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EVIDENCE FOR DOPAMINERGIC VASODILATOR INNERVATION OF THE CANINE PAW PAD

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1 In chloralose-anaesthetized dogs pretreated with guanethidine and pancuronium, electrical stimulation (0.2 to 5 Hz) of the peripheral end of the cut tibial nerve caused a frequency-dependent increase in femoral blood flow which was restricted to the paw pads.

2 This neurogenic vasodilatation was not attenuated by atropine, mepyramine plus burimamide, indomethacin or propranolol. It was, however, attenuated in a dose-dependent manner by intra-arterial administration of the dopamine receptor antagonist, ergometrine (0.05 to 0.5 mg).

3 The effect of ergometrine could not be explained by non-specific effects on axonal conduction or transmission or by vasospasm of the blood vessels of the paw-pads.

4 In dogs with intact tibial nerves, a pharmacologically similar dilator response localized to the paw-pads could be elicited by electrical stimulation of loci in the ipsilateral diencephalon and mid-brain. This response was not due to inhibition of adrenergic vasomotor tone and was abolished by systemic ganglion blockade or by tibial nerve section as well as by femoral arterial administration of ergometrine.

5 It is suggested that the vasculature of the canine paw pads is innervated by a population of autonomic axons which utilize dopamine or a related substance as a transmitter substance and activation of which causes vasodilation.

Introduction

The limb vasculature of cats and dogs receives dilator nerves of at the least three types. The skeletal muscle vascular bed is supplied by cholinergic axons (Folkow & Uvnäs, 1948; Eliasson, Lindgren & Uvnäs, 1953). A second, non-cholinergic innervation to both skeletal muscle and to skin vessels can also be demonstrated, the effects of which can be antagonized by histamine H_1-receptor antagonists (Brody, 1966; Tuttle, 1966; Graham & Lioy, 1973; Lang, Bell, Conway & Padanyi, 1976), suggesting that histamine could be the peripheral transmitter involved. A third population of nerves produces dilator responses which are resistant to antagonism both by atropine and by histamine H_1 and H_2-receptor antagonists (Beck, Pollard, Kayaalp & Weiner, 1966; Zimmerman, 1968; Brody & Schaffer, 1970; Ballard, Abboud & Mayer, 1970; Lang *et al.*, 1976). The site of action of this last group of nerves is predominantly within the vasculature of the paw (Zimmerman, 1968; Brody & Schaffer, 1970; Rolewicz & Zimmerman, 1972).

The canine femoral vascular bed is known to contain vasodilator receptors for dopamine which are distinct from β-adrenoceptors (Bell, Conway, Lang & Padanyi, 1975), and recently it has been demonstrated that these dopamine receptors are localized predominantly within the vasculature of the paw pads (Bell & Stubbs, 1978). It was therefore of interest to determine whether the pharmacological characteristics of the atropine- and antihistamine-resistant neurogenic dilator response in the paw are consistent with its mediation by dopamine.

Methods

Cardiovascular monitoring

Adult mongrel dogs of either sex weighing 8 to 25 kg were anaesthetized with α-chloralose (70 mg/kg, i.v.), following induction with thiopentone sodium and were artificially respired under positive pressure, using minute volumes derived from a standard nomogram (Harvard Apparatus). Blood flows through one or both femoral arteries were recorded by means of cuff-type electromagnetic flow probes and a Devices 2-channel blood flow meter. The flow probes were selected from those with luminal diameters of 2.7, 3.3 or 3.9 mm so as to produce approximately 10% con-

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striction of the arteries. Zero flow calibration was obtained by occlusion of the femoral artery distal to the probe. In several animals, following termination of the experiment, the arterial segment from which flow had been recorded, with the flow probe in place, was cannulated and perfused with Krebs solution using a roller pump. Over the range of flows measured *in vivo* (50 to 500 ml/min) there was a linear and accurate (to within 5%) relation between the flowmeter output and actual arterial flow as measured by timed collection of Krebs solution in a graduated cylinder. Systemic blood pressure was measured via a catheter inserted into a branch of the right femoral artery and heart rate was monitored from lead I or II ECG through a cardiotachometer. All parameters were recorded simultaneously on a Beckman R411 Dynograph or a Grass 7B polygraph. Local injections of drugs into the vascular bed of the leg were made through a cannula passed up to the bifurcation of the aorta from a cutaneous femoral branch. The total volume of injection and saline (0.9% w/v NaCl solution) was maintained at 1 ml or less: this volume of saline alone had no effect on recorded blood flow.

Peripheral nerve stimulation

The peripheral ends of the cut tibial and fibular nerve trunks were stimulated via silver Dastré electrodes placed on the tibial trunk just above the ankle joint and on the fibular trunk where it passes over the lateral head of the gastrocnemius muscle. Pancuronium bromide (0.1 mg/kg, i.v.) was administered to prevent interference with blood flow by skeletal muscle activation. The site of stimulation was covered by a paraffin pool and 1 ms pulses from a Grass SD9 stimulator were applied at frequencies of 0.2 to 5 Hz and supramaximal voltage (20 to 30 V) for 5 to 10 s.

Central nervous system stimulation

The dogs were immobilized in a stereotaxic apparatus (Narashige) and coaxial stimulating electrodes (Rhodes Medical Instruments NE 100) were passed down vertically through a window in the skull into the left diencephalon and midbrain between 2 and 5 mm lateral to the midline, to an initial depth of 25 mm below the cortical surface. Biphasic pulses from a Grass SD9 stimulator were applied at a frequency of 60 Hz for periods of 20 s, with a pulse duration of 2 ms and a constant current between 0.3 and 1.0 mA. The electrodes were subsequently lowered in 1 to 2 mm steps and stimulation repeated at each level. Only increases in blood flow which occurred without concomitant skeletal muscle activation, tachycardia or increased blood pressure were accepted as active dilator responses. Sites which produced active dilatation were generally restricted to a vertical range of 1 to 2 mm, indicating that there was little current spread from the stimulating electrode. At the conclusion of each experiment, electrolytic lesions were made at the site of stimulation, and coronal sections of the brain examined as described previously (Lang *et al.*, 1976).

Localization of vascular responses

Tight bandage ligatures around the ankle or just above the metacarpal paw pad, were used to occlude the circulation to the whole paw or to the paw pads respectively. When an ankle ligature was applied while arterial pressure was monitored in the dorsal pedal artery of the paw, the pressure recorded fell rapidly to less than 10 mmHg and no pulsations were detectable. Excision of the keratinous outer layer of the paw pads resulted in arterial oozing from the subpapillary tissue. This ceased on application of a ligature just above the metacarpal pad and recommenced if the ligature was released once again within 60 s. It was therefore considered that ligatures at either site effectively cut off the arterial circulation to more distal areas of the limb, and that the amounts by which recorded femoral flow fell during ligation would approximate the blood flows to such areas. Furthermore, ligation for several minutes during and after a period of nerve stimulation could be used to determine whether the vessels participating in the nerve-evoked response were proximal or distal to the point of ligation. In several experiments where dilator responses were abolished by ligation, similar periods of ligation did not reduce responses evoked within a few seconds after releasing the ligature. In addition, releasing the ligature within 10 s following nerve stimulation was associated with a flow increase which was considerably greater than the small reactive hyperaemia seen with equivalent periods of ligation in the absence of stimulation, and which was similar in time course to a normal nerve-evoked response. Thus it was considered that local hypoxia due to the periods of ligation used did not itself interfere with the nerve-evoked responses.

Drugs used

All animals were pretreated with guanethidine sulphate (10 mg/kg, i.v. 2 h before or 5 mg/kg s.c. 18 and 2 h before the start of experimentation) in order to prevent dilator responses due to central inhibition of adrenergic discharge and to enable dilations during peripheral nervous stimulation to be studied without interference from concomitant vasoconstriction. These regimens of guanethidine treatment appeared to abolish completely neural release of noradrenaline, as routine administration of hexamethonium bromide

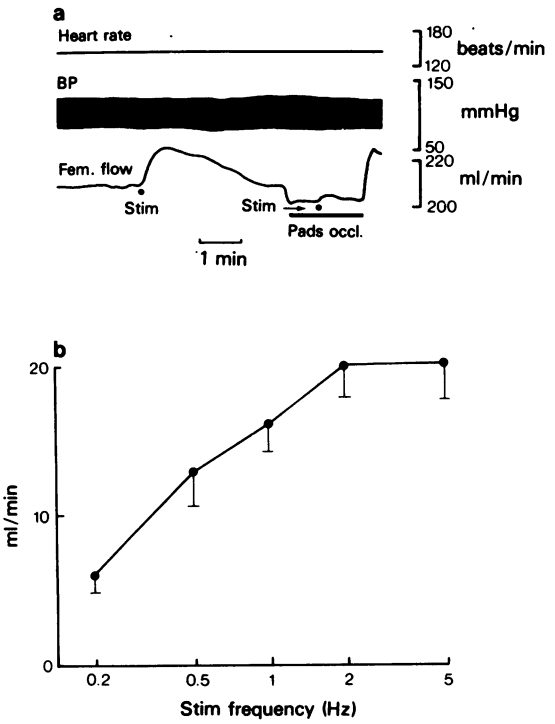

Figure 1 (a) Heart-rate, blood pressure and mean left femoral blood flow in an anaesthetized dog pretreated with guanethidine, atropine and pancuronium. At the black dots (● Stim) the peripheral end of the cut left tibial nerve was stimulated electrically for 5 s at 2 Hz. The femoral dilator response to stimulation was localized to the vasculature of the paw pads, as it was almost abolished when the circulation to this area was occluded with a bandage ligature (Pads occl.) during stimulation. (b) Frequency-dependence of the femoral dilator response to tibial nerve stimulation (expressed as ml/min flow increments), over the frequency range 0.2 to 5 Hz. Each point represents the mean of values from 5 dogs. The vertical lines represent one s.e. mean.

(10 mg/kg, i.v.) at the end of each experiment caused no detectable increase in resting femoral flow and little (less than 10 mmHg) or no fall in systemic blood pressure. In addition, vasoconstrictor responses to peripheral nerve stimulation could be elicited before, but not 30 min after, administration of 10 mg/kg guanethidine.

Except where otherwise specified, atropine methonitrate (0.4 mg/kg i.v.) was administered at the beginning of each experiment. Following this treatment, femoral dilator responses to injected acetylcholine perchlorate (0.5 to 1 μg, i.a.) were absent for the entire period of the experiment. Peripheral responses to electrical stimulation which were obtained were therefore considered to be noncholinergic.

Other drugs used were mepyramine maleate (May & Baker), propranolol hydrochloride (ICIANZ), isoprenaline hydrochloride (Sigma), glyceryl trinitrate (Wellcome), indomethacin (Merck, Sharpe & Dohme), ergometrine meleate (Wellcome), burimamide hydrochloride (Smith, Kline & French) and the bradykinin-potentiating nonapeptide pyro-Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (BPP_{9a}; SQ 20,881; Engel, Schaeffer, Gold & Rubin, 1972).

Results

For 40 dogs used, mean arterial blood pressure was 108 ± 3.6 mmHg (mean ± s.e. mean). Resting femoral blood flow was 216 ± 21.3 ml/min and resting flow through the paw pads, as judged from the fall in flow during tightening of a ligature above the metacarpal pad, was 24 ± 2.5 ml/min. This is similar to the values reported by previous workers (Ballard *et al.*, 1970; Baker, 1972) and corresponds to a flow of approximately 60 ml/min per 100 g paw.

Dilator responses to peripheral nerve stimulation

Tibial nerve Electrical stimulation of the peripheral stump of the cut tibial nerve caused a frequency-dependent increase in femoral blood flow which was maximum at about 2 Hz (Figure 1), and which was not accompanied by appreciable or consistent changes in blood pressure or heart rate. In 19 dogs studied the mean flow increase produced by stimulation at 2 Hz for 5 s was 26 ± 2.8 ml/min. Most of this response was localized in the vasculature of the paw pads as occlusion of the paw pad circulation during the period of stimulation reduced the amplitude of the response to 5 ± 1.3 ml/min. Occlusion of the circulation to the rest of the paw abolished any residual response. Responses to tibial stimulation were characteristically quite prolonged, restoration of resting flow levels following a 5 s period of stimulation taking several minutes (Figure 1).

Fibular nerve Stimulation of the peripheral end of the cut fibular nerve also produced a frequency-dependent increase in femoral blood flow. Under the usual conditions of our study, where atropine was administered at the beginning of the experiment, the localization of these responses was similar to those produced by tibial stimulation. If, on the other hand, the fibular nerve was stimulated in the absence of atropine and with the paw circulation occluded, then a short-lived dilator response occurred within the leg vasculature. This response was abolished by atropine, and presumably represented activation of sympathetic cholinergic axons supplying skeletal muscle vessels.

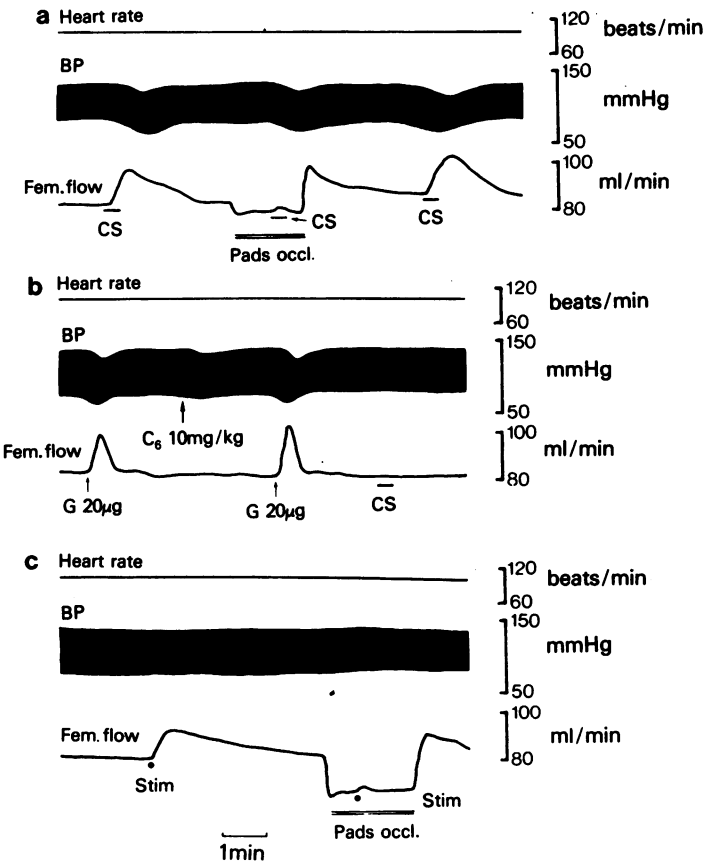

Figure 2 Heart rate, blood pressure (BP) and mean left femoral blood flow (Fem. flow) in an anaesthetized dog pretreated with guanethidine, atropine and pancuronium. (a) Electrical stimulation of the central nervous system in the left diencephalon (CS) for the periods of the short horizontal bars produced an increase in left femoral blood flow, which was localized to the vasculature of the pads as it was abolished when the circulation to this area was occluded with a bandage ligature during stimulation (Pads occl.). (b) Systemic ganglion blockade with hexamethonium (C_6, 10 mg/kg) abolished the dilator response to central stimulation without affecting dilator responses to intra-arterial glyceryl trinitrate (G, 20 øg) or altering resting blood pressure or femoral blood flow. (c) Following ganglion blockade, dilator responses localized to the paw pad circulation could be elicited by electrical stimulation of the peripheral end of the cut left tibial nerve (● Stim).

Dilator responses to central nervous system stimulation

Stimulation of various loci in the left diencephalon and midbrain in dogs pretreated with guanethidine and atropine produced in some animals left femoral dilator responses in the paw pads, and in others dilator responses which occurred either evenly throughout the whole paw or in the leg. Only animals which exhibited dilator responses at least 80% of which were localized in the paw pads were used in the present study. In 21 such animals, the mean increase in femoral flow produced by central stimulation was 18 ± 2.3 ml/min and was accompanied by a fall in blood pressure of between 5 and 20 mmHg, but by no change in heart rate (Figure 2). The depressor response to stimulation was not secondary to the paw pad vasodilatation, as it was not reduced substantially by occlusion of the circulation of all four paws during stimulation and was of variable latency relative to the dilator responses. Its origin therefore remains uncertain. Bilateral femoral dilator responses were observed in only 1 of 5 dogs in which both left and right femoral flows were recorded. In four animals tested, hexamethonium (10 mg/kg i.v.) abolished both the dilator and the depressor responses to central stimulation, without alteration in resting femoral flow

or blood pressure or in femoral reactivity to intra-arterial injections of 5 to 50 μg glyceryl trinitrate (Figure 2). In 5 of a further 6 animals, the dilator responses to central stimulation were abolished by section of the tibial nerve trunk just above the ankle. In the sixth dog tibial section reduced but did not abolish the response: the response remaining was abolished by section of the fibular nerve trunk below the knee. Subsequent to hexamethonium or to nerve section, and in the presence of pancuronium, stimulation of the peripheral stump of the tibial nerve produced an increase in femoral flow which was localised predominantly in the paw pad circulation and was similar in magnitude to that previously evoked by central stimulation (Figure 2).

Peripheral pharmacology of the neurogenic paw pad-localized vasodilatation

β-Adrenoceptor involvement Dilator responses to central (3 dogs) or tibial nerve stimulation (2 dogs) were compared before and after administration of the β-adrenoceptor antagonist, propranolol (0.05 to 0.1 mg/kg, i.v.). This treatment greatly reduces femoral dilator responses to intra-arterial isoprenaline (Bell & Mya, 1977). In each of the present experiments femoral responses to local administration of 0.1 and 0.5 μg isoprenaline were reduced in amplitude by at least 80% after propranolol. However, in no instance were responses to stimulation altered in amplitude by more than 5%.

Histamine receptor involvement Responses to central (5 dogs) or tibial nerve (2 dogs) stimulation were compared before and after combined administration of the histamine H_1-receptor antagonist, mepyramine (2 mg/kg, i.a.) and the histamine H_2-receptor antagonist, burimamide (2 mg/kg i.a.). This treatment markedly reduces dilator responses of the femoral bed to intra-arterial histamine and abolishes responses to activation of certain non-cholinergic dilator nerves which supply both the leg and the paw (Lang *et al.*, 1976). In the present experiments femoral responses to local administration of histamine (2 and 20 μg) were reduced in amplitude by at least 80% after combined antihistamine administration. However, responses to stimulation were not altered in amplitude by more than 5% in any animal.

Kinin involvement Femoral dilator responses to bradykinin (0.5 μg, i.a.) and to central (4 dogs) or to tibial nerve (2 dogs) stimulation were compared before and after infusion into the femoral artery of the bradykinin-potentiating nonapeptide SQ 20,881, at a rate of 500 μg/min. In the presence of this peptide, dilator responses to bradykinin were increased in amplitude and duration by 200 to 400%. (mean amplitude increase; 320%) but those to electrical stimulation were not changed by more than 10% in either amplitude or duration.

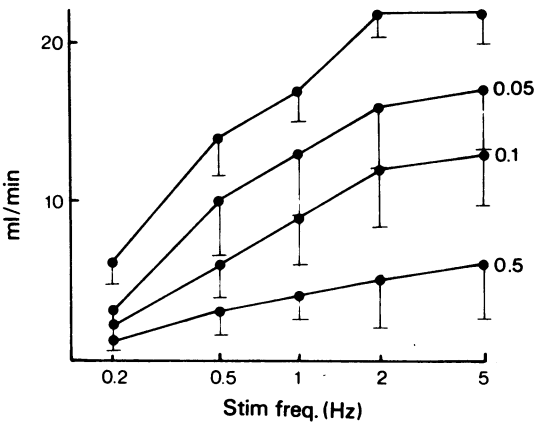

Figure 3 Frequency-response curves for pad-localized dilator responses of the dog femoral vasculature (expressed as ml/min flow increments) to tibial nerve stimulation at 0.2 to 5 Hz under control conditions and following intra-arterial administration of the dopamine-receptor antagonist, ergometrine, in consecutive doses of 0.05 mg, 0.1 mg and 0.5 mg. Each point represents the mean of values from four dogs. The vertical lines represent one s.e. mean. When tested using a two-tailed, paired Student's *t*-test, the responses to stimulation at all frequencies examined were reduced significantly ($P < 0.02$) by 0.1 mg ergometrine.

Prostaglandin involvement The amplitude and duration of responses to tibial nerve stimulation were unaffected (less than 10% change from pretreatment values) 1 h after systemic administration of the prostaglandin synthetase inhibitor (Vane, 1971), indomethacin (10 mg/kg i.p.), in either of 2 dogs tested.

Dopamine receptor involvement Responses to central (4 dogs) and tibial nerve (5 dogs) stimulation were compared before and after administration of ergometrine in a dose of 0.5 mg, intra-arterially, which has been shown previously to attenuate considerably femoral dilator responses to exogenous dopamine (Bell *et al.*, 1975). Ergometrine reduced the amplitudes of responses to central stimulation from 29 ± 6.0 ml/min to 2 ± 0.3 ml/min and of responses to tibial nerve stimulation from 23 ± 6.1 ml/min to 6 ± 2.2 ml/min. When tested by a two-tailed paired Student's *t* test, the effect of ergometrine was significant ($P < 0.02$) for each set of responses. In another series of 4 dogs, increasing doses of ergometrine from 0.05 mg to 0.5 mg intra-arterially produced progressive attenuation of responses to tibial nerve stimulation (Figure 3). Administration of ergometrine was associ-

ated with a variable degree of reduction in total resting femoral blood flow and with a sustained increase in arterial blood pressure. It therefore seemed possible that it might attenuate neurogenic dilator responses in the paw pads by causing spasm of these vessels. In 6 dogs, the local vasoconstrictor effect of graded doses of ergometrine (0.05 to 0.5 mg i.a.) on resting paw pad flow (as judged by the fall in total femoral flow during brief application of a tight occlusive bandage above the metacarpal pad) was compared with its effect on dilator responses to tibial nerve stimulation. While in 2 of these dogs the amount by which resting pad flow fell after ergometrine paralleled the amount by which nerve-evoked responses were reduced in amplitude, in the remaining 4 animals nerve-evoked responses were always affected more than was resting pad flow. In three of these experiments, a total of 0.85 mg ergometrine reduced nerve-evoked responses from 12, 18 and 21 ml/min to 1, 0 and 6 ml/min respectively without altering resting flow by more than 1.5 ml/min in any instance. In the fourth experiment, the lower doses of ergometrine (total 0.35 mg) reduced the nerve-evoked response from 17 ml/min to 11 ml/min with no measurable change in resting flow, but addition of a further 0.5 mg ergometrine which lowered the amplitude of the nerve-evoked response to 5 ml/min also reduced resting flow from 18 ml/min to 12 ml/min. Thus although variable results were obtained, it was clear from these experiments that, at least in some animals, profound attenuation of neurogenic dilatation occurred without concomitant reduction in resting pad blood flow.

In 3 dogs isometric twitch responses of the flexor digitorum longi were recorded during stimulation of the fibular nerve at 0.5 Hz. Injections of up to 2 mg ergometrine intra-arterially had no observable effect on the amplitude of the response. By contrast, injection of pancuronium (20 μg/kg, i.a.) caused complete neuromuscular blockade within a few seconds. In a further 6 dogs the paw circulation was occluded with a ligature above the ankle, and cholinergic dilator responses in the leg to fibular nerve stimulation (2 Hz) were monitored. Injection of 0.5 mg ergometrine (i.a.) had a variable but small depressant effect on these responses, reducing the amplitude from 18 ± 3.1 ml/min to 14 ± 2.2 ml/min. It therefore seemed unlikely that the depressant effect on tibial dilator responses of ergometrine could be explained by non-specific depression of axonal conduction or transmitter release.

Discussion

Electrical stimulation of the peripheral end of the cut tibial nerve produced an increase in femoral blood flow which was not secondary to skeletal muscle activation or to inhibition of adrenergic vasomotor tone, and was not attenuated by systemic administration of atropine. This vasodilator response occurred predominantly within the vasculature of the paw pads. Experiments involving central nervous system stimulation indicated that the tibial dilator axons had connections with the midbrain through autonomic ganglia. The responses obtained could therefore be regarded as due to autonomic axon rather than to sensory axon activation. In addition, they were not affected by the carboxypeptidase inhibitor, SQ 20,881 when administered in a concentration that greatly potentiated femoral dilator responses to bradykinin, indicating that they were not secondary to kinin release from paw pad sweat glands (Hilton & Lewis, 1957).

Use of other pharmacological agents demonstrated that the dilator response to tibial stimulation was not due to neural release of histamine, prostaglandins or to β-adrenoceptor activation. However, it was attenuated in a dose-dependent manner by the alkaloid, ergometrine, which we have shown to act in the canine vascular system as an antagonist at dopamine receptors in doses that do not affect vasodilator responses to acetylcholine, isoprenaline, histamine, bradykinin, 5-hydroxytryptamine (Bell *et al.*, 1975), adenosine triphosphate or prostaglandin E_2 (C. Bell & W.J. Lang, unpublished observations).

The depressant effect of ergometrine on tibial dilator responses could not be ascribed to nonspecific depression of axonal conduction or transmission, or to vasospasm of the blood vessels of the toes sufficient to prevent active dilatation. Its effect is therefore compatible with the concept that the transmitter liberated by the dilator axons in the tibial nerve is dopamine or a related substance. Such a view is consistent with the observation that the majority of femoral dopamine receptors are localized in the vasculature of the paw pad (Bell & Stubbs, 1978). It is also supported by histochemical and biochemical data which indicate that the arteriovenous anastomoses of the paw pads are innervated by a population of non-adrenergic axons containing high levels of dopamine (Bell, Lang & Laska, 1978a).

In view of the many biochemical and pharmacological characteristics which are common to both adrenergic and dopaminergic neurones (Vogt, 1969; Schumann & Kroneberg, 1970), it was striking that we appear to be able to evoke transmitter release from dopaminergic terminals after guanethidine treatment sufficient to prevent completely release of transmitter from adrenergic axons. The adrenergic neurone-blocking action of guanethidine is associated with active axonal uptake of the drug (Mitchell & Oates, 1970) and displacement of vesicular amine (Cass & Spriggs, 1961; Boullin, Costa & Brodie, 1966). It appears that one or both of these processes occurs

less effectively in dopaminergic axons, as biochemical and histochemical data have demonstrated that guanethidine does not deplete transmitter stores in dopaminergic terminals at doses that do deplete stores in adrenergic terminals (Evans, Singer, Armstrong, Saunders & Burnstock, 1975; Bell, Lang & Laska, 1978a, b). In contrast, reserpine is effective in causing transmitter depletion in both neurone types (Lidbrink, Jonsson & Fuxe, 1974; Bell *et al.*, 1978a, b). In the context of the present experiments, it is therefore of note that sciatic nerve stimulation has been reported to cause marked vasodilatation in the dog hind paw following guanethidine pretreatment, but not following reserpine treatment (Ballard *et al.*, 1970).

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