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THE SPINAL ORIGIN OF THE MOTOR AND INHIBITORY INNERVATION OF THE RAT ANOCOCCYGEUS MUSCLES

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SUMMARY

1. A preparation is described whereby the responses of the two anococcygeus muscles can be recorded *in vivo* in the pithed rat and the autonomic outflows to the muscle selectively stimulated in the spinal canal.

2. Motor responses are obtained from stimulation at two levels; an upper extending from T 11 to L 3 and a lower from L 6 to S 2. Stimulation between these levels, i.e. between L 3 and L 6, produces no response. The response to stimulation at both upper and lower levels is abolished by phentolamine. The response to stimulation in the upper region is abolished by hexamethonium and is, therefore, presumably preganglionic; the response to stimulation at the lower level is resistant to hexamethonium and presumably post-ganglionic. Stimulation at levels above T 11 causes contraction after a delay, by liberating catecholamines from the adrenal medulla. This effect is blocked by both phentolamine and hexamethonium.

3. If the adrenergic motor nerves are blocked and the muscle tone raised by a combination of guanethidine and tyramine, stimulation between L 5 and S 2 produces inhibition. The inhibitory outflow, therefore, overlaps the motor outflow but extends one segment more rostral (L 5). Stimulation restricted to this L 5 segment even in the presence of a normal unblocked motor innervation causes inhibition. The inhibitory response is blocked by hexamethonium or mecamylamine but desensitization and 'escape' occurs. This desensitization is less than that observed in the vas deferens when its motor nerves are similarly stimulated in the spinal cord.

4. It is concluded that inhibitory fibres to the anococcygeus arise in the spinal cord and are organized in the pattern of the autonomic nervous system with a peripheral synapse. The site of origin of these inhibitory fibres is different from the motor adrenergic fibres to the muscle.

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INTRODUCTION

Stimulation of intramural nerves in the rat anococcygeus muscle produces contraction from excitation of the dense adrenergic innervation. If muscle tone is first raised, stimulation produces inhibition (Gillespie, 1972). The nature of this inhibitory innervation and of the transmitter involved is unknown. The present experiments were intended to answer two questions. First, do the inhibitory nerves arise in the spinal cord and, if so, does their origin correspond with that of the motor adrenergic nerves? Secondly, if a separate inhibitory innervation exists, is it part of the autonomic nervous system with a characteristic two neurone efferent pathway?

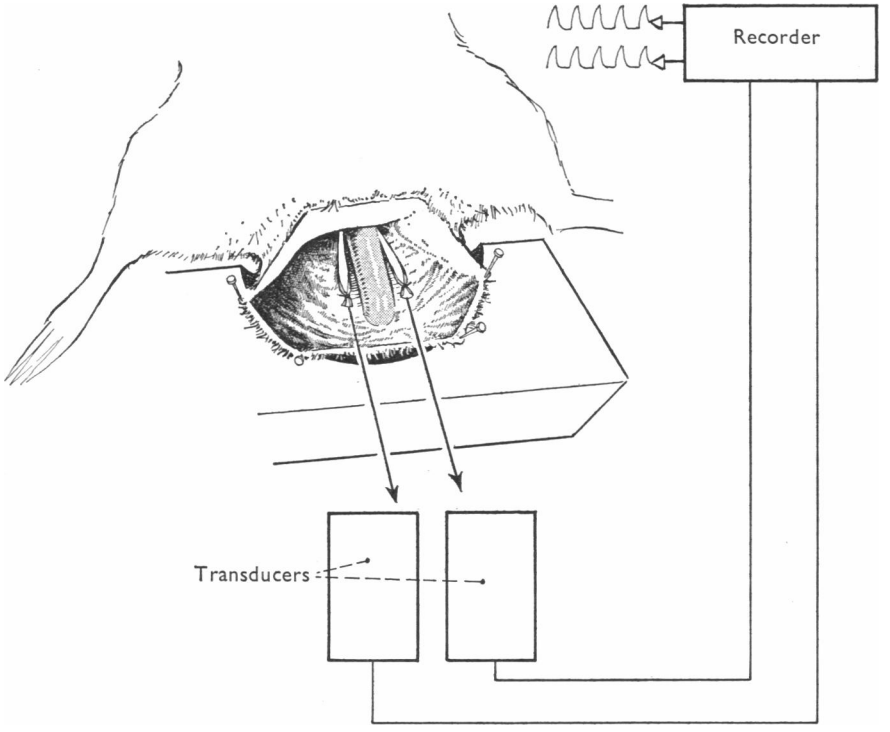

Fig. 1. The arrangement to record *in vivo* the tension response of the anococcygeus muscles in the rat. The scrotum is opened and pinned to a shaped wax block to form a pouch which is filled with liquid paraffin. Each anococcygeus is attached by thread to an isometric transducer.

METHODS

Male Wistar rats (250–300 g) were anaesthetized with a mixture of halothane and nitrous oxide and pithed by the method of Gillespie, MacLaren & Pollock (1970). In this technique the pithing rod is a stainless-steel wire sliding in a fine teflon tube. Both wire and tube can be independently withdrawn to any position in the spinal canal, and the spinal outflow at that region stimulated through the stainless-steel inner wire. The length of wire exposed determines the number of segments stimulated. The position of the steel electrode within the canal was checked by radiography. A silver indifferent electrode was inserted under the skin parallel to the vertebral column. The spinal nerve roots were stimulated electrically by 1 msec pulses of supramaximal voltage at frequencies between 0·1 and 100 Hz, with an electrode length of 5 or 10 mm. Intravenous pancuronium bromide (1 mg/kg) was given to prevent muscle twitching. The two anococcygeus muscles join together on the ventral surface of the colon just short of the anal margin to form a ventral bar. The muscles were exposed by an incision in the scrotum just anterior to the anal margin, and the edges pinned on to a wax block to form a sac which was filled with liquid paraffin. The two muscles were then separated by splitting the ventral bar and each was attached by a thread to a Grass FTO 3 isometric transducer, and a tension of 1 g applied (Fig. 1). In some experiments the muscles were not separated and the tension in the two muscles together was recorded.

The rat's temperature was monitored by a rectal thermometer and maintained at 36° C by a tungsten lamp. Blood pressure was recorded from one carotid artery and one femoral vein was cannulated for drug administration. In some experiments the other femoral vein was cannulated for slow infusion of a tyramine hydrochloride solution (1 mg/ml. solution given at a rate of 10–40 μg/min). Muscle tension, blood pressure and heart rate were displayed on a Grass Polygraph.

The following drugs dissolved in normal saline were used. Doses refer to the salts. Atropine sulphate (B.D.H.); guanethidine sulphate (Ciba); hexamethonium bromide (Koch-Light); D-lysergic acid diethylamide tartrate (Sandoz); mecamylamine hydrochloride (Merck, Sharp and Dohme); (−)-noradrenaline bitartrate (Koch-Light); pancuronium bromide (Organon); phentolamine mesylate (Ciba); tyramine hydrochloride (Sigma).

RESULTS

The motor response

The spinal origin of the motor nerves

Responses to stimulation of successive 5 mm intervals along the spinal canal from S 2 to C 3 are shown in Fig. 2. On withdrawing the stimulating electrode, motor responses were obtained at two positions; first, between S 2 and L 6 and again between L 3 and T 11, with a gap between L 6 and L 3 (Fig. 2*a*). The maximal response was similar in size in both regions (4·9 g $\pm$ 0·2 g per single muscle). Responses were rapid in onset, starting within 1 sec of stimulation, and quick to decline at the end of the stimulation period. Beyond T 11 these rapid responses disappeared to be replaced by delayed responses which began after 10–15 sec, i.e. after end of stimulation. These responses are presumed to be due to

circulating catecholamines released from the adrenal medulla (Fig. 2*b*). No responses were obtained higher than T 2.

Regions below S 2 could not be separately stimulated since the narrowness of the canal prevented entry of the teflon tube which isolates the stimulating electrode. The precise lower limit of the lower motor outflow could not, therefore, be defined but experiments with finer teflon and the electrode extruded to the end of the vertebral canal suggest that outflows to the anococcygeus do not extend much beyond S 2.

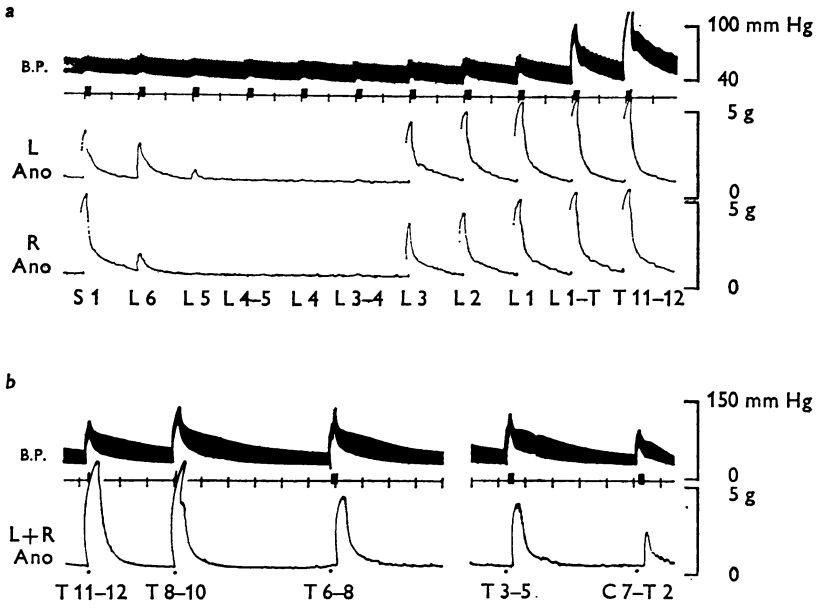

Fig. 2. The effect of stimulating at different levels of the vertebral canal on the response of the anococcygeus muscle and the blood pressure in the pithed rat. Stimulation was at 30 Hz for 10 sec with supramaximal voltage and is indicated by a dot below the trace. *a*, In this rat the tension in the left (L) and right (R) muscles was recorded separately. A 5 min exposure of the stimulating electrode was used, and levels from S 1 to T 11–12 examined. Motor responses were obtained from two regions, at L 6 and S 1 and again at and above L 3. Between L 3 and L 5 no response was obtained from the anococcygeus. *b*, In this rat the combined tension of the two anococcygeus muscles was recorded at higher levels of the vertebral canal explored with a 10 mm electrode exposure. At T 8–10 a second, delayed component appears in the response, and at higher levels this completely replaces the fast response coincident with stimulation seen at lower levels. The delay averages about 15 sec so that the response begins after the end of stimulation and is presumably due to catecholamine release from the adrenals. Time 1 min.

Frequency characteristics

The effect of 10 sec periods of stimulation at frequencies from 0·1 to 100 Hz was examined in both regions giving motor responses. Motor responses were obtained at all frequencies with a maximum at 30 Hz. At frequencies above 10 Hz, the response from the upper and lower

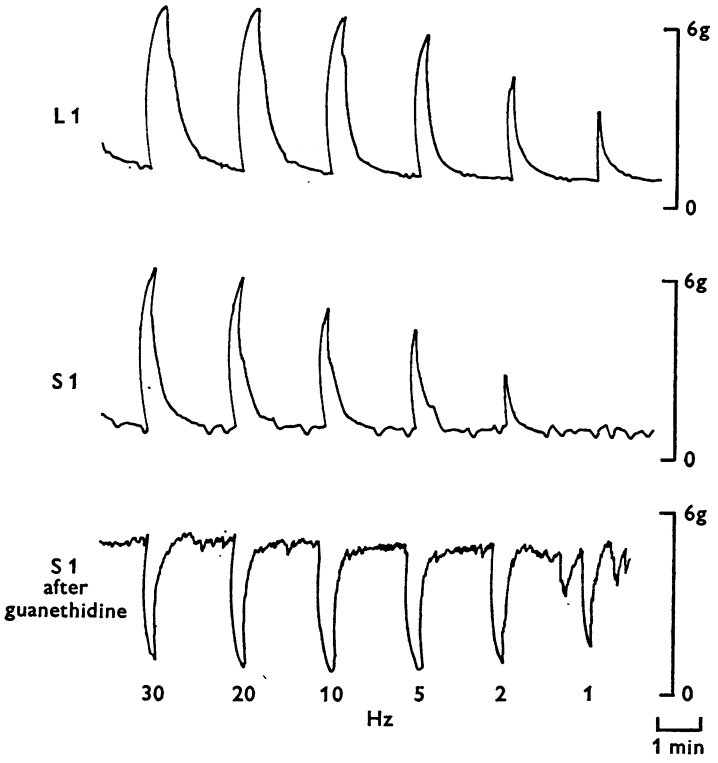

Fig. 3. Frequency characteristics of the motor and inhibitory responses in the anococcygeus muscle of a pithed rat from stimulation in the vertebral canal. The upper record shows the motor response from stimulation at L 1, the middle the motor response from stimulation at S 1, and the lower the inhibitory response from stimulation at S 1 in the presence of guanethidine (10 mg/kg). The frequency of stimulation is shown on the bottom record but applies to all. The duration of stimulation was 10 sec and the electrode exposure 5 mm.

regions were equal and had reached their maximum within the stimulation period of 10 sec. At low frequencies, responses were larger from stimulation in the upper than in the lower region and had not reached their maximum amplitude within the 10 sec of stimulation (Fig. 3).

When longer stimulation periods were used to compensate for this, the most effective frequency remained at 30 Hz, but frequencies as low as 0·5 Hz could eventually produce a plateau tension of some 70% of the maximum at 30 Hz.

Single pulses produced responses of approximately 10% of the maximum tension and summation of tension was seen at frequencies at or above 0·1 Hz (Fig. 4). At higher frequencies, up to 50 Hz, repetitive stimulation produced a smooth contraction well maintained for a 3 min period.

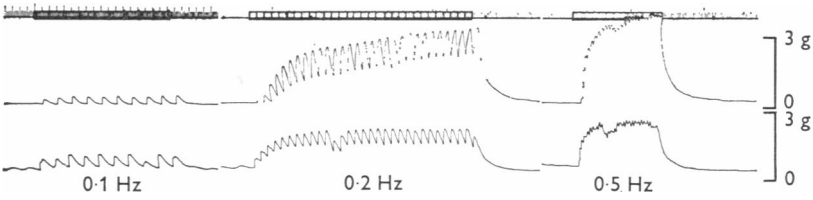

Fig. 4. The separate responses of the left and right anococcygeus muscles in the pithed rat to stimulation in the vertebral canal at L 1–2, at the frequencies shown at the bottom of each panel. At 0·1 Hz the responses to individual stimuli do not summate; at 0·2 Hz and 0·5 Hz summation occurs and if sufficient time is allowed, large tensions are developed. Time marker 1 and 5 sec.

Effect of blocking drugs

Responses from both motor regions were abolished by phentolamine (2 mg/kg) (Fig. 5*a*).

A short-lived inhibition of the motor response from the upper (L 3–T 11) region was produced by 1 mg/kg of hexamethonium, and the response completely abolished by 5 mg/kg. The motor response from the lower (S 2–L 6) region was unaffected by these doses of hexamethonium (Fig. 5*b*). Mecamylamine (5 mg/kg) gave similar results to hexamethonium. Atropine (1 mg/kg) and LSD-25 (400 μg/kg) had no effect on the motor responses from either region.

The motor response from stimulation higher in the spinal canal and due to liberation of catecholamines from the adrenals was abolished by phentolamine (2 mg/kg) and reduced by 70% by hexamethonium (5 mg/kg).

Drugs producing motor responses

In vitro the inhibitory response of the anococcygeus is seen only after raising the tone of the muscle (Gillespie, 1972). The actions of three drugs potentially capable of raising tone were examined *in vivo* to see which

was most able to produce and maintain a steady level of tone on which the inhibitory nerve response could be displayed and studied.

Single injections of noradrenaline (4 μg/kg) and tyramine (500 μg/kg) both produced motor responses which lasted 5–10 min and had no effect on the motor responses to stimulation. A single injection of guanethidine (10 mg/kg) produced a maximal motor response which was well maintained for 25–40 min after which spontaneous activity started and tension

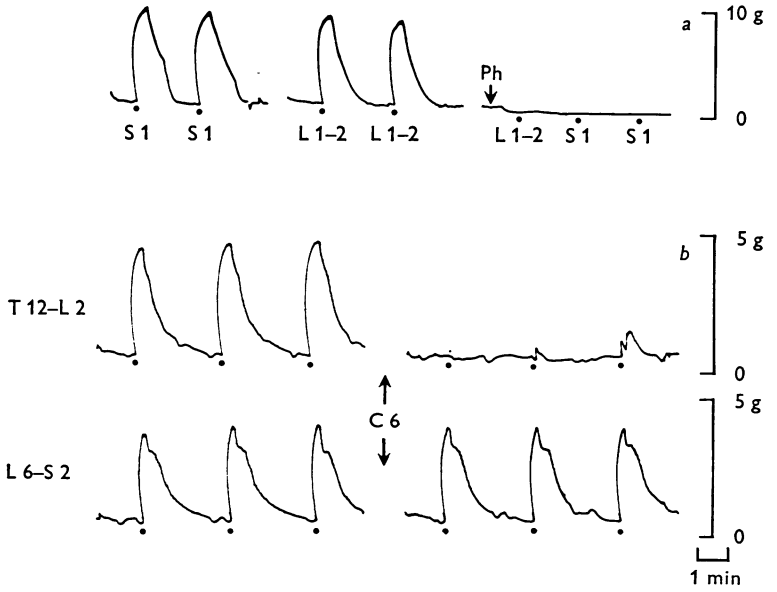

Fig. 5. *a*, This shows the effect of phentolamine (Ph: 2 mg/kg) on the motor response of the rat anococcygeus to stimulation in the vertebral canal at S 1 and at L 1–2. Phentolamine blocks both responses. *b*, This shows the effect of hexamethonium (C 6: 5 mg/kg) on the motor response of the anococcygeus in another rat to stimulation in the vertebral canal in two similar positions, L 6–S 2 and T 12–L 2. Hexamethonium blocks the motor response from the rostral stimulation without affecting that from the caudal. Stimulation was for 10 sec at 30 Hz. Electrode exposure, (*a*) 5 mm, (*b*) 10 mm.

gradually declined. After guanethidine, motor responses to stimulation in both spinal regions were abolished, but adrenal responses were little altered.

Phentolamine (2 mg/kg) abolished motor responses to noradrenaline, tyramine and guanethidine.

A slow infusion of tyramine (1 mg/ml. solution given at a rate of 40 μl./min) produced a maximal motor response well maintained for the

duration of the infusion, even when this was continued up to 2 hr. Slower rates of infusion of tyramine (20 μg/min and 10 μg/min) produced sub-maximal motor responses which were also well maintained.

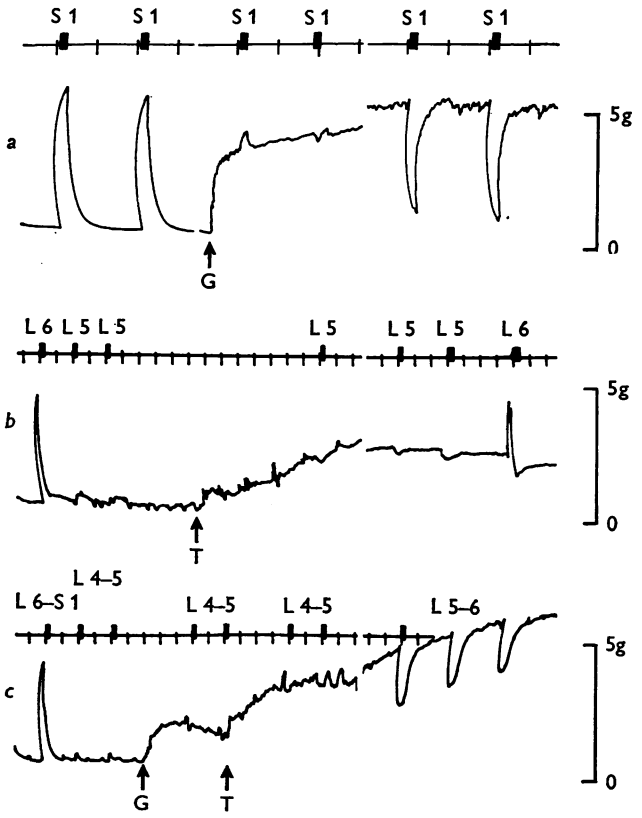

Fig. 6. Effect of guanethidine and tyramine on the anococcygeus muscle of the rat, and its response to nerve stimulation in the vertebral canal at the position shown above the time trace on each record. Time 1 min. *a*, The motor response to nerve stimulation before guanethidine (G: 10 mg/kg) was converted to inhibition in the presence of that drug which itself produced a prolonged rise in muscle tone. Tension was recorded from both muscles. Electrode exposure 5 mm. *b*, In another rat a slow infusion of tyramine (T: 20 μg/min) caused a maintained rise in tone but did not reverse the motor response to stimulation at L 6. Stimulation at L 5, however, which before tyramine was ineffective, caused inhibition in the presence of that drug. Tension was recorded from a single muscle. Electrode exposure 5 mm. *c*, In a third rat guanethidine (G: 5 mg/kg) caused only a small rise in tone; a tyramine infusion (T: 20 μg/min) further increased this. This record also shows the sharp boundary of the inhibitory outflow. Stimulation at L 4–5 produced little inhibition whereas at L 5–6 large inhibitory responses were obtained. Record from a single muscle. Electrode exposure 10 mm.

The inhibitory response

Spinal origin

When the tone of the muscle was raised by guanethidine (10 mg/kg) stimulation in the region L 5–S 2 produced inhibitory responses. In individual experiments, stimulating at 30 Hz for 10 sec, this inhibition

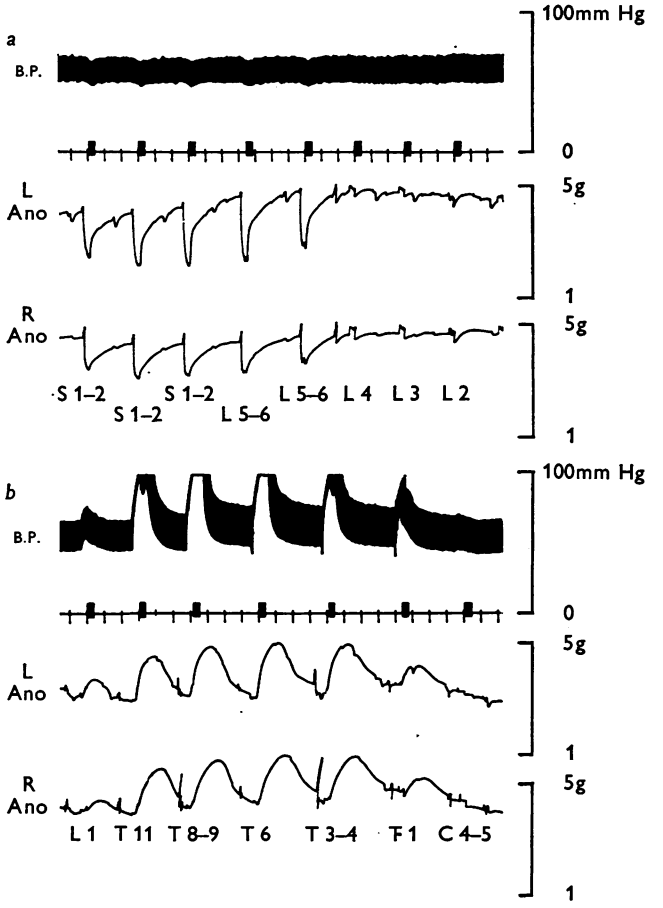

Fig. 7. Responses of the anococcygeus and the blood pressure in the pithed rat to stimulation in the vertebral canal at 30 Hz at the levels shown below each record. An initial injection of guanethidine (G: 5 mg/kg) was given to reverse the response to stimulation to inhibition. The two sets of records are successive and represent exploration of the reponse to stimulation between S 1–2 and C 4–5. *a*, Shows that the inhibitory response is confined to stimulation in the region S 2–L 5. *b*, Shows that more rostral stimulation in the thoracic region can restore the motor response as a consequence of liberating catecholamines from the adrenal gland. At these levels there is also a large vasopressor response. Time 1 min.

could amount to 90% of the induced tone (Fig. 6*a*). The average inhibition in all experiments was 60%.

When the tone was raised with tyramine (20 μg/min), which did not abolish motor responses, stimulation in the upper end of this region (L 5) produced small inhibitory responses, whereas stimulation in the lower part of the same region (L 6–S 2) produced a biphasic response, contraction followed by relaxation (Fig. 6*b*).

From these results, guanethidine was clearly the better drug on which to study inhibitory responses, presumably because of its neuronal blocking action on the adrenergic motor nerves in addition to its indirect symthomimetic action in raising tone. Guanethidine had one disadvantage; after 25–40 min the tone began to fall and rhythmic activity appeared. In these circumstances it was difficult to demonstrate inhibition. The possibility was, therefore, investigated of reinforcing the motor action of guanethidine with a slow infusion of tyramine, thus combining the neurone blocking action of the former with the steady induced tone of the latter.

Guanethidine (5 mg/kg) was given and a slow infusion of tyramine (20 μg/min) started 5 min later. Tone in the anococcygeus was raised and could be maintained for 2 hr, producing optimum conditions for studying the inhibitory response (Fig. 6*c*). Under these conditions, the spinal origin of the inhibitory fibres was determined by stimulating at successive 5 mm intervals along the spinal canal as shown in Fig. 7. Inhibitory responses were obtained only between S 2 and L 5. No inhibitory responses were found at the upper region giving motor responses, or at any higher level up to C 4. The region giving inhibitory responses (S 2–L 5) overlapped with the lower region giving motor responses (S 2–L 6) but the former extended one segment more rostral. Stimulation in the region corresponding to the efferent fibres to the adrenals still produced a delayed contraction from catecholamine liberation.

Frequency characteristics

The inhibitory response *in vivo* showed the same high sensitivity to low frequencies of stimulation as was noticed *in vitro* (Gillespie, 1972). Maximum responses were obtained with a 10 sec period of stimulation between 2 and 5 Hz (Fig. 3). Responses were rapid in onset and well maintained over a 20 sec period.

Effect of blocking drugs

Clearly nerve fibres mediating inhibition in the anococcygeus were located in the spinal cord. The question of whether this pathway to the

muscle was interrupted by a ganglion relay was investigated by examining the effects of hexamethonium and mecamylamine.

Hexamethonium or mecamylamine (5 mg/kg) completely abolished the inhibitory response. This is shown for hexamethonium in Fig. 8. When recovery from this inhibition occurred, a second similar dose had little effect in renewing the block. If, initially, a small (1 mg/kg) dose of hexamethonium was given, a transient reduction in the response was observed.

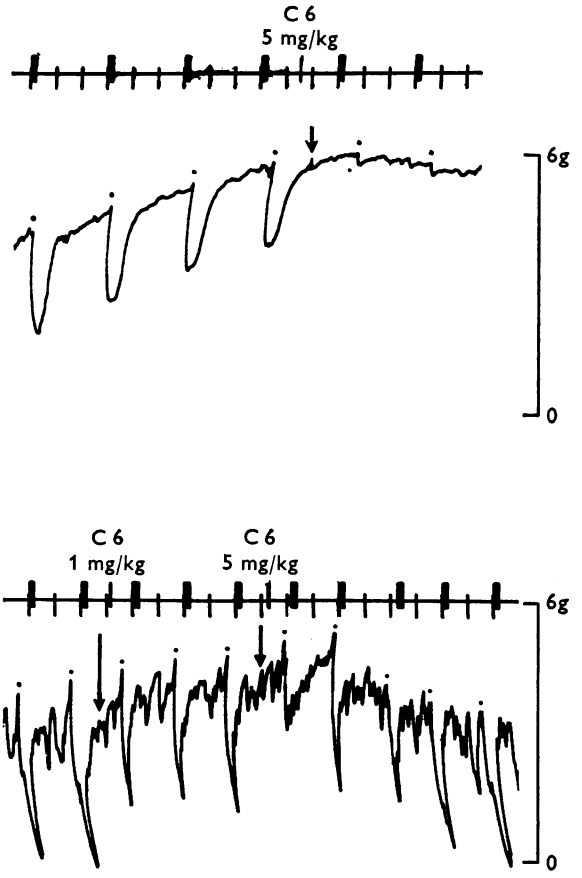

Fig. 8. Effects of hexamethonium (C 6) on the inhibitory responses of the anococcygeus of the pithed rat to stimulation at 30 Hz for 10 sec in the vertebral canal at L 6–S 1. The start of each stimulation period is indicated by a dot and the injection of hexamethonium by an arrow. In the upper record a single dose of hexamethonium (5 mg/kg) almost completely abolishes the inhibitory response. In the lower trace a smaller dose of hexamethonium (1 mg/kg) caused a small reduction in the size of the inhibitory response and a subsequent larger dose (5 mg/kg) produces only a transient block of the response. Time 1 min.

On recovery from this, the tissue was insensitive to subsequent large (5 mg/kg) doses of the drug.

Atropine was also studied since there is the possibility of muscarinic receptors in ganglia (Steinberg & Hilton, 1966). Atropine (1 mg/kg) had no effect on inhibitory responses.

DISCUSSION

Of the two questions posed at the outset of these investigations, the answer to the first is clear. There is an inhibitory nerve outflow from the spinal cord to the anococcygeus muscle whose frequency and drug sensitivity resembles that of the nerves involved in the inhibitory response *in vitro* (Gillespie, 1972). It seems most unlikely that this inhibitory outflow could be the sympathetic adrenergic outflow liberating some additional transmitter whose inhibitory effect is uncovered by blocking the release of noradrenaline by guanethidine, for the following reasons. First, the level of origin of the motor and inhibitory fibres differs. The preganglionic motor fibres arise from L 3–T 11 whereas the inhibitory preganglionic fibres arise from S 2–L 5. Secondly, in the presence of tyramine, when the motor responses to stimulation either of the preganglionic outflow at L 3–T 11 or the post-ganglionic at S 2–L 6 are not blocked, stimulation at L 5 produces a pure inhibitory response, i.e. adrenergic blockade is not an essential requirement before inhibition can be demonstrated. These results are supported by the observation that destruction of the adrenergic innervation by 6-hydroxydopamine abolishes the motor adrenergic response but leaves the inhibitory response unaltered (Gibson & Gillespie, 1973).

The second question, whether the inhibitory outflow is organized in the pattern of the autonomic nervous system, hinges round the action of the ganglion blocking agents hexamethonium and mecamylamine. The effects of these two drugs are similar. They abolish the adrenergic motor response to stimulation in the upper (preganglionic) region. This block is reasonably long lasting and when recovery takes place a second dose of ganglion blocking drug will restore the block. On the inhibitory response the evidence for ganglion blockage was less convincing. If a large dose of drug was given, the inhibitory response was convincingly blocked and remained so for some time. As the response returned, however, a second large dose produced a short-lived inhibition only. If a small initial dose were used, it itself produced only slight transient block but was effective in producing desensitization, so that a second large dose was almost ineffective.

The doubt these results threw on the presence of a ganglion in the

peripheral pathway made us examine the sensitivity of another organ with an adrenergic motor innervation from the lumbar cord – the vas deferens. This was found to be as easily desensitized to the action of hexamethonium as the inhibitory pathway to the anococcygeus muscle. A large dose caused inhibition of the motor response which quickly 'escaped', whereupon a second similar dose had only a slight short-lived action. Since there is certainly a synapse in the motor pathway to the vas deferens (Sjöstrand, 1965) we feel the results consistent with the presence of a synapse in the inhibitory outflow to the anococcygeus muscle.

An observation of some interest was the ability to stimulate the post-ganglionic adrenergic fibres from the lower lumbar–upper sacral region of the spinal canal. In previous investigations of this method of stimulating the spinal autonomic outflows, it had never been possible to stimulate post-ganglionic fibres (Gillespie *et al.* 1970). One explanation for the present exception could have been the position of the anococcygeus muscle directly in front of the vertebral column and having a tendinous origin from the upper coccygeal vertebrae. These conditions might have been particularly favourable for direct stimulation of intramural nerves in a muscle attached to bone overlying the stimulating electrode. This was checked by marking the position of the upper tendinous origin of the anococcygeus muscle with a metal marker and comparing by X-ray the relative positions of this marker and the spinal electrode. There was always a vertical displacement of at least 20 mm so it is unlikely that motor intramural nerves were stimulated within the muscle itself. The ability to stimulate the post-ganglionic motor nerves at least 20 mm from the muscle suggests that in this tissue, unlike the vas deferens, motor innervation is by conventional post-ganglionic 'long' adrenergic neurones.

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