microscope fitted with an HbO 200 mercury vapour lamp, a 3 mm BG12 exciter filter and a K530 barrier filter.

Other drugs used were carbachol (Burroughs Wellcome), guanethidine sulphate (Ciba), (–)-noradrenaline bitartrate (Koch-Light) and phentolamine mesylate (Ciba).

Results

The effect of immunosympathectomy and 6-hydroxydopamine

The control anococcygeus muscle showed characteristic fluorescent nerve fibres distributed throughout the tissue (Fig. 1A) as has previously been reported (Gillespie & Maxwell, 1971). In muscles from animals treated after birth with anti-serum to the nerve growth factor there was a reduction in the density of fluorescent fibres but the muscle was still densely innervated (Fig. 1B). After treatment with 6-OHDA almost all fluorescent fibres had disappeared (Fig. 1C).

Response to noradrenaline and to carbachol

Both noradrenaline and carbachol caused contraction of the anococcygeus. The response to noradrenaline 10^{-7}M was completely abolished by phentolamine $1\text{ }\mu\text{g/ml}$ which has no effect on the response to carbachol $3 \times 10^{-6}\text{M}$. Dose response curves for noradrenaline and carbachol were constructed for control muscles, for muscles from immunosympathectomized animals and for muscles from animals treated with 6-OHDA. These dose-response curves were compared by regression analysis of the straight line part of the curves and the ED50 for each drug and each muscle compared. Figure 2 shows the results. Both immunosympathectomy and 6-OHDA caused a shift in the dose-response curve for noradrenaline to the left,

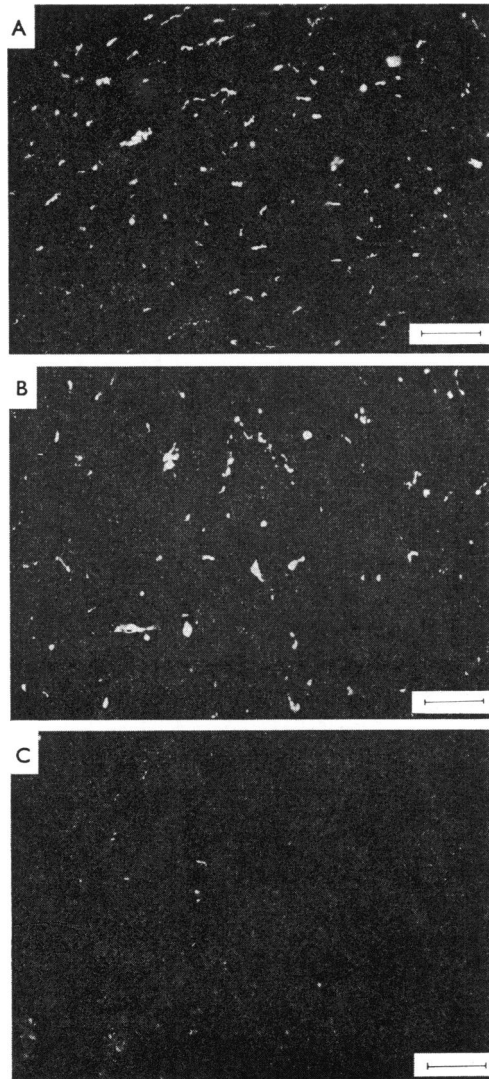


FIG. 1. Transverse sections of the anococcygeus muscle from control rats (A) and from rats treated with immune serum (B) or 6-hydroxydopamine (C). The calibration bar=100 μ m.

a shift which was greater for 6-OHDA than for immunosympathectomy. There was no change in the sensitivity to carbachol in either experimental group.

In these experiments a significant difference between the maximum response of muscles from male and female animals was found (Fig. 3). The contractions both to noradrenaline and to carbachol were significantly greater in muscles from male rats than in those from female. The maximum tension response to noradrenaline was also greater than that to carbachol in male animals (Fig. 3). These differences made it necessary in determining the effect of 6-OHDA and immunosympathectomy to make comparisons between animals of the same sex so that both the control and the experimental groups were subdivided into male and female sub-groups. Only male animals had been treated with immune serum so that only male controls were necessary.

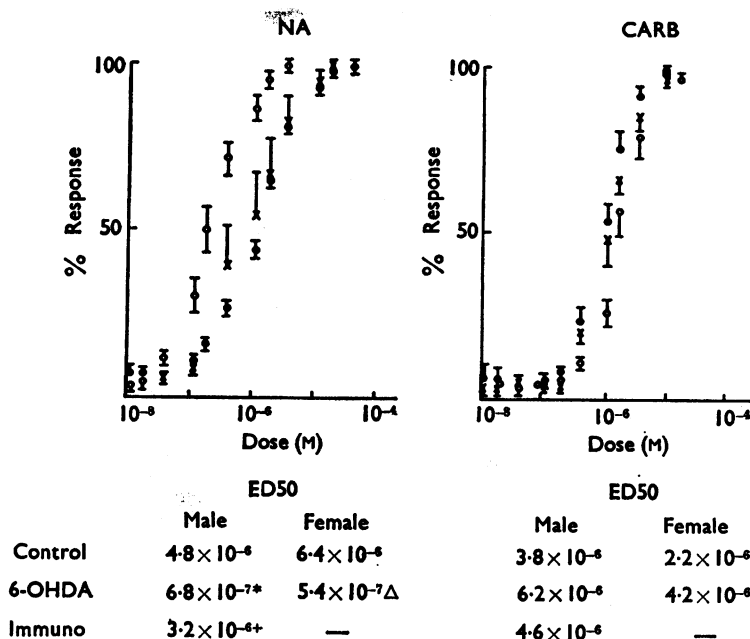


FIG. 2. Dose-response curves of the anococcygeus to noradrenaline (NA) and carbachol (CARB) in male rats. ●—●, Control; ○—○, 6-OHDA; ×—×, immunosympathectomized. The doses shown are molar. The ED₅₀ values calculated from these curves for male rats and from similar curves for female rats are given below. There is a significant shift to the left in the dose-response curve for noradrenaline in the immunosympathectomized animals and a greater shift in the animals treated with 6-hydroxydopamine (* $0.001 < P < 0.01$; + $0.01 < P < 0.02$; $\Delta P < 0.001$.) Bars represent \pm S.E.M.

Response to field stimulation and to guanethidine

The response to field stimulation before and in the presence of guanethidine is shown in Figure 4. In previous work on this preparation, guanethidine was found to be the most suitable drug to uncover the inhibitory response since it acted both as an indirect sympathomimetic to raise tone and at the same time blocked the motor adrenergic neurone; Fig. 4C shows this effect. In immunosympathectomized animals and especially in animals treated with 6-OHDA, guanethidine though still effective as an adrenergic neurone blocking agent against any residual motor response to field stimulation was quite unable to cause an increase in tone (Fig. 4I, 6-OH). Carbachol was, therefore, used to maintain a steady tonic contraction on which the inhibitory response could be displayed.

In muscles from normal animals, field stimulation before guanethidine caused contraction with a maximum response at 30 Hz. Guanethidine 10^{-5} M caused an increase in tone and reversed the response to field stimulation, to inhibition. In immunosympathectomized animals there was no measurable reduction in the maximum motor response to field stimulation and no change in the optimum frequency of stimulation. When the tone of the muscle was raised with carbachol, field stimulation produced an inhibitory response as great as in control animals (Fig. 4I). In animals treated with 6-OHDA, the motor response was almost abolished and there was a shift in the optimum frequency to between 50 and 100 Hz. In the presence of carbachol, however, field stimulation still produced inhibition (Fig. 4, 6-OH). The magnitude of the inhibition in Fig. 4, 6-OH appears less than that

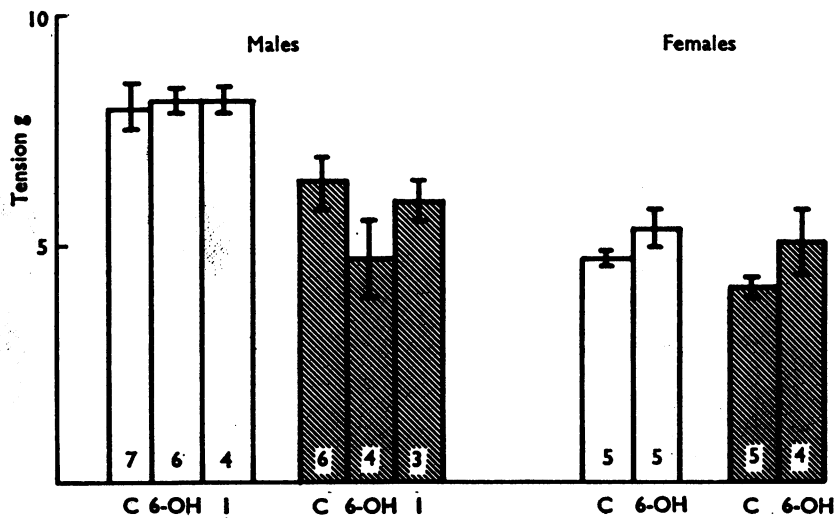


FIG. 3. Histogram of the maximum responses of the rat anococcygeus to noradrenaline (clear columns) and carbachol (hatched columns) in male and female control (C) rats together with the effect of immunosympathectomy (I) and 6-hydroxydopamine (6-OH). The numbers of animals in each group are at the bottom of each column. Bars represent \pm S.E.M.

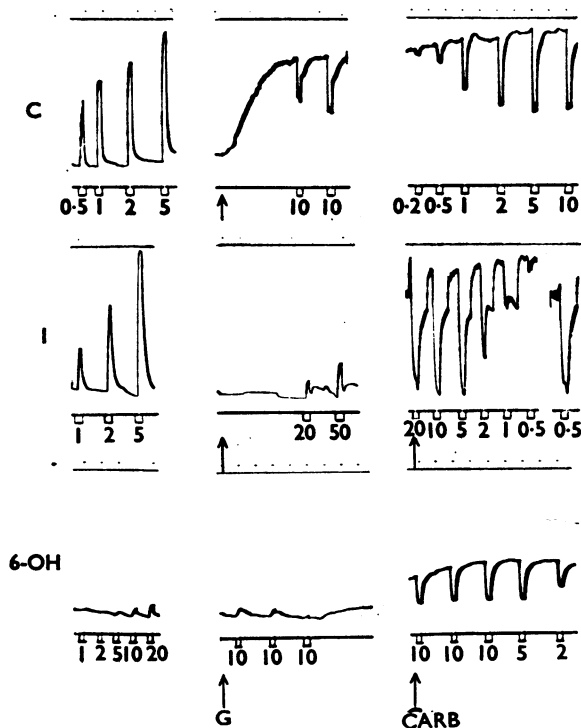


FIG. 4. Records of the response of the anococcygeus muscle to field stimulation when muscle tone is low in the absence of drugs and when the tone has been raised either by guanethidine (G) or by carbachol (CARB). The upper records are from a control animal (C), the middle from an immunosympathectomized animal (I) and the lower from an animal given 6-hydroxydopamine (6-OH). The frequency and duration of stimulation in Hz is given below each record. Time marker 1 minute.

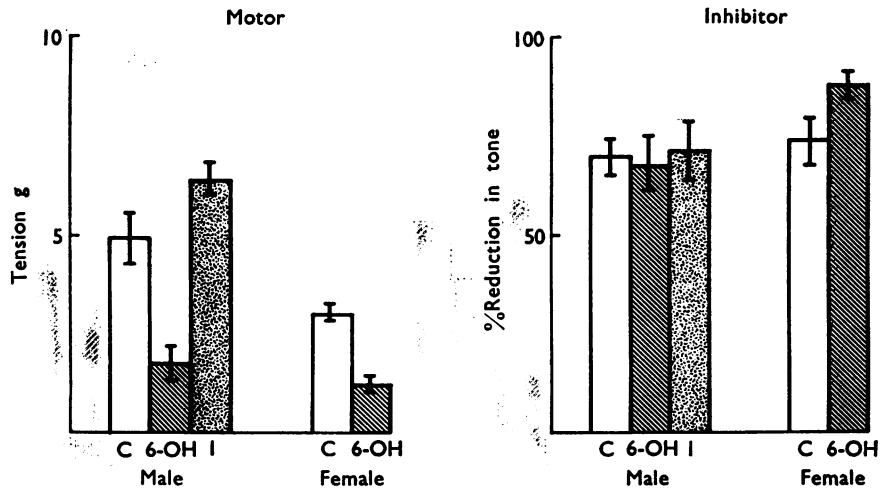


FIG. 5. The effect of immunosympathectomy (I, stippled columns) or 6-hydroxydopamine (6-OH, hatched columns) on the maximum motor and inhibition response to field stimulation. The control response is represented by the clear columns. Since the maximum response is less in muscles from female animals the results in these are shown separately. In both males and females 6-hydroxydopamine reduces the motor but has no effect on the inhibitor response. The bars represent \pm S.E.M.

of Fig. 4C and I. This was simply an expression of the degree of tone achieved by the carbachol. Where this was greater, then the inhibition was appropriately greater. For this reason we have expressed the magnitude of the inhibitory response in terms of the % reduction in the tone induced by carbachol or guanethidine and Fig. 5 summarizes the results of all the experiments expressed in this way.

Discussion

The resistance of the inhibitory response to destruction of the adrenergic nerves, particularly by 6-OHDA, suggests that the inhibitory transmitter does not come from these nerve endings. Taken in conjunction with the recent evidence that the inhibitory fibres can be stimulated in the spinal cord and that their origin from the spinal cord is at a different level from that of the motor adrenergic fibres (Gillespie & McGrath, 1972) it is fairly clear that a distinct inhibitory nerve pathway to this muscle exists.

Immunosympathectomy was relatively ineffective in destroying the adrenergic nerves to the muscle. This is perhaps not surprising since there appears to be a variation in sensitivity of the neurones to the immune serum, related perhaps to their degree of maturity, such that the caudally placed neurones rising in the pre-vertebral ganglia are more resistant to destruction (Vogt, 1964). Other factors such as the blood flow to the organ and, therefore, the fraction of the 6-OHDA dose delivered to it probably also influences the degree of destruction (Iversen, Glowinski & Axelrod, 1966). This was suggested in two experiments in which the dose of 6-OHDA was halved and both the vas deferens and the anococcygeus examined histologically and their response to field stimulation *in vitro* measured. After 6-OHDA the anococcygeus fibres had disappeared and the muscle's response to field stimulation was greatly diminished whereas the density of adrenergic innervation measured histologically in the vas deferens was unaltered as was its response to stimulation.

In these experiments there were three measures of adrenergic motor denervation; first, the disappearance histochemically of the fluorescent terminals; secondly, the loss of the indirect sympathomimetic action of guanethidine and, finally, the reduction or loss of the response to field stimulation of these nerves. Of these, the loss of the indirect sympathomimetic action of guanethidine was easily the most sensitive, and the response to field stimulation the least sensitive, measure of the change in innervation. For example, immunosympathectomy produced no change in the response to field stimulation some slight reduction in the density of adrenergic nerve terminals histochemically but almost complete loss of the tone-raising action of guanethidine (Fig. 4I). It may be that guanethidine though able to cause a continuous slow leak of transmitter cannot produce the high concentrations achieved by the nerve impulse, so that any slight change in the relationship between nerve terminal and muscle can have a considerable effect on the indirect sympathomimetic action of guanethidine with little or no action on the response to nerve stimulation.

In this tissue, loss of the adrenergic nerves by the action of 6-OHDA causes a hypersensitivity to noradrenaline but not to carbachol. Such a specific hypersensitivity resembles that seen in the denervated nictitating membrane (Tsai, Denholm & McGrath, 1968) rather than the non-specific hypersensitivity in the spinal cat reported by Trendelenburg & Weiner (1962). We made no investigation to determine the pre- or post-synaptic origin of this hypersensitivity in the anococcygeus but it would be consistent with an abolition of neuronal uptake as suggested by Tsai *et al.*, 1968. We found no evidence for an increase in the maximum response accompanying the hypersensitivity as has been reported by Muir & Pollock (1972) as a consequence of the chronic administration of morphine.

The different maximum responses observed in muscles from male and female rats is probably related to muscle size. The muscle in the male rat is noticeably broader than that in the female and forms a pronounced ventral bar in front of the colon, a feature hardly developed in the female. Presumably this is related to the retractor penis muscle which takes origin from this ventral bar. It is necessary to take account of these differences in experimental design.

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(Received August 22, 1972)