INHIBITORY NEUROTRANSMISSION

IN

THE BOVINE RETRACTOR PENIS

AND

THE RAT ANOCOCCYGEUS MUSCLE

A thesis presented for the degree of Master of Science

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SUMMARY

1. The aims of this study were twofold. First to try to detect the non-adrenergic non-cholinergic (NANC) neurotransmitter in the bovine retractor penis by bioassay and secondly to study the effects of various drugs which might reveal its relationship with nitric oxide and endothelium-derived relaxing factor (EDRF). This second objective was pursued in both the bovine retractor penis and rat anococcygeus muscles.

2. The attempt to detect the NANC inhibitory neurotransmitter released by field stimulation from the donor bovine retractor penis muscle and detected by a variety of test tissues (bovine retractor penis, rat anococcygeus and rabbit aortic strip) arranged in several different test systems failed.

3. Superoxide dismutase (10 u./ml. up to 400 u./ml.) had no effect on NANC inhibitory relaxation and had no measurable effect in protecting the NANC neurotransmitter.

4. L-Arginine, a precursor of EDRF, did not potentiate NANC inhibitory relaxation in the BRP either fresh or cold stored up to ten days.

5. L-Monomethyl-N-arginine (L-NMMA, a competitive inhibitor of L-arginine in endothelial cells) and D-monomethyl-N-arginine (D-NMMA) up to 3×10^{-4}M. were without effect on NANC inhibitory relaxation of the

bovine retractor penis muscle. But at higher concentration of 10^{-3}M. L-NMMA inhibited the NANC relaxation and further raised guanethidine-induced tone, an effect partially reversed by L-arginine 10^{-3}M.

6. L-NMMA (10^{-5}M. up to 3×10^{-4}M.), but not D-NMMA (10^{-5}M. up to 3×10^{-4}M.), raised tone and inhibited the NANC inhibitory relaxation on the rat anococcygeus muscle in a dose-dependent manner, an effect competitively reversed by L-arginine (3×10^{-5}M. up to 10^{-3}M.) or by washing out the L-NMMA.

7. L-NMMA up to 6×10^{-4}M. was without effect on the relaxation induced by nitric oxide on the rat anococcygeus or the rabbit aortic preparations suggesting the inhibition of the response to nerve stimulation was not post-synaptic.

8. L-NMMA, but not D-NMMA, dose-dependently (10^{-5}M. up to 3×10^{-4}M.) inhibited the EDRF relaxation induced by acetylcholine in the rabbit aortic preparations, an effect reversed by L-arginine or washing out L-NMMA.

9. L-Canavanine, an inhibitor of nitric oxide production in macrophages, at concentration up to 3×10^{-4}M. had no effect on NANC relaxation of the BRP preparation. But at higher concentration of 10^{-3}M. it inhibited the NANC relaxation, an effect partially reversed by L-arginine 10^{-3}M.

10. L-Canavanine up to 2×10^{-3}M. exhibited no effect on NANC inhibitory relaxation on the rat anococcygeus preparation.

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INTRODUCTION

Body function is controlled and integrated mainly by the nervous system and the endocrine system. The control in the nervous system depends primarily on the rapid electrical transmission of information in the nerve fibres, then between the nerve cells, and finally between the axone terminal of the last neurone in the chain and the effector and their cells it innervates. The signals across junctions are usually carried by chemical rather than electrical impulses. This chemical transmission takes place through the release of small amount of neurotransmitter from the nervous fibre terminals into the region of the synapse or junction. The chemical neurotransmitter diffuses to the postsynaptic cell and binds to a specialized receptor. The nerve system can be divided into two major functional subdivisions, the autonomic division and the somatic division. The autonomic nervous system in turn consists of two major portions, the sympathetic division and the parasympathetic division, originally based on anatomical differences. On the other hand, a different classification of the autonomic nervous system can be made, based on the neurotransmitter released from the nervous fibre terminals. Classically the autonomic nervous system consisted of a cholinergic nerve division and a noradrenergic division, based on the release of either acetylcholine or noradrenaline. With only a few exception the cholinergic system corresponds to the parasympathetic division and the noradrenergic with the sympathetic division (Dale & Gaddum 1930; Dale 1935; Dale & Feldberg 1936). Modern scientific developments in biochemistry, physiology and pharmacology however have provided the evidence for the existences or coexistences of many other neurotransmitters i.e. a variety of peptidergic neurotransmitters as substance P,

purinergic neurotransmitter by ATP and dopaminergic neurotransmitter. These have broken down the classical concept the autonomic nervous system only consisted of cholinergic nerve and noradrenergic nerve.

Part of the evidence for the existence of these other neurotransmitters was a nerve response which was unaffected by either adrenergic or cholinergic blocking agents. Such responses and the nerves producing them are referred to as non-adrenergic non-cholinergic (NANC) responses and nerves. The non-adrenergic non-cholinergic nerve innervation exists broadly in most peripheral smooth muscles, largely in gastrointestinal tract, cardiovascular system, urinary reproductive system and glands. The NANC nerve innervation on bovine retractor penis muscle and rat anococcygeus muscle are the good examples. In 1972, Gillespie first reported that the rat anococcygeus muscle appeared to possess many of the desirable features of an ideal innervated preparation of smooth muscle for both pharmacological and physiological study. A dense adrenergic innervation is distributed throughout the whole muscle and an NANC inhibitory innervation originating in the sacral cord several spinal segments from the sympathetic outflow was also discovered (Gillespie 1972; Gillespie & McGrath 1973). These inhibitory nerves were not an example of cotransmission by adrenergic nerves since destruction of the latter by 6-hydroxydopamine had no effect on the NANC inhibitory response (Gibson & Gillespie 1973). In 1974 Klinge & Sjostrand reported a similar NANC inhibitory innervation in the bovine retractor penis muscle (a continuation of the anococcygeus muscle, Gillespie & Martin 1980). In the BRP excitatory fibres derives from the lumber sympathetic outflow. Most fibres emanate from the sacral ganglia of the sympathetic chain and reach the muscle via the pudic nerve. Some fibres seem to run in the hypogastric nerve and a few fibres may follow the pelvic parasympathetic nerves. The

excitatory neurotransmitter of the sympathetic fibres in both muscles is noradrenaline. The neurotransmitter of NANC inhibitory nerves in both tissues remains unknown. The requirements for a substance to be a neurotransmitter should be first it is present in the nerve fibres, secondly it is released by nerve stimulation, thirdly it should mimic the nerve response to electrical stimulation, drugs which potentiate or block the nerve response should have the same effect on the putative neurotransmitter, the mode of action of neurotransmitter of nerve should be the same, and finally combines with its receptor specifically. The rat anococcygeus is a particularly significant tissue in deciding an possible candidates as NANC neurotransmitter since few potential candidates can cause relaxation in this tissue. The most obvious example is that while noradrenaline cause contraction through α-adrenoceptors so too does acetylcholine acting through conventional muscarinic receptors. A cholinergic innervation is therefore ruled out. Isoprenaline fails to cause relaxation and at high doses it will cause contraction through α-receptor. A neurotransmitter acting through β-receptor (which appears to be lacking in this species) is also excluded. For similar reasons histamine, 5-HT , dopamine, glycine, glutamate, ATP and a variety of peptides can be excluded. Only bradykinin and some related peptides and VIP cause relaxation at low doses. However, drugs such as haemoglobin and ethonal which are known to block the response to NANC nerve stimulation do not block the response to these peptides (Gillespie et al. 1981a; Bowman et al. 1982b). Only three potential candidates cause relaxation, act through a similar mechanism and are blocked by haemoglobin, there are nitric oxide (NO), the endothelium-derived relaxing factor (EDRF) and an inhibitory factor (IF) extracted from the BRP.

HISTORICAL REVIEW OF NANC NERVE INNERVATION

In 1883 Sertoli first studied the retractor penis muscle of several species both in vitro and in vivo. He found the muscle exhibited spontaneous activity. When the muscle was electrically stimulated, it produced contraction and also sometimes relaxation. In warm environment it relaxed and in cold contracted. De Zilwa (1901) studied the dog retractor penis in vitro. He also found that the smooth muscle exhibited spontaneous activity and the tone increased in low but decreased in high temperature. The muscle stimulated electrically produced contraction when it was in low tone but relaxation in high tone.

Langley and Anderson (1895) reported that in rabbit atropine could not inhibit the penile erection produced by electrical stimulation of the sacral parasympathetic outflow via the pelvic nerves. Bayliss and Starling (1899) reported that vagal stimulation in the dog small intestine caused first relaxation and then a following powerful contraction. Both the relaxation and the contraction were unaffected by atropine. Henderson and Roepke (1933), using penile perfusion in dog, found physostigmine potentiated the effect of pelvic nerve stimulation on venous flow, while atropine had almost no effect. In 1934 they also reported that in the guinea pig the motor response of the urinary bladder to parasympathetic nerve stimulation was slightly reduced by low doses of atropine but the large residual motor effect was insensitive to even high doses. Oppenheimer (1938) studied the cat retractor penis in vivo. He found the inhibitory frequency-response curve to parasympathetic stimulation was also hyperbolic. Acetylcholine mimicked the effect of this parasympathetic stimulation. Atropine abolished the effect of acetylcholine but not that of the parasympathetic stimulation. He believed the relaxation of the retractor penis was caused by the release of the

neurotransmitter acetylcholine. The resistance to atropine of the parasympathetic response was due to the proximity theory, the theory of Dale and Gaddum (1930) and reinvoked by Ambache (1955). The proximity theory was that the muscle cell membrane is in such close proximity to the nerve terminals as to deny access to exogenous blocking agents. Electron-microscopical studies disproved this theory because large gaps was found between the nerve terminal and the muscular cell membrane where neurotransmitter was released and acted on the target membrane. Goldenberg (1965) found that in cat retractor penis injected nicotine produced relaxation which was blocked by hexamethonium but not by atropine. He believed the relaxation was not due to acetylcholine but due to an unknown neurotransmitter released from the parasympathetic nerves stimulated by nicotine. Henderson and Roepke (1934) from their work on the bladder also suggested that atropine resistance could be best explained by the major release of a neurotransmitter other than acetylcholine or noradrenaline.

The experimental proof of the existence of NANC nerves were delayed because no selective powerful adrenergic blocking drugs was available. When adrenergic neurone blocking drugs were introduced and applied to analyse the response of stomach to nerve stimulation, it was found they had no effect on the inhibitory response to vagal stimulation in the guinea pig stomach (Martinson et al. 1963) and to transmural stimulation in the guinea pig and mouse (Paton & Vane 1963). Non-adrenergic inhibitory nerves were suggested based on the observation that the nerve stimulation of the guinea pig taenia coli exhibited a potent inhibitory response which was not blocked by bretylium (Burnstock et al. 1963; 1964). Since then non-adrenergic inhibitory nerves have been reported in the stomach (Martinson 1965; Campbell 1966), the small intestine (Holman & Hughes 1965; Day & Warren 1967; 1968; Weston 1971; Hirst & McKirdy 1974) and

the large intestine (Bianchi et al. 1968; Small 1972).

Luduena and Grigas (1966) studied the pharmacology of the isolated dog retractor penis by field stimulation. They reported the response of field stimulation on the untreated muscle was contraction which was blocked by phentolamine, piperoxan, bretylium, guanethidine and local anaesthetics. Bretylium and guanethidine also gradually induced a developing contraction and then the stimulation of the muscle exhibited an inhibitory relaxation which was neither abolished by atropine or scopolamine nor potentiated by physostigmine. Hemicholinium, hexamethonium and dichlorosoproterenol (DCl) had no effect on the inhibitory relaxation. Acetylcholine, histamine, nicotine and 5-hytroxytryptamine contracted the muscle. The muscular contraction induced by acetylcholine or nicotine was abolished by atropine or hexamethonium respectively. These might suggest an non-cholinergic innervation in the dog retractor penis. There was no evidence for the link between cholinergic and adrenergic neurotransmitters. They believed either excitatory sympathetic or inhibitory parasympathetic postganglionic fibres were activated by field stimulation.

Klinge (1970a) studied the response of the isolated bull retractor penis muscle to drugs. Adrenaline and noradrenaline at low concentrations produced vigorous muscular contraction which was abolished by phenoxybenxamine. Bradykinin at very low concentration contracted the muscle. Acetylcholine, histamine and 5-hytroxytryptamine also contracted the muscle but acetylcholine at high concentrations irregularly contracted the muscle. The muscle also exhibited contraction to a variety of nicotinic and muscarinic agonists in high concentrations such as propionylcholine, carbamylcholine, bethanechol, butyrylcholine, nicotine and methacholine. There were many reports showed the evidence for the existence of

NANC nerve innervation. They were the retractor penis muscle of bull, dog and horse (Klinge 1970a; Klinge & Sjostrand 1974; Ambache & Killick 1978), the anococcygeus muscle of rat, mouse, rabbit and cat (Gillespie 1972; Gillespie & McGrath 1973; 1974; Creed et al. 1977), the rabbit rectococcygeus (King et al. 1977; King & Muir 1977; 1981), the guinea pig taenia coli (Bennet et al. 1966), the lower oesophageal sphincter muscle of opposum (Rattan et al. 1977), the oesophagus (Lund & Christensen 1969), the guinea pig gall bladder (Davison et al. 1978), the guinea pig trachea (Coleman 1973; Coburn & Tomita 1973), the rabbit portal vein (Hughes & Vane 1967), the penile artery of bull (Dorr & Brody 1967; Klinge & Sjostrand 1974). Luduena and Grigas (1972) reported the dog retractor penis in vitro produced contraction in response to exogenous adenosine triphosphate (ATP) and prostaglandin $F_{2\alpha}$, and γ-aminobutyric acid (GABA) had was no effect. Prostaglandin E_1 on other hand did relax the muscle and for this reason they suggested it might be the parasympathetic neurotransmitter in this tissue.

Klinge and Sjostrand (1974) reported a detailed study of the effects of drugs and nerve stimulation on isolated smooth muscle strips of the retractor penis and the penile artery of bull. They found the retractor penis exhibited rhythmic spontaneous activity and often sustained tonic contraction. Field stimulation produced a monophasic, biphasic or triphasic response (inhibitory and / or excitatory) depending on the muscle tone. All of these effects were abolished by tetrodotoxin or local anaesthetics and due therefore to nerve stimulation. The excitatory response to field stimulation was abolished by α-adrenergic neuron blocking agents or α-adrenoceptor blocking drugs and enhanced by inhibition of neuronal noradrenaline uptake. The effects of adrenaline, noradrenaline and isoprenaline on the muscle were blocked by α- and β-adrenoceptor blocking agents. The

inhibitory response to field stimulation was uncovered by adrenergic neurone blocking drugs. This inhibitory response was unaffected by antimuscarinic, ganglionic blocking, neuromuscular blocking drugs, antihistamines and serotonin antagonists. It was also not blocked by botulinum toxin or hemicholinium or potentiated by physostigmine. Acetylcholine at high concentrations produced contraction of the muscle, suppression of the excitatory response to field stimulation and a brisk short lasting relaxation. The first two effects of acetylcholine were emulated by pilocarpine and abolished by antimuscarinic drugs. The relaxation was sometime proceeded by a rapid contraction, resembled the effect of transmural nerve stimulation, prevented by hexamethonium and emulated by nicotine. The relaxation induced by nicotine was abolished by ganglionic blocking agents or local anaesthetics. Histamine and 5-hydroxytryptamine also produced muscular contraction. The retractor penis was contracted by ATP. A number of other nucleotides and nucleosides had no effects or weak effects similar to those of ATP. GABA, glycine, glutamic acid, aspartic acid, alanine, valine, tyrosine, N-acetyltyrosine, tryptophan, cysteine, histidine, proline, hydroxyproline, glutamine, asparagine, methionine, and lysine were all without effects. Prostaglandins E_1 and E_2 relaxed the retractor penis but prostaglandin $F_{2\alpha}$ contracted it. The inhibitory response of the retractor penis to field stimulation was not suppressed by prolonged exposure to inhibitors of prostaglandin synthesis, an observation excluding $PGF_{2\alpha}$ as the neurotransmitter as suggested by Luduena and Grigas (1972). Substance P and minute concentration of bradykinin contracted the retractor penis. Posterior pituitary hormone had no effect on it. They believed the motor nerves of retractor penis muscle were adrenergic. There was also an inhibitory nerve innervation in the muscle. The neurotransmitter released from the inhibitory nerves relaxed the muscle by direct action and was most probably a substance other than acetylcholine, isoprenaline, histamine, 5-HT,

substance P, bradykinin, pituitary hormones, a prostaglandin, any of the nucleotides, nucleosides or amino acid studied as described above. The relaxation induced by acetylcholine was probably due to nicotinic stimulation of the inhibitory nerves.

The anatomical relationship between the non-adrenergic inhibitory neurones and the rest of the peripheral autonomic nerve system has been studied mainly in the gut. The cell bodies of non-adrenergic inhibitory neurones lie in Auerbach's plexus (Burnstock et al. 1966). In the gastrointestinal tract they are intrinsic neurones confined to the gut wall and with a mainly cholinergic input. In the stomach (Martinson 1965; Campbell 1966; Bulbring & Gershon 1967) and the small intestine (Bayliss & Starling 1899; Goldenberg & Burns 1968), the intramural inhibitory neurones are controlled by the pre-ganglionic vagal parasympathetic nerves. Klinge (1970b; 1970c) reported a high content of noradrenaline, a large amount of acetylcholine and exhibition of acetylcholinesterase activity in the retractor penis of bull. Abundance of adrenergic fibres and acetylcholinesterase-positive fibres were exhibited by histochemical studies (Klinge et al. 1970b; 1970c).

In the taenia coli of guinea pig the inhibitory relaxation was poorly sustained if continuous stimulation was applied (Burnstock et al. 1966). In the sphincteric muscle especially the internal anal sphincter the response to continued stimulation was well maintained (Rayner 1979). In the cat stomach relaxation mediated by non-adrenergic inhibitory nerves was not readily fatigued (Abrahamson 1973).

Stimulation of the pelvic nerve neither brought about mechanical inhibition (

Garry & Gillespie 1955) nor inhibitory junction potentials on the smooth muscle cells of the large intestine (Furness 1969) although transmural stimulation caused inhibitory junction potentials in both circular and longitudinal layers (Furness 1969). These inhibitory junction potentials were reduced by hexamethonium (C_6) suggesting an intrinsic cholinergic drive (Furness 1970). Electrical and mechanical responses in the guinea pig taenia coli and urinary bladder were reduced by lowering the external calcium (Burnstock et al. 1978) implying the transmitter release was calcium dependent and the transmitter itself was probably held in membrane-bound storage vesicles, whose contents were released by exocytosis. In the gut-related anococcygeus muscle responses were potentiated by tetraethyl ammonium which was known to prolong the nerve action potentials so allowing more time for calcium entry (Gillespie & Tilmisany 1976). Another NANC nerve innervated tissues is anococcygeus, which extends to the retractor penis (Gillespie & Martin 1980; Gillespie 1987). The anococcygeus was introduced as an ideal pharmacological preparation by Gillespie (Gillespie 1972) although there were some reports about the similar structures in the end of last century (Langley & Anderson 1895). Gillespie (1972) reported that the rat anococcygeus muscle had a dense adrenergic innervation distributed throughout the whole muscle but with no obvious cholinergic innervation and no evidence of localised cholinesterase staining histochemically. This muscle contracted in responses to the noradrenaline, acetylcholine, furmethide, 5-hytroxytryptamine, but not to histamine. Isoprenaline and ATP had no effect at low concentration but caused contraction at high concentration. Phentolamine and atropine respectively abolished the effects of noradrenaline and acetylcholine. When stimulated by field stimulation, this muscle produced powerful contraction, which was blocked by phentolamine, guanethidine but not atropine or hexamethonium. After the tone of the muscle was raised by guanethidine, 5-

HT or furmethide, field stimulation of the muscle uncovered the inhibitory relaxation which was insensitive to hexamethonium but abolished by tetrodotoxin. This inhibitory response to nerve stimulation was neither abolished by atropine, phentolamine, phenoxybenzamine, propranolol, hexamethonium or lysergic acid diethylamide, nor mimicked by acetylcholine, isoprenaline or ATP although histamine caused contraction and isoprenaline relaxation in rabbit and acetylcholine and isoprenaline relaxed the muscle in cat (Gillespie & McGrath 1974). Gibson and Gillespie (1973) reported that the inhibitory relaxation induced by field stimulation of the muscle against tone raised by carbachol was not abolished by the partial denervation of adrenergic neurones by immunosympathectomy or virtually complete destruction of the adrenergic neurones by 6-hydroxydopamine pre-treatment. Gillespie and McGrath (1973) also reported that the motor and the inhibitory nerve fibres innervated the anococcygeus had their origin in different regions of the spinal cord. The motor fibres were typically sympathetic with their origin in the upper lumbar region. The NANC inhibitory nerves anatomically were typical parasympathetic nerves with origin on the sacral cord. This reinforced the earlier observation that destruction of the sympathetic nerves with 6-hydroxydopamine had no effect on the NANC fibres and established the latter as an anatomically district nerve fibre with an unknown neurotransmitter.

The mode of action of these NANC nerves has been studied. Creed et al. (1975) reported the contraction of rat anococcygeus due to the release of the noradrenaline by field stimulation or guanethidine or addition of exogenous noradrenaline into the bath was accompanied by depolarisation of the cellular membrane to above -20 mV. and a fall in cellular membrane resistance. These indicated the noradrenaline released acted by increasing the permeability of the membrane ion channel(s) probably to sodium. But the relaxation induced by inhibitory NANC

nerve stimulation was not accompanied by any measurable change of the cellular membrane potential or resistance. The response to NANC nerve stimulation in the rabbit anococcygeus was accompanied by a large hyperpolarisation. In the latter muscle the mechanism of inhibition could be the closure of voltage sensitive calcium channels in response to hyperpolarisation. In the rat muscle the absence of hyperpolarisation suggests relaxation must be the consequence of biochemical rather than electrical coupling.

INHIBITORY FACTOR (IF)

The smooth muscle inhibitory factor (IF) extracted from the BRP was considered a promising candidate for the NANC inhibitory neurotransmitter first because it was extracted from tissue containing these nerves and secondly it possesses many properties required of such an inhibitory neurotransmitter.

IF was first extracted by Ambache et al. (1975) from the bovine retractor penis muscle by a dilute solution of hydrochloric acid. It was a thermolabile, water-soluble and ether-insoluble substance which powerfully inhibited the BRP and mimicked closely the inhibitory response to NANC relaxation of BRP and whose action was unaffected by drugs blocked conventional transmitters. They believed it could be the NANC inhibitory neurotransmitter in the BRP.

In this laboratory these results were verified and extended (Gillespie & Martin 1978; Gillespie & Martin 1980; Gillespie et al. 1981b). The most important observation was that the IF as extracted from the BRP lacks biological activity.

This appeared only after a brief exposure to acid at pH 3 or less. The material reported by Amboche had of course already been activated by the dilute acid used in the extraction. IF was also soluble in methanol, less so in ethanol and was insoluble in acetone. It existed in the two forms mentioned above, a relatively stable but pharmacologically inert which was converted to an unstable but much more powerful inhibitory compound by a brief exposure to acid before neutralization and assay. Its molecular weight was estimated approximately at between 500 and 1000 Daltons by ultrafiltration of acid extracts of the BRP through a series of membranes with different molecular retention values. It is not ATP (Bowman et al. 1979), a peptide (Gillespie et al. 1981a) or a fatty acid (Gillespie & Martin 1980).

IF mimics the effects of NANC nerve stimulation in the BRP and the powerful NANC nerve innervated tissues, the penile artery, the guinea pig taenia coli and trachealis muscle, not only in the degree of the relaxation but also the time course of response (Bowman & Gillespie 1983; Crossley & Gillespie 1983). The mode of action of IF and nerve stimulation was identical in BRP and penile artery but not in taenia coli since haemoglobin which blocked both the response to nerve stimulation and the IF in the BRP and rat anococcygeus did not block the response to nerve stimulation in the taenia coli (Bowman & Gillespie 1983). Both IF and nerve stimulation produce membrane hyperpolarisation (Byrne & Muir 1985). They caused relaxation by raising cyclic GMP level, an effect blocked by haemoglobin, N-methylhydroxylamine (Bowman & Drummond 1984) and methylene blue (Bowman et al. 1986) and potentiated by a selective inhibitor of cyclic GMP phosphodiesterase M & B 22948 (Bowman & Drummond 1984). Ethanol also selectively inhibited the relaxation on the rat anococcygeus and BRP to NANC nerve stimulation and to IF without effect affecting the

relaxant response to isoprenaline or even to other agents such as sodium nitroprosside which stimulated guanylate cyclase or to the phosphodiesterase inhibitor isobutylxanthine which prolonged the action of cyclic GMP. The mechanism of action of ethonal might be to reduce calcium binding to membrane or a reduced efficiency of receptor coupling (Gillespie et al. 1982). The possibility that the IF like other neurotransmitter was held in storage vesicles was examined (Hunter 1982). BRP muscle was homogenised and separated into the conventional P_1, P_2, P_3 and S_3 fraction. The assumption was that if there are specific storage vesicle the IF would merely be in the P_3 fraction. The results showed activity in all fraction but with a higher concentration in P_3 suggesting storage in association with some form of particle. Martin et al. (1980) on the other hand reported there was evidence that IF extracted from BRP is nitrite, whose acid-activated derivative is stabilized nitric oxide. If this is so then the IF may be an artifact unrelated to the neurotransmitter though the stabilising factor might be related.

So far IF fulfils many of the requirements as the NANC inhibitory neurotransmitter in BRP and rat anococcygeus but with some exception as IF needs for acid activation and could be extracted from various tissues which with or without NANC innervation (Gillespie & Martin 1980). Also so far it failed to detect the release of neurotransmitter from the NANC nerve.

ENDOTHELIUM-DERIVED RELAXING FACTOR (EDRF)

Because the IF possesses several properties in common with the NANC nerves such as the ability to stimulate guanylate cyclase and its block by haemoglobin, it was regarded as a promising NANC inhibitory neurotransmitter. The experimental difficulties in detecting its release by nerve stimulation and the failure to identify it chemically lead the research to investigate some substances which also have properties in common with the relaxation produced by NANC nerve stimulation. These were EDRF and nitric oxide both like the NANC nerves and the IF are unstable, short acting relaxants acting through guanylate cyclase and blocked by haemoglobin.

EDRF was first recognised by Furchgott et al. (1980) in experiments with isolated preparations of arteries (rings, transverse strips or helical strips). In such preparations the well known relaxant effect of acetylcholine was strictly dependent on the presence of endothelial cells on the intimal surface of the preparations. This discovery resolved the paradox that acetylcholine though a powerful vasodilator in vivo often produces no relaxation or even contraction on isolated preparations of arteries in vitro. The explanation was that their endothelial cells were unintentionally rubbed off. This initial observation with acetylcholine has lead to the recognition that many other potent vasodilators also require endothelial cells to produce their effect.

EDRF is powerful relaxant on vascular smooth muscle, destroyed by haemoglobin and borohydride acting through guanylate cyclase, has a short half-life and is chemically unstable (De Mey & Vanhoutte 1983; Griffiths et al. 1984). Gillespie and Shen (1988) reported the EDRF as well as IF powerfully relaxed

vascular and some non-vascular smooth muscles.

EDRF can be released from vascular endothelial cell stimulated by acetylcholine, bradykinin, substance P and some related peptides, ATP and ADP, histamine, serotonin, thrombin, arachidonic acid and the calcium ionophore A23187 (Furchgott & Zawadzki 1980; Furchgott 1981; Altura & Chand 1981; Chand & Altura 1981) although there are differences between species and different blood vessels. After removal of the endothelial cells, the vasoconstriction by noradrenaline and serotonin is significantly more powerful (Cocks & Angus 1983) indicates the accompanying release of EDRF by these agonists. EDRF also inhibits platelet aggregation and adhesion to vascular endothelium (Azuma et al. 1986; Radomski et al. 1987a; 1987b; 1987c).

Drugs and experimental conditions which inhibit the endothelium-dependent relaxation are anoxia, ETYA (5,8,11,14-Eicosatetraynoic acid, an inhibitor of lipoxygenase), quinacrine (an inhibitor of arachidonic acid release), p-bromophenacylbromide (BPB, an irreversible inhibitor of phospholipase A_2), NDGA (nordihydroguiaretic acid, lipoxygenase inhibitor and antioxidant), hydroquinone, methylene blue and haemoglobin (De Mey & Vanhoutte 1983; Furchgott & Zawadzki 1980; Bowman & Gillespie 1981; Gruetter et al. 1981a; 1981b; Furchgott et al. 1983; Singer & Peach 1983).

Drugs potentiating the effect of EDRF are M & B 22948, the selective inhibitors of cyclic GMP phosphodiesterase (Martin et al. 1986).

The action of EDRF is mediated through stimulation of the soluble guanylate cyclase and the consequent elevation of cyclic GMP within the smooth muscle (

Diamond & Chu 1983; Furchgott & Jothianandon 1983; Rapoport & Murad 1983a; 1983b).

Strong experimental evidence that EDRF is nitric oxide has been produced (Palmer et al. 1988a; 1988b). Sufficient nitric oxide is released from endothelial cells to account for the biological activities of EDRF (Palmer et al. 1987). Recently the same group have produced evidence that the Nitric oxide is synthesized by vascular endothelial cells from the terminal guanidino nitrogen atom(s) of the amino acid L-arginine (Palmer & Moncada 1989; Palmer et al. 1988b; Schmidt et al. 1988b).

The vascular relaxation by EDRF is inhibited by the inhibitors of L-arginine-utilizing enzyme L-monomethyl-N-arginine and L-canavanine (Palmer et al. 1988b; Schmidt et al. 1988a). The release of nitric oxide from the cultured endothelial cells is also blocked by L-NMMA (Palmer et al. 1988b). This inhibitory action of L-NMMA is appearently a competitive substrate inhibition since it can be reversed by L-arginine.

Nitric oxide is rapidly oxidized in presence of oxygen and water. This oxidation is greatly enhanced by the presence of superoxide anions present in all aqueous solution containing oxygen in solution and particularly in solution oxygenated with 95 % O_2. It is by destroying these anions that superoxide dismutase protects EDRF from breakdown (Gryglewski et al. 1986; Rubanyi 1986).

Although EDRF and the IF possess any properties in common, the IF does not act through the release of EDRF since removing the endothelium from an aortic strip which abolishes the relaxant effect of acetylcholine does not also abolishes

the relaxant effect of the IF. There are also some properties of the IF which are appearently incompatible with it being EDRF (NO) such as a molecular weight of between 500 to 1000 (Gillespie et al. 1981b), difficulties in diffusing through cell membrane (Gillespie & Shen 1988) and its relatively high concentration in the washed high speed pellet from tissue homogeneous.

For the reasons given above EDRF, IF and nitric oxide are similar in many of their properties. The IF is perhaps the most promising NANC neurotransmitter because it mimics the action and effects of NANC nerve stimulation. If, bearing in mind that the possible relationship with nitric oxide, the release of the NANC inhibitory neurotransmitter could be detected, this would pave the way to a similar study of its properties and its likely identification. If detection is impossible then indirect studies of the relationship between the responses to nerve stimulation, to EDRF and nitric oxide may through light on the nature of the NANC inhibitory neurotransmitter. These are the aims of this project.

MATERIALS AND METHODS

In this project these isolated preparations, the bovine retractor penis (BRP) muscles, the rat anococcygeus muscles and rabbit aortic strips, were used. These preparations were used first in a series of experiments trying to identify by bioassay the release of neurotransmitter from field stimulated non-adrenergic non-cholinergic (NANC) nerves. A second group of experiments examined the effects of drugs on the response to NANC nerve stimulation and a comparison with the response to endothelium-derived relaxing factor (EDRF) and nitric oxide.

THE BOVINE RETRACTOR PENIS (BRP) MUSCLE

THE ANATOMY OF THE BOVINE RETRACTORS PENIS MUSCLE

The paired retractors penis muscle of bulls have their origin from the ventral surface of the first and second coccygeal vertebrae. From their origin behind the colon the muscles run ventrally to pass the anal canal, one on either side of the colon, and proceed along the ventral surface of the bulbocavernousus muscle to the distal part of the penis where a broad insertion takes place. The paired muscles maintain the characteristic sigmoid flexure of the relaxed penis (Fig. 1). The bovine retractors penis muscles were obtained from Glasgow Abattoir. The BRP muscles were from bullocks of different breeds, weighing 200 to 1000 kg. The whole penis was removed complete with the paired retractors penis muscle

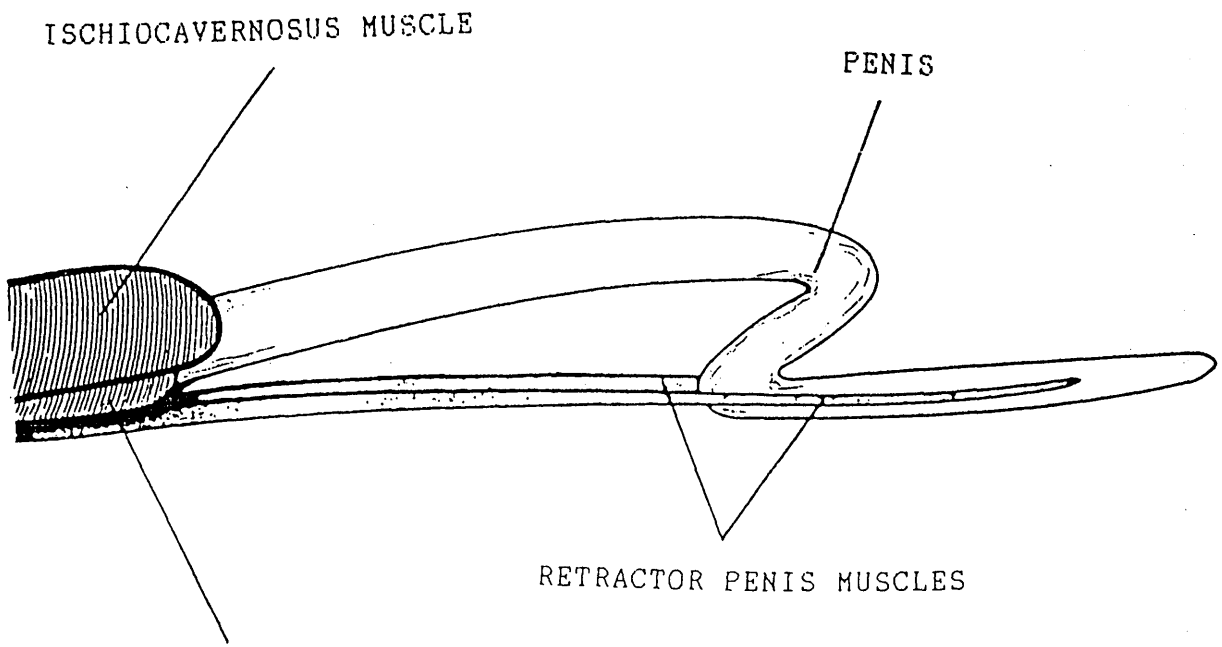


Fig. 1 Schematic diagram of the contracted retractor penis muscles maintaining the relaxed penis of the bull with its characteristic sigmoid flexure.

as far as the ischiocavernosus muscle and the bulbocavernosus muscle. To ensure that only one muscle from each animal was used, the BRP was dissected in the abattoir and only one of the each paired retractors penis muscle was used and transferred in a bottle containing fresh made-up Krebs' solution to the laboratory within 3 hours of slaughter. In the laboratory the muscles were cleared of connective tissues and stored in Krebs' solution or Tyrodes solution at about + 4°C in the refrigerator until required. Muscles were either used immediately or after storage for periods up to 2 days after slaughter. There was no any indication of disadvantage of a few hours' delay in obtaining the BRP muscles from the abattoir after slaughter or their storage for 3 days in the Krebs' or Tyrodes solution. In some experiments in order to exhaust the endogenous arginine in the BRP muscles, the muscles were purposely stored for periods up to 10 days. In these cold stored experiments four BRP muscle strips from four different bullocks were used on each successive day of the 10 day period.

THE BOVINE RETRACTOR PENIS MUSCLE PREPARATION

The BRP muscles were placed on a glass petri dish (partly filled with sylgard) containing Krebs' solution and the muscles were pinned out. The connective sheath covering the muscles was removed to expose the muscle fibre bundles. Parallel bundles of muscle fibres were selected and longitudinal strips, 10 to 20 mm. in length and about 1.5 to 2 mm. in width, were cut. In experiments to release the NANC inhibitory neurotransmitter and to detect this by bioassay much longer preparations 20 to 40 mm. in length and 4 to 6 mm. in width were sometimes used to increase the amount of the neurotransmitter released. In a series of experiments to test whether L-arginine was a physiological precursor of the NANC inhibitory neurotransmitter it was necessary to exhaust the

endogenous arginine in the preparations. To do this preparations were stored in the cold in sterile Tyrodes solution for up to ten days with the sterile Tyrodes solution changed daily. Because a four channel pen recorder was used, it was convenient to have four preparations for each day's experiment. To provide this ten preparations were cut from each of four fresh muscles and one preparation from each put into ten sterile tissue cultured bottles (Fig. 2) containing 20 ml. of newly made up Tyrodes solution which had been sterilised by filtration through an ultrafilter (Sartorius Minisart sterile 0.45 μm. max. 7 bar). The BRP muscle strips were washed with sterile Tyrodes solution before they were put into the sterile culture bottles. Each day the Tyrodes was removed and replaced with fresh solution. All of these procedures, dissection, preparation of bottles and filling and refilling bottles, was carried out in a sterile hood. Between manipulations the bottles were stored in a refrigerator at 4° C. Tyrodes solution was preferred to Krebs' because of its phosphate buffer which maintained a constant pH. Krebs' solution, if not gassed with 5 % CO_2, quickly becomes alkaline and the calcium phosphate precipitates out. No antibiotics were used partly because streptomycin at least is known to be able in skeletal muscle to interfere with neurotransmission.

On each experimental day one bottle containing the four preparations each from a different animal was placed in a hot water reservoir and slowly heated to 37° C and then kept at this temperature for 30 to 45 minutes. This allows the Na^+ / K^+ ATP'ase pump to be restored and restore the normal intracellular ionic balance by pumping out Na^+ in exchange for K^+. After this equilibration period the preparations were removed and each end tied with 5:0 weight braided silk. One end was kept short for tying the preparation to a hook in the organ bath, the other was left long for connection to a Grass FT 03 tension transducer. Each

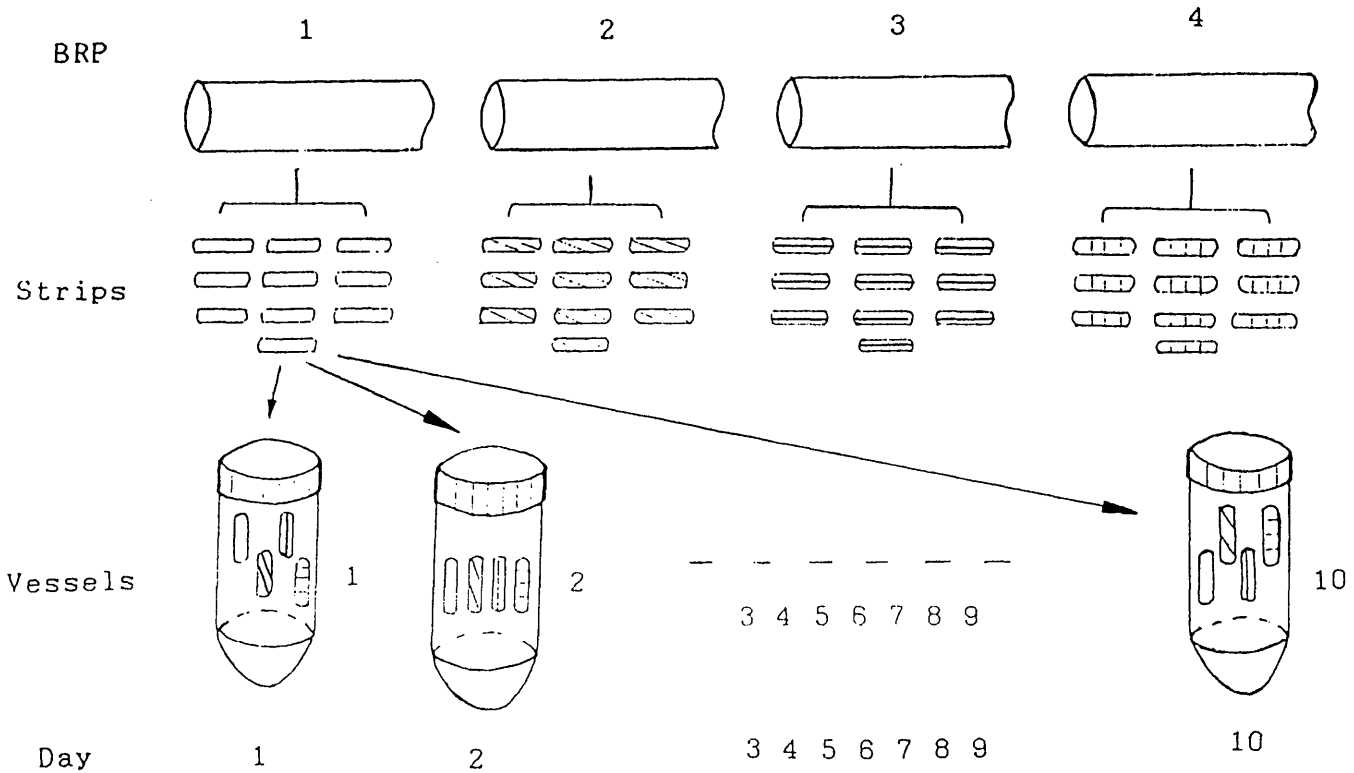


Fig. 2 Schematic diagram of the arrangement for the BRP muscle strips used up to 10 days. Four different BRP muscles from four bullocks were cut into 10 strips and equally distributed throughout 10 sterile tissue cultured vessels containing bacteria-free Tyrodes solution. For further details see the text.

preparation was suspended in a 10 ml. organ bath containing Krebs' physiological saline at 36° C and aerated with 5 % CO_2 in O_2. In some experiments studying the release of the NANC inhibitory neurotransmitter smaller 1 ml. baths were used. When the responses to the stimulation of intramural nerve fibres were studied, one end of the strip was tied to a silver / silver chloride hook electrode and the muscle strips passed through the silver / silver chloride ring electrode embedded in Araldite (Burn and Rand 1960) with the long thread tied to a Grass force displacement isometric transducer. (Figure 3). The ring electrodes consisted of a coil of 0.5 mm. diameter silver wire, 4 mm. in length and of inside diameter 3 mm. embedded in Araldite epoxy resin, with a hook of silver wire 4 mm. directly below the coil forming another electrode. During the experiments in which the responses to exogenous drugs only were studied, the BRP preparations were mounted on glass hooks.

In all experiments the BRP muscle strips were set up with no applied tension and allowed to equilibrate for 10 to 15 minutes and then given 1 gm. resting tension by repeated stretch of the strips controlling the amount of stretch by the tension displayed on the recorder. The tone of the BRP muscle strips were raised by addition of guanethidine (10^{-5}M.) which has the additional advantage of blocking the motor adrenergic nerves.

ARRANGEMENTS OF THE PREPARATION IN THE DETECTION OF NANC NEUROTRANSMITTER

In the detection of the NANC neurotransmitter in the BRP muscles some other arrangements of the preparations were used purposely in an attempt to increase the quantity of the neurotransmitter released, reduce the dilution or to reduce the

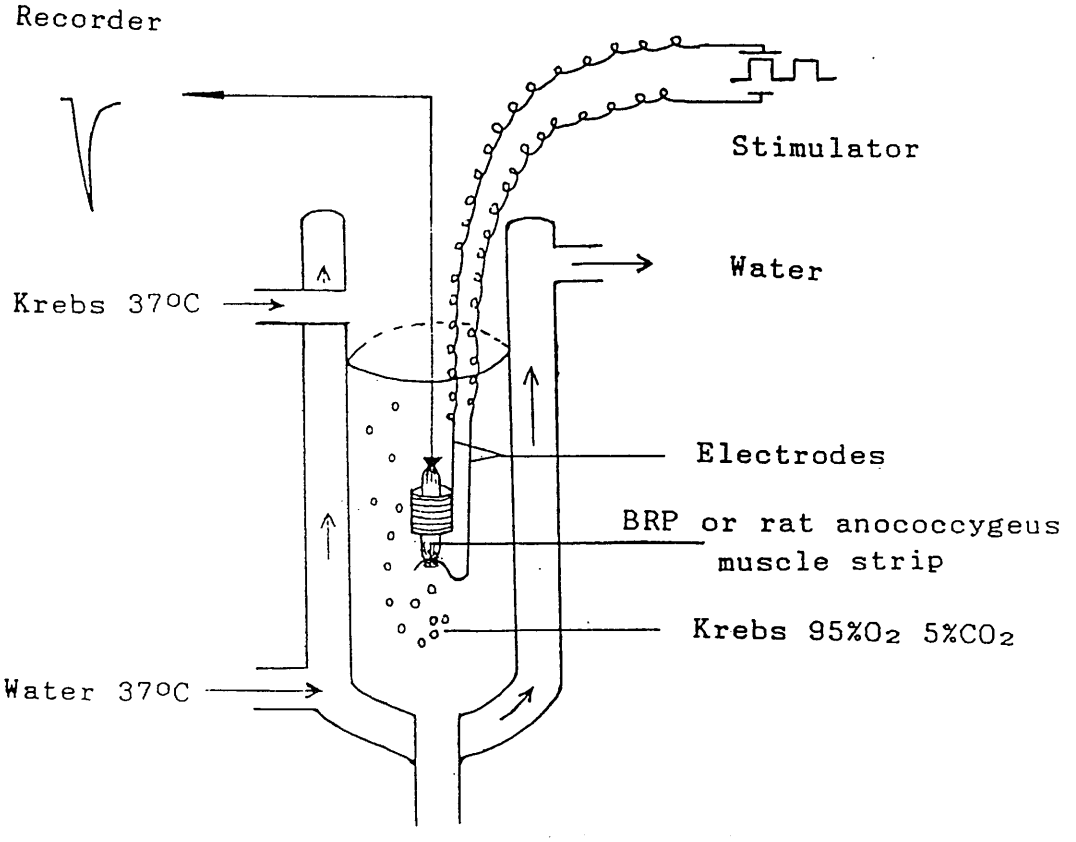


Fig. 3 A diagram of the arrangement for field stimulation of the BRP or rat anococcygeus muscle strip in the isolated organ bath.

delay between release and detection.

First, a donor BRP muscle strip was mounted inside the ring electrodes in a organ bath (1 ml. or 10 ml.) described as above and stimulated by field stimulation (Fig. 4). A test BRP muscle strip was mounted above the donor BRP muscle strip and the ring electrodes 1 to 10 mm. or just outside the ring electrodes but close to the donor BRP muscle strip at the same level. Sometimes a second test strip, the rabbit thoracic or abdominal aortic transverse strip, was also used and similarly placed just above and inside the ring electrode. These preparations were 2 mm. wide rings which were cut through to produce open strips about 10 mm. long.

Secondly, a cascade was used. The arrangement is in Figure 5. A donor BRP muscle strip was tied on the hook electrode with another end passing through the ring electrode and tied with a long braided silk thread (EP 1) to the force transducer. A test BRP or rat anococcygeus muscle strip was tied below the donor strip and attached by a thread to another force transducer. All of these preparations were mounted inside a jacketed organ bath to maintain a warm and water-saturated environment. The jacket was circulated with water at 40° C. The Krebs' solution, previously heated to 37^{0}C and saturated with 5% CO_2 in 95% O_2, flowed along a glass tube just above the donor strip and dropped on and superfused the donor strip. Then the superfusate was collected by a small glass tube just below the donor strip and superfused the test strip below. The constant flow of Krebs' solution was maintained by a Watson-Marlow constant flow pump. Flow speeds of 1, 2, 4, 6, 8 and 10 ml. per minute were studied.

Finally, a closed cascade (Fig. 6) was used. In the lower chamber the donor

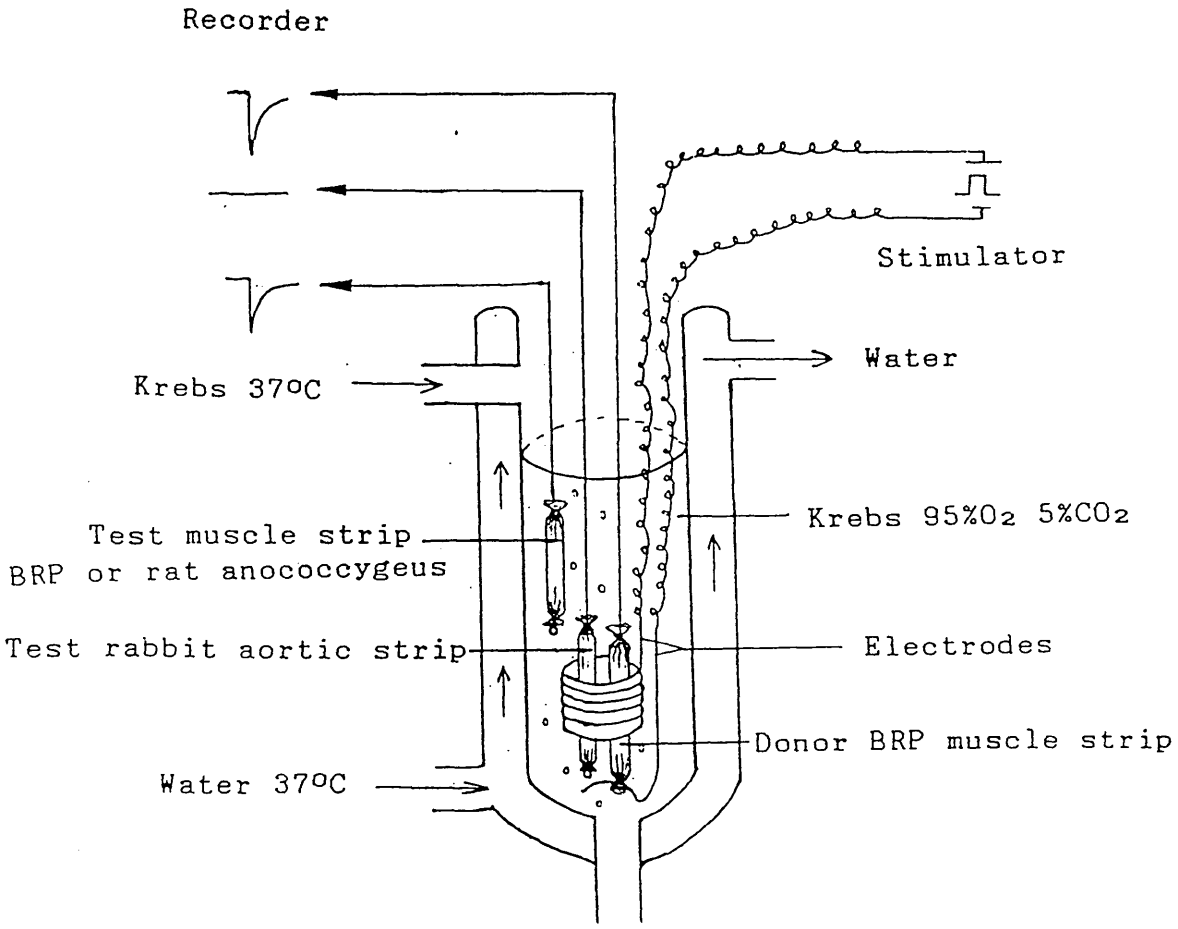


Fig. 4 A diagram of arrangement for the bioassay to detect NANC neurotransmitter released from the donor BRP muscle strip and detected by the close test rabbit aortic strip and another test BRP or rat anococcygeus muscles strip. For further details see the text.

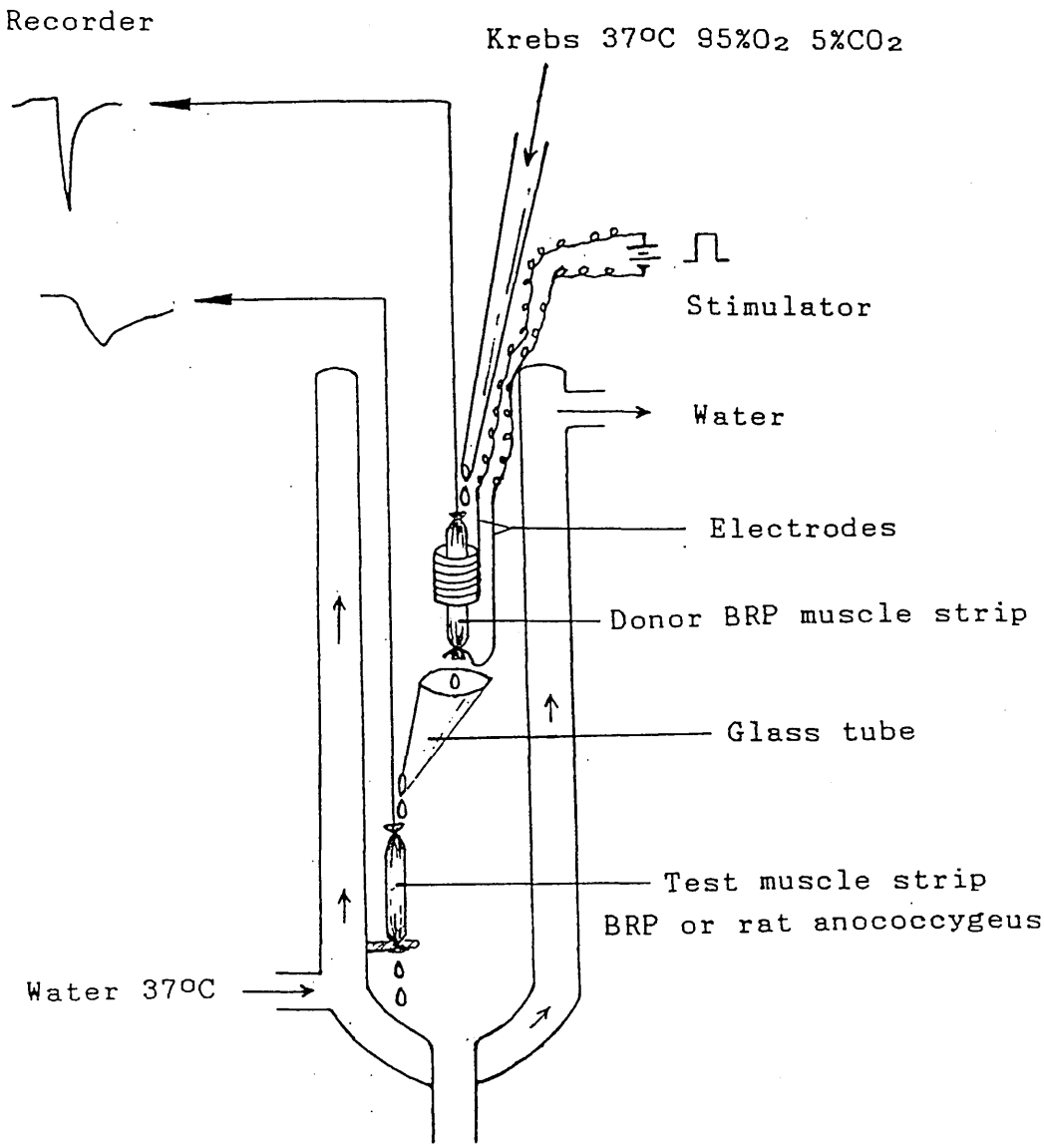


Fig. 5. A diagrammatic representation of the open cascade system used to release NANC neurotransmitter from a donor BRP muscle strip and to detect the neurotransmitter by a test BRP or rat anococcygeus muscle strip immediately below the donor strip.

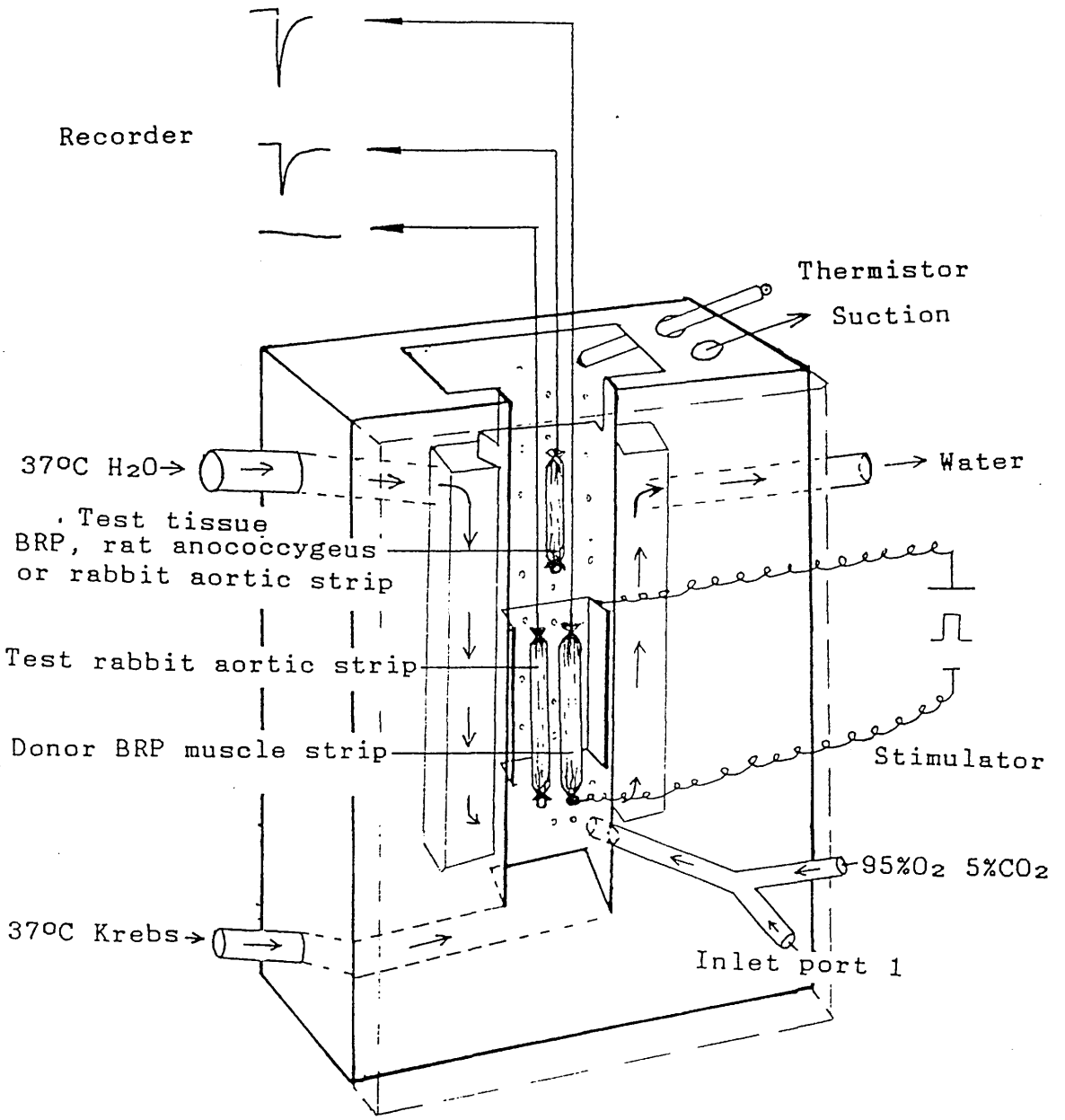


Fig. 6. A diagrammatic representation of the closed cascade system used to release neurotransmitter from a donor BRP muscle strip and to detect the neurotransmitter immediately on a very close test rabbit aortic strip and a test BRP or rat anococcygeus muscle strip above the donor strip in a chamber. Drugs can be added into the chamber either through the inlet port 1 or into the Krebs solution. The cascade was jacketed with channels through which water 37°C was pumped to maintain the constant temperature.

muscle strip was tied to small Ag / AgCl hook electrode while the second electrode was a thin plate of Ag / AgCl 25 mm. long extending over the three walls of the chamber. The other end of the donor muscle strip was tied to the force transducer. The test BRP muscle strip was mounted in a second chamber immediately above the ring electrode. The volume of these chambers was approximately 1 ml. Sometimes another test rabbit thoracic or abdominal aortic strip was mounted very close to the donor BRP muscle strip inside the ring electrode at the same level or just above the donor BRP strip and the ring electrode (Figure 6). The speed of the superfusion flow was studied at different levels of 0.5, 1, 2, 4, 6, 8, 10, 12 and 15 ml. per minute respectively, and some experiments were done with stop-flow. The constant flow was carried out and maintained by a Watson-Marlow pump. The motor adrenergic nerves were blocked and the tone of the BRP and rat anococcygeus muscle strips was raised by guanethidine (10^{-5}M.). Where the rabbit aortic strip was used as a second test tissue, the tone of the donor BRP strip, the test BRP or rat anococcygeus strip and the test rabbit aortic strip was raised by NA (10^{-6}M.), with EDTA (10^{-5}M.) and ascorbic acid (10^{-4}M.) to protect NA from being destroyed by oxidation. Occasionally Phentolamine (10^{-5}M.) was used to block the adrenergic excitation and tone raised with 5-HT (10^{-5}M.). The drugs were added into the Krebs' solution reservoir when superfusion was used. In other experiments to prove the system capable of detecting the release of a known neurotransmitter noradrenaline both the donor and the test strips were mounted under 1 g resting tension but without adding agonist drugs to raise tone or drugs to block the adrenergic nerves. In these circumstances field stimulation of the donor tissue caused the release of noradrenaline and contraction. The released noradrenaline could then be detected as contraction in the test tissue.

THE RAT ANOCOCCYGEUS MUSCLE

THE ANATOMY OF THE RAT ANOCOCCYGEUS MUSCLE

The paired rat anococcygeus muscles arise from tendinous origins on the first two coccygeal vertebrae in the midline of the pelvic cavity. At their origin they lie close together behind the colon. They then pass on either side of the colon, merge onto the ventral surface of the colon and unite together as a longitudinal ventral bar which passes into the skin of the perineum. In some rats some fibres of the anococcygeus muscles form a short transverse bar merging on the ventral surface of the colon. The paired muscles may consist of thousands of individual smooth muscle cells merged in parallel bundles. Each smooth muscle cell has an irregular outline because of numerous caveolae and projections (Gillespie & Lullman-Rauch 1974). The extrinsic nerves pass in a branch of the perineal nerve on either side to enter the deep surface of each muscle just short of the formation of the ventral bar (Figure 7).

THE RAT ANOCOCCYGEUS MUSCLE PREPARATION

The rat anococcygeus muscles were dissected and removed as described by Gillespie (1972). Mainly, 250 to 450 gm. male Wistar rats were killed by inhaling carbon dioxide or by stunning and exsanguination. The abdomen was opened and the vasa deferentia, seminal vesicles, testes, cremaster, bladder and urethra were also removed. After the removal of the fatty tissue adhering to the muscles of the hindlimbs, the skin of the perineum was reflected back. With care not to damage the underlying colon, the bulbo-cavernosus and ischiocavernosus muscles were cut through the symphysis pubis split and the two cut end of the pelvic arch

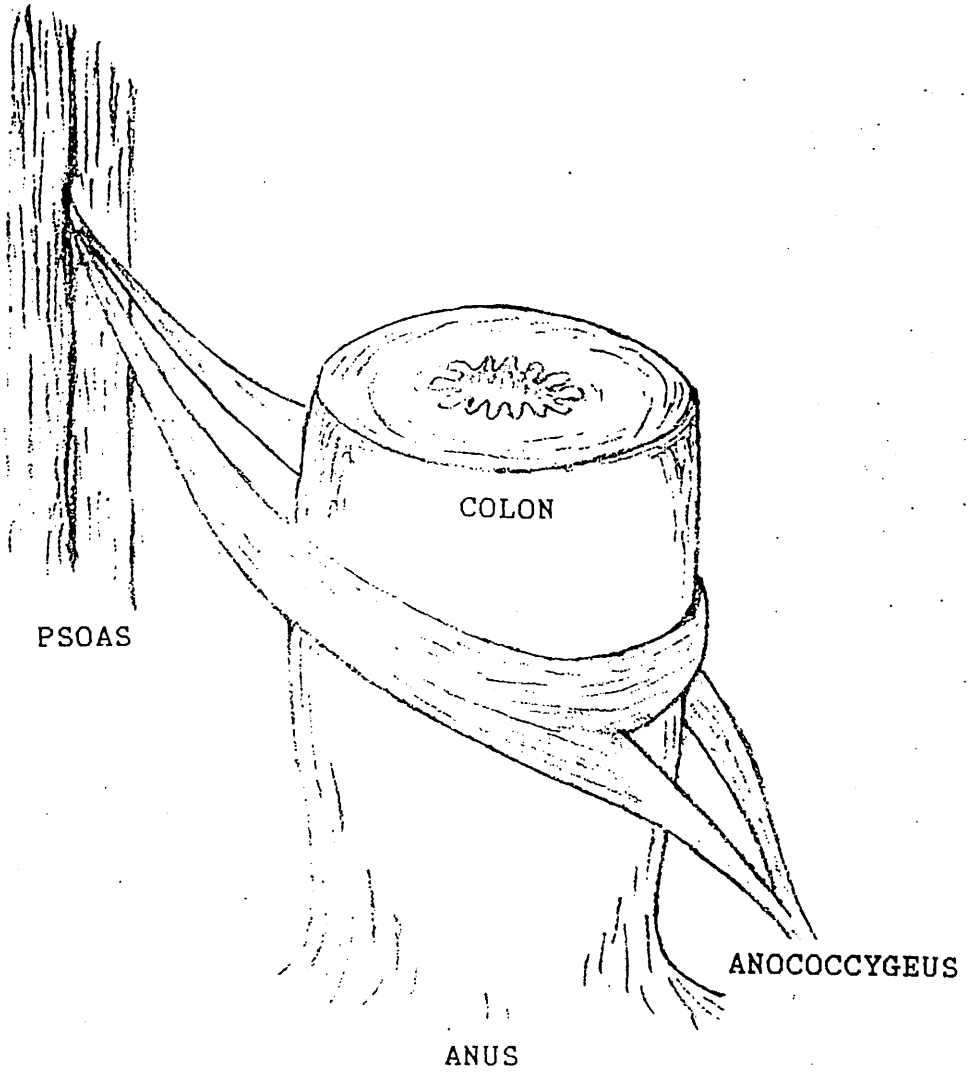


Fig. 7. The schematic drawing of the male rat anococcygeus muscles in situ.

forced apart. By this method the pelvic cavity was very clearly exposed. The perineum was stretched apart and pinned on a wooden block on which the rat was lying. Then the ventral anococcygeus was very clearly visible, locating close to the skin of the perineum. The fat and the attached tissues were carefully removed. After the colon was cut through at the level of the pelvic brim and the pelvic portion was pulled forward ventrally, the pair of anococcygeus muscles came into view. The anococcygeus muscles were cleared, their tendinous ends tied and the muscles removed between the tendinous coccygeal connections and the perineal end of the ventral bar, a length of about 30 to 40 mm. The muscles were transferred to a petri dish containing fresh aerated Krebs' solution and pinned out onto a translucent gel covering the bottom of the dish. With very sharp scissor the remaining connective tissues were removed carefully and the transverse bar was cut, the paired anococcygeus muscles were divided into two preparations about 3 to 4 cm length and 2 to 4 mm. width by cutting longitudinally throughout the midline of the longitudinal bar. Both ends were tied with braided silk (EP1), one on the electrode hook in the bath and another with a long thread through the ring electrode on the force transducer. The dissection was completed within 15 to 20 minutes and the anococcygeus muscles were always kept moist by application of the fresh Krebs' solution. The preparations were mounted under 1 gram resting tension by stretch in 10 ml. or 1 ml. baths and bathed in the Krebs' solution and allowed to equilibrate for 60 to 90 minutes before experiments were begun. The tone of the anococcygeus preparations was usually raised by the addition of guanethidine (10^{-5}M.).

THE RABBIT AORTIC STRIP PREPARATION

Rabbit thoracic or abdominal aortic strips were used. New Zealand white rabbits, 2 to 3 kg. of either sex, were killed by inhaling carbon dioxide. The chest and abdomen was opened. The thoracic or abdominal aorta was carefully dissected and then removed into Krebs' solution in a dish. The aorta was gently cut into rings about 2 to 3 mm. width. Then the rings were cut longitudinally into transverse strips. Great care was taken during the whole procedure to avoid unnecessary stretch or contact of instruments with the luminal surfaces of the strips to ensure integrity of the vascular endothelium. In some experiments the endothelium was purposely disrupted by gently rubbing a small moist cotton ball on the luminal surfaces of the aortic strips. In all strips the integrity of the endothelium was judged by the presence (with intact endothelium) or absence (disrupted endothelium) of endothelium dependent relaxation induced by acetylcholine. The tone of the strips was raised by 5-HT (10^{-5}M.) or NA (10^{-6}M.) in the experiments of the detection of the NANC neurotransmitter or by phenylephrine (7.5×10^{-7}M.) to obtain submaximal contraction of the strips in the experiments of the effects of L-NMMA and exogenous L-arginine on the rabbit aortic strips. The strips were allowed to equilibrate 60 to 90 minutes before experiments were begun. After each wash to remove drugs and before the next step of experiments the strips were also allowed to equilibrate until the tone recovered.

TEMPERATURE

The Krebs' solution both in the isolated organ baths and in the tubes linked with the baths was maintained at 37^0C by the circulatory jacket water pumped and heated by a constant heater pump (Techne Tempette Junior TE-8J).

TONE OF THE PREPARATION

Generally, guanethidine 10^{-5}M. is chosen to be used in the study of NANC inhibitory neurotransmission in the BRP and rat anococcygeus as it is the most effective drug in uncovering NANC inhibition because guanethidine meets the two requirements for optimum NANC inhibitory responses, first the tone of the preparation must be raised and secondly the motor adrenergic response blocked.

When rabbit aortic preparation was used as a second testing tissue in the bioassay detection of NANC inhibitory neurotransmitter, the tone of the tissues (BRP, rat anococcygeus and rabbit aortic preparations) was raised by 5-HT 10^{-5}M. with phentolamine 10^{-5}M. to block the excitatory response, as guanethidine is not suitable to raise the tone of rabbit aortic preparation.

In study of effects of drugs on endothelium-dependent relaxation in rabbit aortic preparation, phenylephrine 7.5×10^{-6}M. was used to raise tone of the preparations as it maintained a suitable and much stable tone.

FIELD STIMULATION

Field stimulation of the intermural nerves (Burn & Rand 1960) was applied through the silver / silver chloride electrodes with rectangular shocks of 0.5 millisecond pulse width and supramaximal voltage. The pulses were delivered from Grass S88 stimulator. In the experiments of the pharmacological analysis of the effects of drugs on the responses to field stimulation, the preparations of the BRP muscles were generally stimulated for 20 pulses at 3 minutes' intervals. The frequencies of the stimulations used were usually at 0.1, 0.2, 0.5, 1, 2, 5 and 10 Hz. But with exceptions sometimes, for example continuous higher frequencies were used in an attempt to exhaust the endogenous arginine in the BRP muscles, much exceptions are indicated in the text and the figures.

RECORDING OF MECHANICAL RESPONSES OF THE PREPARATION

Mechanical responses of the smooth muscle strips were recorded isometrically by means of Grass force-displacement transducers FT 03 C coupled to and displayed on a four channel Grass Model 7 polygraph ink-writing recorder.

SOLUTIONS

KREBS' SOLUTION

The composition of the Krebs' solution used was based on that described by Krebs and Henseleit (1932). Namely,

NaCl 118.5 mM.	*KCl* 4.75 mM.	$CaCl_2$ 2.25 mM.
$NaHCO_3$ 25 mM.	KH_2PO_4 1.2 mM.	$MgSO_4$ 1.2 mM.
Glucose 11.1 mM.		

Each component was prepared as a concentrated stock solutions. When the stock solution of $NaHCO_3$ was made up, it was usually bubbled with CO_2 for 1 hours. The Krebs' solution was made up from the stock solutions apart from glucose, which was added into the mixtures as the solid daily. The final Krebs' solution was bubbled with 5 % CO_2 in O_2 for 30 minutes before use. In the experiments to detect NANC neurotransmitter or using NA to raise the tone of the smooth muscle strips, Krebs' solution was made up with deionised distilled water and ascorbic acid (10^{-4}M.) and EDTA (10^{-5}M.) were added.

TYRODES SOLUTION

In those cold stored experiments where preparations were kept in the cold for up to ten days, Krebs' solution was unsuitable since in the absence of the 5 % CO_2 it gradually becomes alkaline and the calciumion precipitated. For these experiments Tyrodes solution was used. The composition of the Tyrodes solution (pH

7.4) in grams per 1000 ml. was

NaCl 8.0	*KCl* 0.2	$CaCl_2$ 0.2	$MgCl_2$ 0.1
$NaHCO_3$ 1.0	NaH_2PO_4 0.05	*Glucose* 1.0	

The solution was made up daily with distilled deionised water.

DRUGS AND COMPOUNDS USED

Drugs and compounds used were added into the isolated organ baths in the smallest possible volumes (50 to 300 μM.) usually by a microsyringe. The drug solutions were generally made up as concentrated stock solutions (10^{-2}M.) in deionised distilled water. Serial dilutions to the enquired concentrations were made up from the stocks in 9 % saline or distilled deionised water. All the concentrations in the text and the figures were the final concentrations in the baths.

Acetylcholine Chloride	Koch-Light
L-Arginine Hydrochloride	Sigma
L-Ascorbic Acid	Koch-Light
L-Canavanine Sulphate	Sigma
EDTA	Koch-Light
Guanethidine Sulphate	CIBA
L-monomethyl-N-arginine	Welcome Research Laboratories
D-monomethyl-N-arginine	Welcome Research Laboratories
Noradrenaline Bitartrate	Koch-Light

Phentolamine Mesylate	CIBA
L-Phenylephrine Hydrochloride	Koch-Light
Serotonin Creatinine Sulphate	Koch-Light
Superoxide Dismutase	Sigma

Acetylcholine, L-arginine, serotonin, L-NMMA, D-NMMA and L-canavanine were all made up as 10^{-2}M. (occasionally 10^{-1}M.) stock solutions. Each stock solution was divided into 1 ml. aliquots put into small plastic containers and stored in the deep freeze for future use.

Noradrenaline was made up as 10^{-2}M. stock solution in glass distilled deionised water previously titrated to pH 3 with 1N. HCl and dilutions were made in the water from the stock.

SOLUTION OF NITRIC OXIDE

The solution of nitric oxide was prepared immediately before use. Gaseous nitric oxide, 99% pure, was obtained from BDH. One end of a soft rubber tube was connected with the cylinder containing nitric oxide with the another end under water. Enough gas was passed trough to clear the air from the tubing. A fine hypodermic needle attached to an air-tight syringe was then used to pierce the tubing wall near the cylinder. 0.65 ml. of nitric oxide gas was drawn into the syringe and then injected at atmospheric pressure into 65 ml. of helium deoxygenated normal saline which filled completely a brown bottle sealed with a rubber seal. This produced a stock solution with a nominal concentration of 4.4×10^{-4}M. in the stock solution. The solution of nitric oxide was freshly made up daily and protected from light.

Normally the solutions of drugs and compounds used during the experiments were kept in ice. Unless otherwise stated all effects of the drugs and the compounds were verified on not less than eight preparations.

ABBREVIATIONS USED IN THE TEXT AND THE FIGURES

Ach	Acetylcholine
ARG	L-Arginine
BRP	Bovine Retractors Penis
t-BRP	Test Bovine Retractor Penis
d-BRP	Donor Bovine Retractor Penis
CAN	L-Canavanine
EDRF	Endothelium-derived relaxing Factor
EDTA	Ethylenediaminetetraacetic Acid
Hb	Haemoglobin
5-HT	Serotonin
NA	Noradrenaline
NANC	Non-adrenergic Non-cholinergic
L-NMMA	L-monomethyl-N-arginine
D-NMMA	D-monomethyl-N-arginine
P	Probability
RAS	Rabbit Aortic Strip
SOD	Superoxide Dismutase
TTX	Tetrodotoxin

STATISTICS

Sets of observations were expressed as the mean +/- one standard error of the mean (s.e.m.) in appropriate tables and charts. The significance of the difference between sets of means was analysed and estimated normally by using the student's t-test. Where the variance was unequal and the student's t-test was invalid, the U-test (Mann-Whitney test) was used instead. The probability more than 0.05 is considered no significant difference, less than or equal to 0.05 is significant, and less than or equal to 0.01 is very significant between sets of means.

RESULTS

THE BIOASSAY DETECTION OF THE NANC NEUROTRANSMITTER

While non-adrenergic non-cholinergic (NANC) nerves are known to innervate the bovine retractor penis muscle (Klinge et al. 1970; Klinge & Sjostrand 1974; Ambache & Killick 1978) and the rat anococcygeus muscle (Gillespie 1972; Gillespie & McGrath 1973; Gillespie & McGrath 1974; Creed et al. 1977), the neurotransmitter is still unknown. Many substances especially some known neurotransmitters were rejected as the non-adrenergic non-cholinergic neurotransmitter (Klinge & Sjostrand 1974). Now the most likely candidates are focused on the endothelium-derived relaxing factor (EDRF), nitric oxide (NO), and the inhibitory factor (IF) extracted from the BRP muscles because of their many properties in common with each other. Among these three candidates the IF is very promising. Many attempts have been made to analyse and classify the non-adrenergic non-cholinergic neurotransmitter on the basis of drug interaction with the NANC response. Other attempts have been made to isolate and purify and chemically identify the IF but with only partial success. If the non-adrenergic non-cholinergic neurotransmitter released from the BRP muscles, on the other hand, could be detected by bioassay, it would obviously pave the way to investigate the properties of the non-adrenergic non-cholinergic neurotransmitter and the effects of drugs on it and its nerves and receptors, to extract and purify the neurotransmitter, and finally to identify the neurotransmitter. Such attempts to detect the neurotransmitter in a Loewi-type experiment have been

made in this laboratory before without success. It was hoped that new information from work on EDRF if appeared to BRP might change this.

THE RELEASE AND DETECTION OF THE NANC INHIBITORY NEUROTRANSMITTER IN THE BRP MUSCLE

BRP muscle strips were mounted in the baths as illustrated in Fig. 4 and tone raised by guanethidine (10^{-5}M). The NANC inhibitory relaxation of the donor BRP muscle strips was induced by field stimulation and detected by relaxation of the raised tone. Many methods were used to try to increase the release of the NANC inhibitory neurotransmitter and to improve its chances of diffusing to the bath fluid to be detected by the test BRP muscles. For example thinner donor BRP muscle strips (15 to 20 mm. length, 2 to 4 mm. width) to improve diffusion or bigger donor BRP muscle strips (20 to 30 mm. length, 4 to 6 mm. width) to increase the total released. The possibility of increasing the sensitivity of the detection system was explored by using different test tissues (BRP muscles, rat anococcygeus muscles or rabbit aortic strips). The field stimulation parameters were carried always through with supramaximal voltage. The numbers of pulses ranged from 10, 20, 50 up to 100 at frequencies 0.1, 0.2, 0.5, 1, 2, 5 and 10 Hz, different durations of stimulation from 10, 20 seconds, or 60 seconds at 0.1, 0.2, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 Hz, or continuous field stimulation of supramaximal voltage at frequencies 5, 10, 20 or 40 Hz for 60 or 120 seconds respectively. None of these gave any indication of a response of the test tissue strips related to NANC inhibitory relaxation of the donor BRP muscle strips (Fig. 8 and Fig. 9)

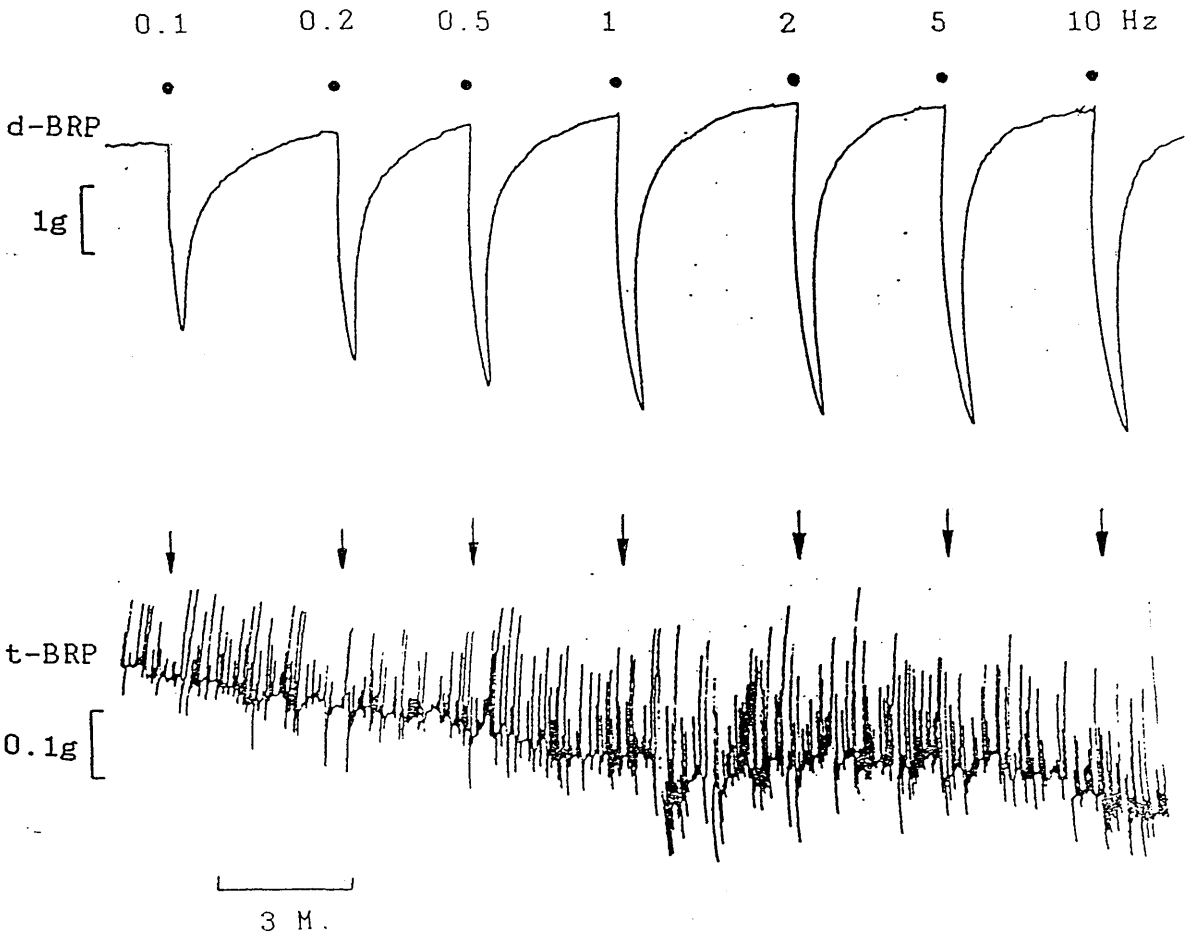


Fig. 8 Compares the response of the donor BRP preparation (d-BRP) to stimulation of its NANC nerves at the frequencies shown above each response with the failure of the test preparation (t-BRP) to show any response. Stimulation was by 0.5 ms. pulses at supramaximal voltage for 10 seconds. Tone was raised in both preparations by guanethidine 10^{-5}M in the Krebs' solution. Bioassay in a bath as in figure 4.

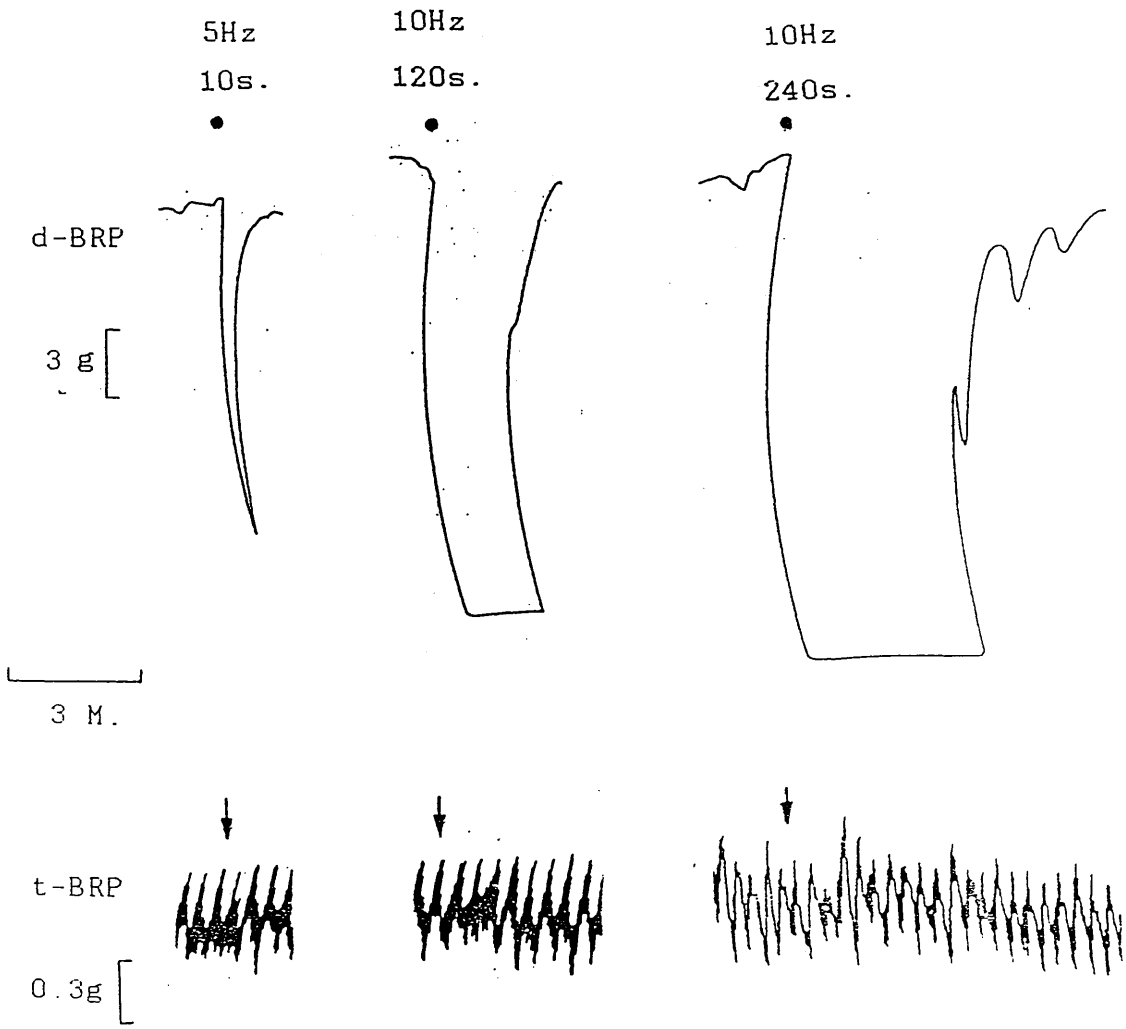


Fig. 9 Compares the response of the donor BRP preparation (d-BRP) to stimulation of its NANC nerves at the frequencies and for duration shown above each response with the failure of the test preparation (t-BRP) to show any response. Stimulation was by 0.5 ms. pulses at supramaximal voltage. Tone was raised in these preparations by guanethidine 10^{-5}M in the Krebs' reservoir. Bioassay in a bath as figure 4.

RELEASE AND DETECTION OF NANC NEUROTRANSMITTER IN A CASCADE SYSTEM

It was possible in the experiments described in the previous section that the dilution of released neurotransmitter by the bath fluid was too great for detection. Therefore a superfusion system greatly reducing the volume was used. In such a system it was possible the NANC inhibitory neurotransmitter could be detected. The arrangement for these experiments is illustrated in Figure 5. Before the experiments were begun, the test and the donor tissues were superfused to equilibrate for 60 to 90 minutes. The Krebs' solution flow rate from the Watson-Marlow constant flow pump was coliberated in advance and temperature adjusted to 37° C by a thermistor controlling the bath temperature. The test tissues were very close to the donor BRP muscle strip in order to shorten the time for the superfusate dropping on the test tissue. Different test tissues (BRP muscle, rat anococcygeus muscle or rabbit aorta), differently sized donor BRP muscle strips (20 to 40 mm. length, 5 to 10 mm. width for the bigger strips or 15 to 20 mm. length, 2 to 4 mm. width for the thinner strips), different parameters of the field stimulation (higher or lower frequencies, shorter, longer or continuous stimulation), and different output of the Krebs' superfusate flow (0.5, 1, 2, 4, 6, 8, 10, 12 or 15 ml. per minute) were studied. No inhibitory response of the test tissues was even observed in spite of powerful and maintained NANC inhibitory relaxation of the donor tissue in response to field stimulation. Drugs to raise tone in the donor and test tissues were added to the Krebs' solution in the reservoir. Where the BRP was used as the only test tissue, guanethidine (10^{-5}M) was used. With the rabbit aortic strip as a second test tissue phentolamine (10^{-5}M) to block α adrenergic excitation plus serotonin (10^{-5}M) was used.

THE RELEASE AND DETECTION OF THE NANC NEUROTRANSMITTER IN A CHAMBER SYSTEM

The closed cascade illustrated in Figure 6 had been used in this laboratory very successfully to demonstrate and study the release of EDRF from rabbit aortic endothelium. Since there may be a relationship between the NANC neurotransmitter and EDRF, it was felt to be worthwhile testing whether similar conditions would be successful with the NANC neurotransmitter. The chamber containing 1 ml. Krebs' solution and the arrangements for the test and the donor tissues are illustrated in Fig. 6. Where the test preparations were the BRP and rat anococcygeus muscle strips, these were always above the ring electrode and the donor BRP muscle strip to prevent escaping current stimulated their own NANC nerves. When the test preparation was rabbit aortic strip, this could be maintained very close to the donor BRP tissue because in the absence of NANC nerves the rabbit aortic strip had no inhibitory response to the field stimulation. The tone of the test and the donor strips was raised by guanethidine (10^{-5}M), or 5-HT (10^{-5}M) with phentolamine (10^{-5}M), or noradrenaline (10^{-6}M) added into the Krebs' solution reservoir. Again, many methods were used to try to detect the NANC inhibitory neurotransmitter i.e. differently sized donor BRP muscle strip (30 to 40 mm. length, 3 to 4 mm. width to release more NANC neurotransmitter, or 15 to 30 mm. length, 2 to 3 mm. width to increase diffusion), different flow rates of the Krebs' superfusate solution (0, 0.5, 1, 2, 4, 6, 8, 10, 12 or 15 ml. per minute) and different parameters of the field stimulation (numbers of pulses carrying from 10, 20, 50 or 100 respectively at frequencies 0.1, 0.2, 0.5, 1, 2, 5 and 10 Hz, or a fixed time of the stimulation of 10 or 20 seconds at frequencies 0.1, 0.2, 0.5, 1, 2, 5 and 10 Hz, or continuous field stimulation for 60, 120 or 180 seconds at 2, 5 or 10 Hz, with supramaximal voltage and 0.5 milliseconds' pulse duration). All failed to successfully detect any

NANC inhibitory neurotransmitter released from the donor BRP muscle by the test tissues (Fig. 10 and Fig. 11).

As a control the same system and the conditions were used to demonstrate release of the endogenous neurotransmitter noradrenaline by field stimulation from the same donor BRP muscle and to compare this with known concentrations of exogenous noradrenaline (10^{-9}M) by the test tissues. Both BRP and rabbit aortic strips as the test tissues were successful in detecting noradrenaline released from the donor BRP muscle by field stimulation (Fig. 12 and Fig. 13). The amount released was less than 10^{-9}M noradrenaline showing that the test system was very sensitive at least to another known neurotransmitter noradrenaline.

In these experiments both endogenous and exogenous noradrenaline was prevented from being destroyed in the Krebs' solution by use of distilled deionised water to make up the Krebs and addition of EDTA (10^{-4}M) and ascorbic acid (10^{-5}M) to chelate any remaining heavy metal ions such as iron that could chelate the oxidation of noradrenaline. It was possible the failure to detect the NANC inhibitory neurotransmitter was due to a similar destruction of the NANC neurotransmitter and only if this could be prevented would it be possible to detect it. It is difficult to decrease such problem since the chemical nature of the NANC neurotransmitter is unknown. However, there are reasons to think it related to EDRF and nitric oxide which are rapidly destroyed by superoxide anions. Superoxide dismutase which protects EDRF might therefore both potentiate the response of the donor tissue and allow detection in the test tissues. This possibility was therefore investigated.

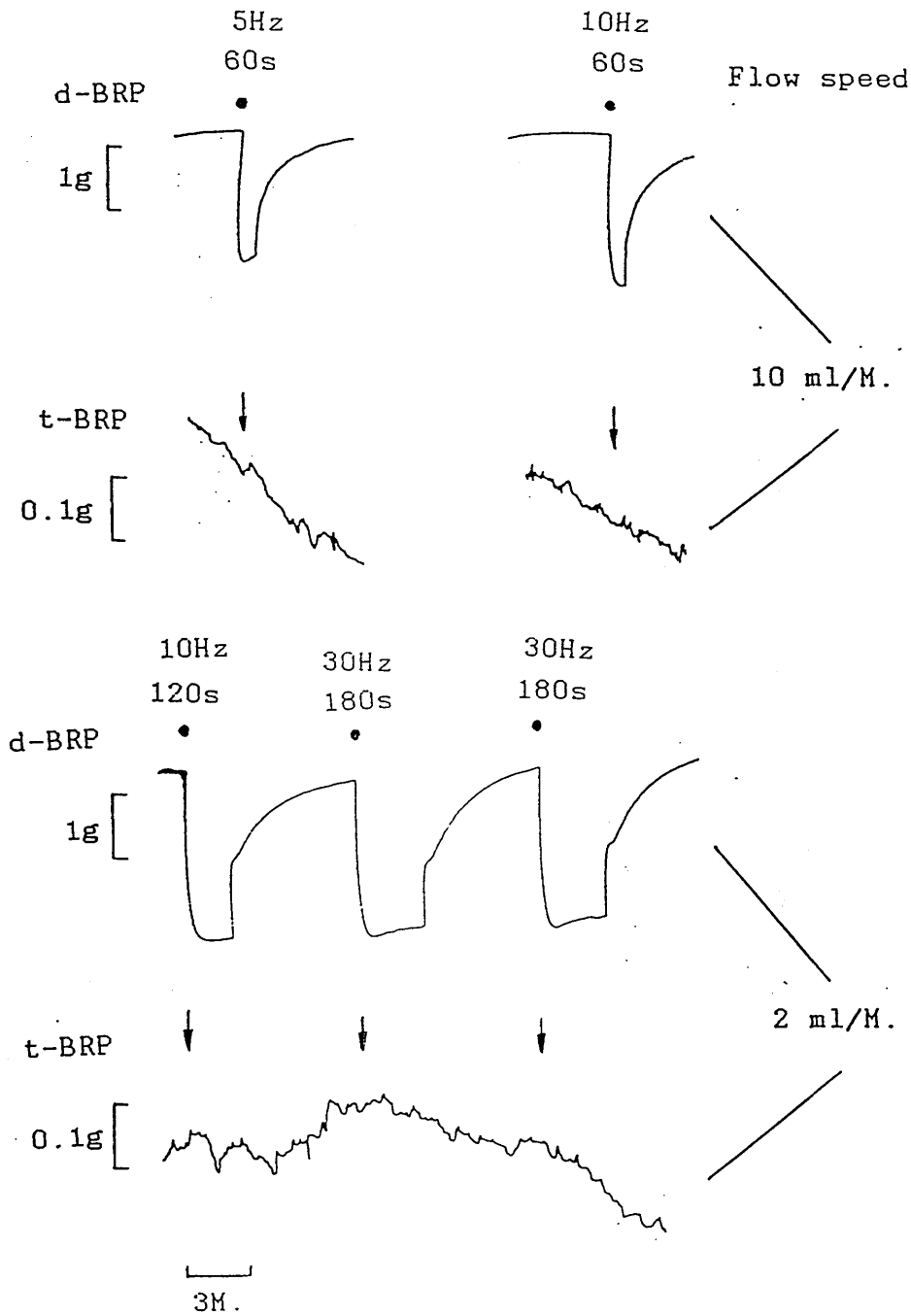


Fig. 11 Compares the response of donor BRP (d-BRP) preparation to stimulation for periods of time varying from 60 to 180 seconds and at frequencies of 5, 10 or 30 Hz with the failure of the test BRP (t-BRP) to response in any combination of conditions. Flow rates were either 10 ml. / min. (upper part of records) or 2 ml. / min. (lower part of records). Tone was raised by guanethidine 10^{-5}M. in the Krebs' solution. Bioassay in a closed cascade as in figure 6.

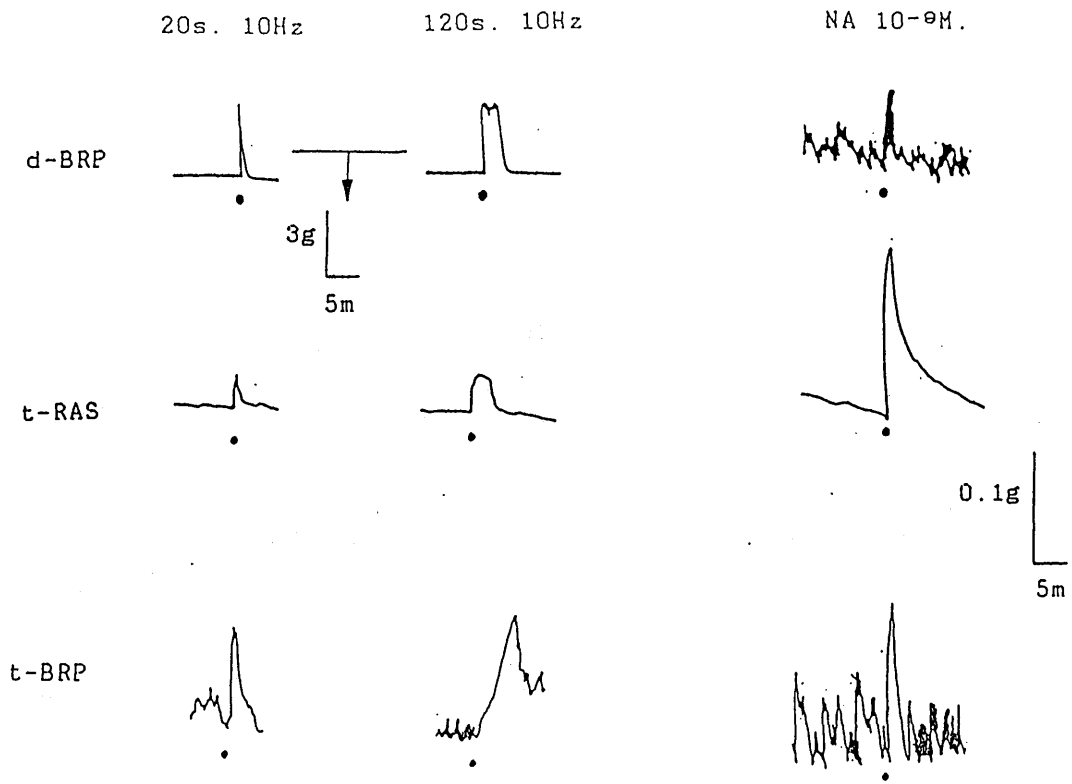


Fig. 12 A closed cascade system with a donor BRP (d-BRP) and two test tissues, the rabbit aortic strip (t-RAS) and the BRP (t-BRP). The donor BRP was stimulated for 20 or 120 seconds at 10 Hz and in the absence of tone or guanethidine contracts in response to stimulation of its adrenergic motor nerves. Both test preparations also contract and the contraction is greater with longer period of stimulation. The third penal shows the response of the three tissues to and injection of 1 ng. of noradrenaline. The response exceeds that to the released transmitter. Flow rate was 10 ml. / min.

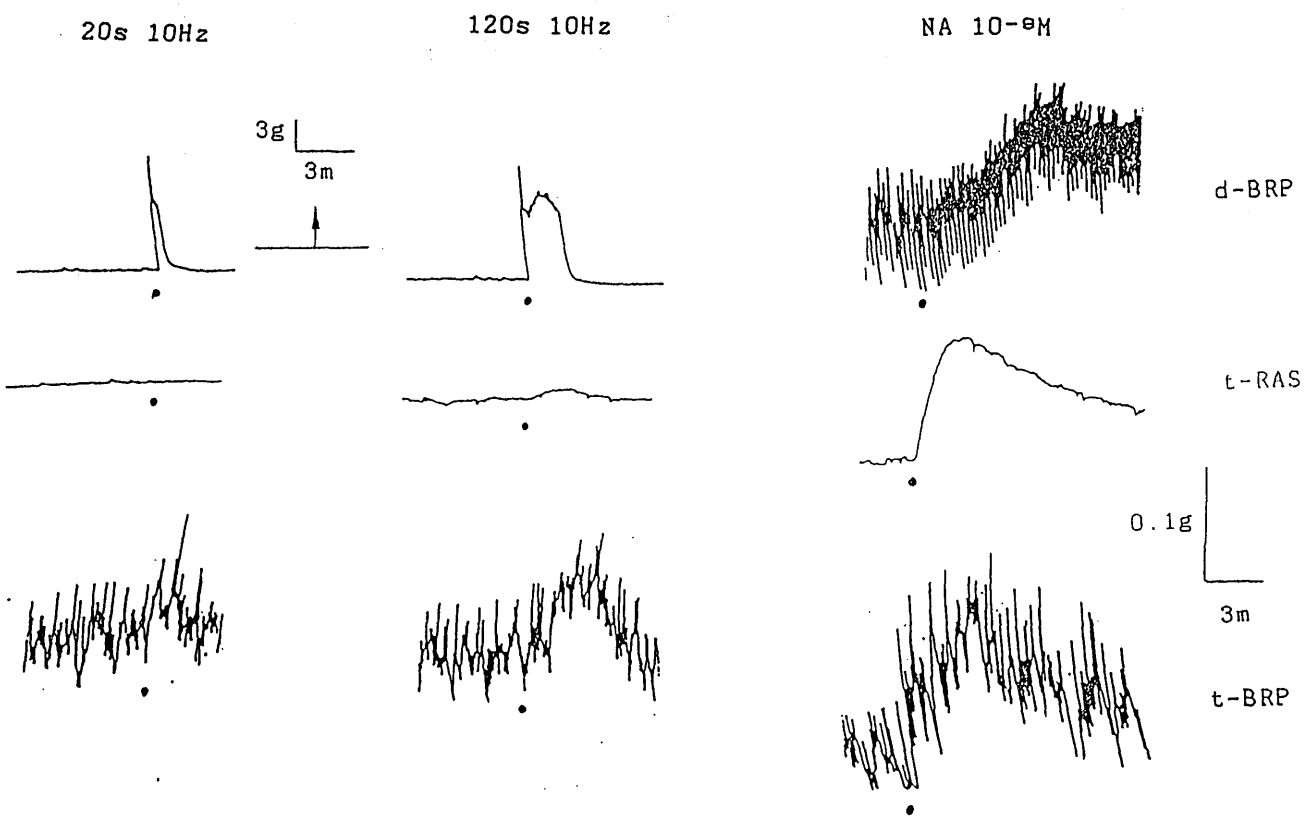


Fig. 13 The effect of flow rate on the detection of noradrenaline released from a donor BRP (d-BRP) and detected by a test rabbit aortic strip (t-RAS) and a test BRP (t-BRP). The arrangements including the frequency and duration of field stimulation were identical with the experiment illustrated in figure 12 but the flow rate was reduced to 2 ml. / min. Though the response of the donor BRP tissue is as great the response of the test tissues as much smaller in amplitude though prolonged. The same effect is seen with the injection of 1 ng. of exogenous noradrenaline.

THE EFFECT OF SUPEROXIDE DISMUTASE ON THE NANC INHIBITORY RESPONSE

EDRF is inactivated by superoxide anion in the effluent from superfused endothelial cells. Superoxide dismutase enhances the amount of EDRF released from the endothelial cells in the effluent (Gryglewski et al. 1986; Rubanyi & Vanhoutte 1986) because Superoxide dismutase destroys the superoxide anion (Apps & Harnden 1985). If the NANC neurotransmitter is nitric oxide, it was very likely that superoxide dismutase would prolong the half-life and potentiate the response.

THE EFFECT OF SUPEROXIDE DISMUTASE ON THE ISOLATED BRP MUSCLE IN A BATH

The BRP muscle strips were mounted in 10 ml. organ baths shown in Fig. 3. The tone of the strips was raised by guanethidine 10^{-5}M. The NANC inhibitory relaxation of the BRP muscle strips was induced by field stimulation on the NANC nerves. The effect of different concentrations of superoxide dismutase on the BRP muscle was studied at different frequencies, numbers of pulses and durations of field stimulation at supramaximal voltage and 0.5 milliseconds of pulse duration. There were no effects of the superoxide dismutase on the NANC inhibitory relaxation at final concentrations of 10, 20, 40, 80, 100, 200, 300 and 400 u./ ml. on responses to 10, 20, 50 or 100 pulses, or for 10 or 20 second of stimulation at 0.1, 0.2, 0.5, 1, 2, 5 and 10 Hz frequencies even higher frequencies of 16, 32, 64 and 128 Hz. Also superoxide dismutase had no effect on the tone of the BRP muscles. These results suggested that superoxide dismutase had no protective effect on the NANC inhibitory neurotransmitter. Fig. 14 and Fig.

15 are the typical examples of the effect of superoxide dismutase on the NANC inhibitory relaxation of BRP muscles.

THE EFFECT OF SUPEROXIDE DISMUTASE ON THE BIOASSAY OF THE TEST AND THE DONOR TISSUES IN A BATH

In spite of the failure of superoxide dismutase to potentiate the response to nerve stimulation, it might still protect that protein of the NANC neurotransmitter diffusing to the bath. It was possible that superoxide dismutase had no effect on the NANC inhibitory relaxation because the neuromuscular junctions of the NANC nerves are very tight so that the NANC inhibitory neurotransmitter available for superoxide dismutase to act was too short before the combined either with its specific receptor or diffused into the cell to act on guanylase cyclase. It was therefore considered worthwhile to see whether superoxide dismutase could protect the NANC inhibitory neurotransmitter released by a donor BRP muscle and allow it to be detected on the test tissues (BRP muscle, rat anococcygeus muscle or rabbit aorta).

In a 1 ml. isolated organ bath a donor BRP muscle strip and one or two test tissue strips (BRP muscle, rat anococcygeus muscle or rabbit aorta) were mounted as shown in Fig. 4 and Fig. 6. The tone of the tissues was raised by guanethidine (10^{-5}M), or if the rabbit aortic strip was used as a second test strip, 5-HT (10^{-5}M) with phentolamine (10^{-5}M) was used. The donor BRP muscle strip was field stimulated and the NANC relaxation displayed. Final concentrations of superoxide dismutase from 10 u./ ml. up to 200 u./ ml. Krebs' solution were used. The presence of the superoxide dismutase neither potentiated the NANC inhibitory relaxation in the donor BRP muscle strip, nor revealed any inhibitory

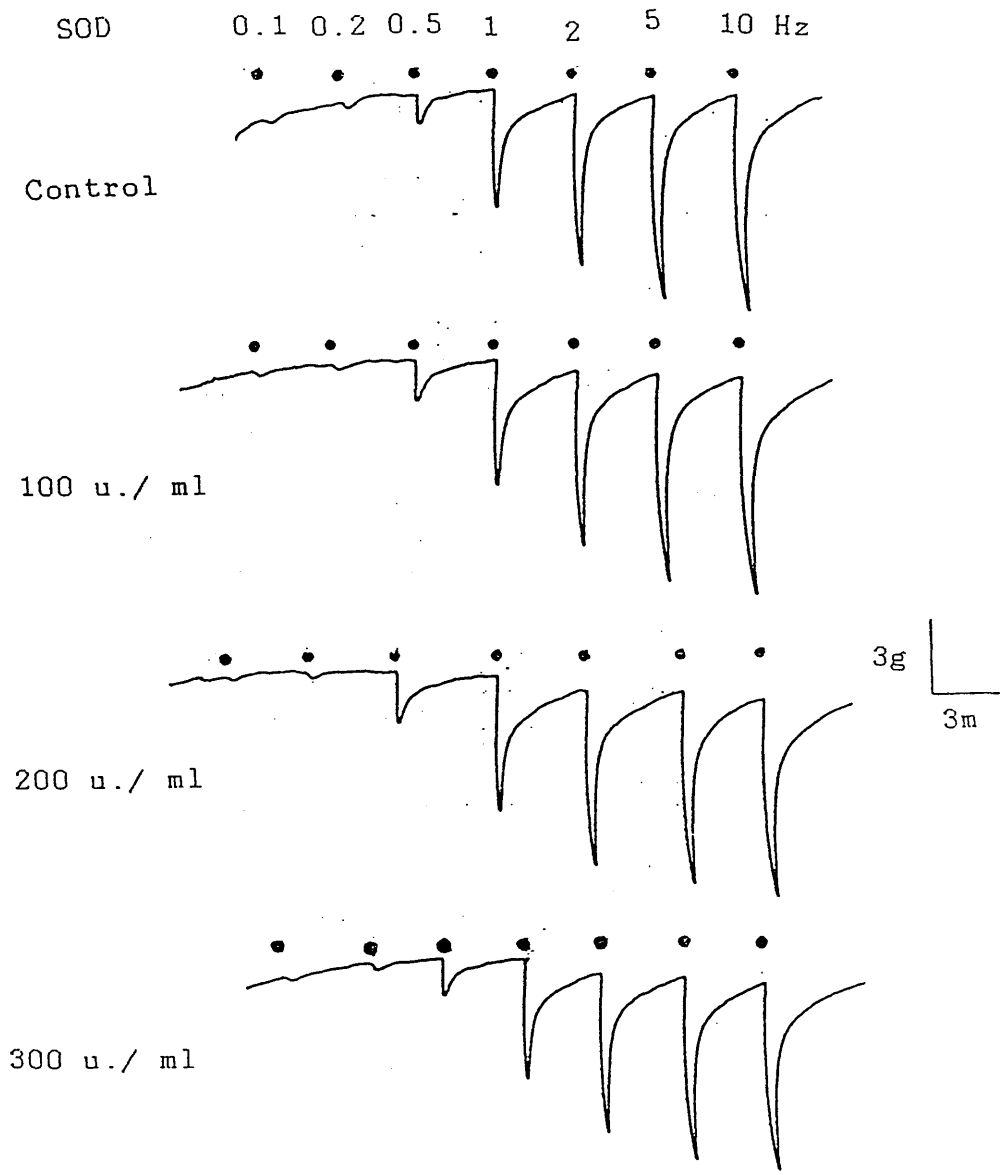


Fig. 14 The effect of superoxide dismutase (SOD) on the NANC inhibitory relaxation of BRP muscles to field stimulation (supramaximal voltage and 0.5 milliseconds for 10 seconds). The records are from a single experiment in which increasing concentrations of 100, 200 and 300 u./ ml. of SOD were added to the bath (Fig. 3) before repeating field stimulation. SOD had no effect on the NANC response. Tone was raised by guanethidine 10^{-5}M. in the bath as Fig. 3.

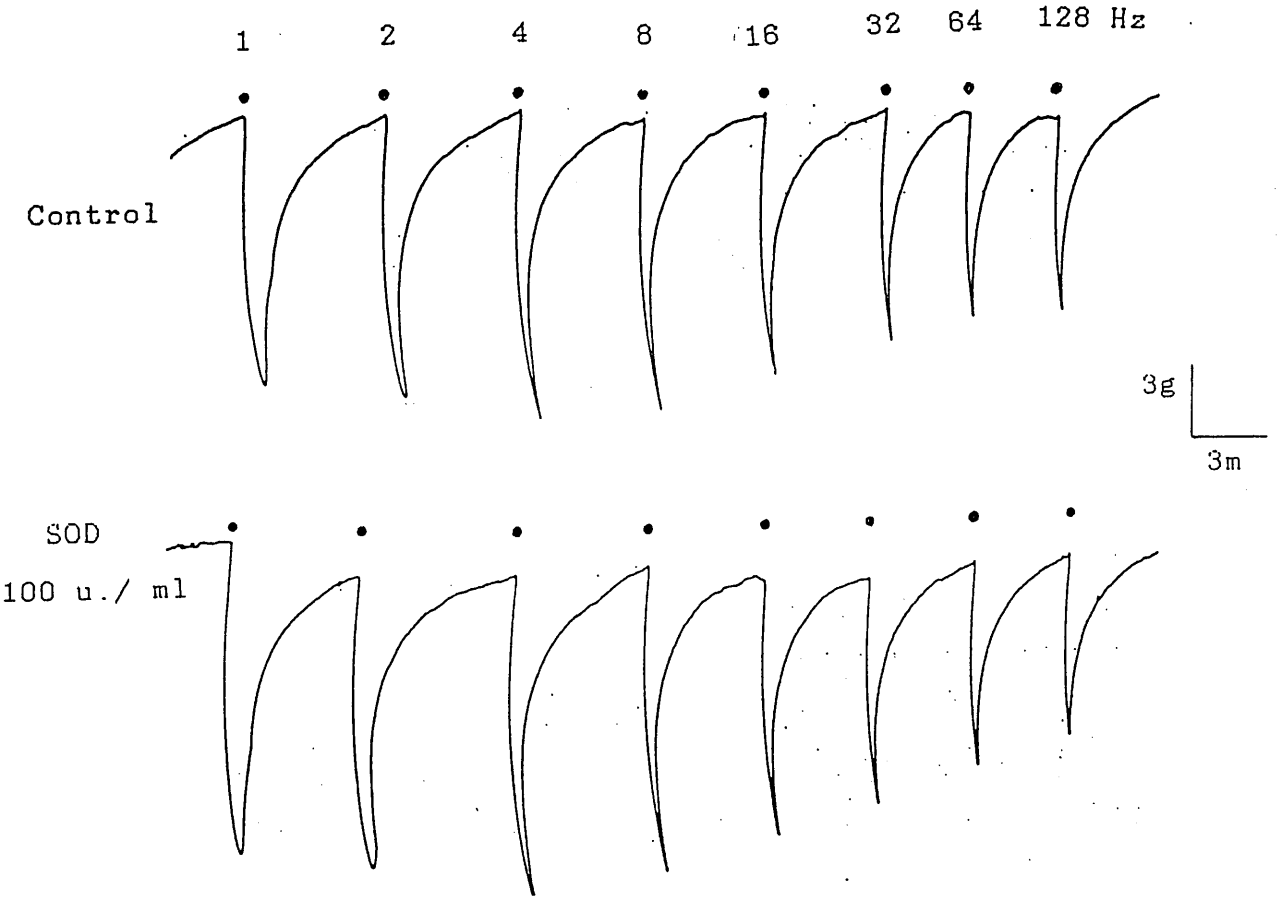


Fig. 15 The effect of superoxide dismutase (SOD) on the NANC inhibitory relaxation of the BRP muscle strip to field stimulation (100 pulses , supramaximal voltage and 0.5 milliseconds' pulse duration) against guanethidine-induced tone. The records are from a single experiment. After the control responses shown in the upper records, SOD 100 u. / ml. was added to the bath and the frequency-response repeated. SOD had no effect on the response.

response in the test preparations as shown in Fig. 16. That superoxide dismutase did not uncover either a potentiation in the donor tissues or protection of the NANC neurotransmitter in the bath suggests that superoxide anion released from the tissues or existing in the oxygenated Krebs' solution did not account for failure to detect the NANC inhibitory neurotransmitter.

ARTIFACTS IN THE DETECTION OF NEUROTRANSMITTER RELEASE

In using bioassay to detect release of NANC neurotransmitter it is necessary to use a high sensitivity of the transducer and polygraph. Such conditions make it easy for artifacts to suggest neurotransmitter is released and detected. In previous attempts to demonstrate neurotransmitter release, the escape of current to the test tissue, changes in pH and the release of chlorine by electrolysis at the electrodes applying the field stimulation to the donor tissue have all caused stimulation related inhibition of the test tissues but not related to NANC neurotransmitter released. In the present experiments the use of Ag / AgCl electrodes eliminated the problem of electrolysis but the escape of current from the donor tissue electrodes to the test tissue still remained a problem. This is illustrated in Figure 17. Stimulation of the donor BRP muscle clearly produced a relaxation of the test tissue. One suspicious feature was the shape of the response which was similar to that in donor BRP tissue and coincided exactly with it. If it had been due to released NANC neurotransmitter, it would be expected to be smaller in magnitude but longer lasting because of the dilution and delay in the bath. An alternative was escape of current from the electrodes stimulating a few nerve fibres in

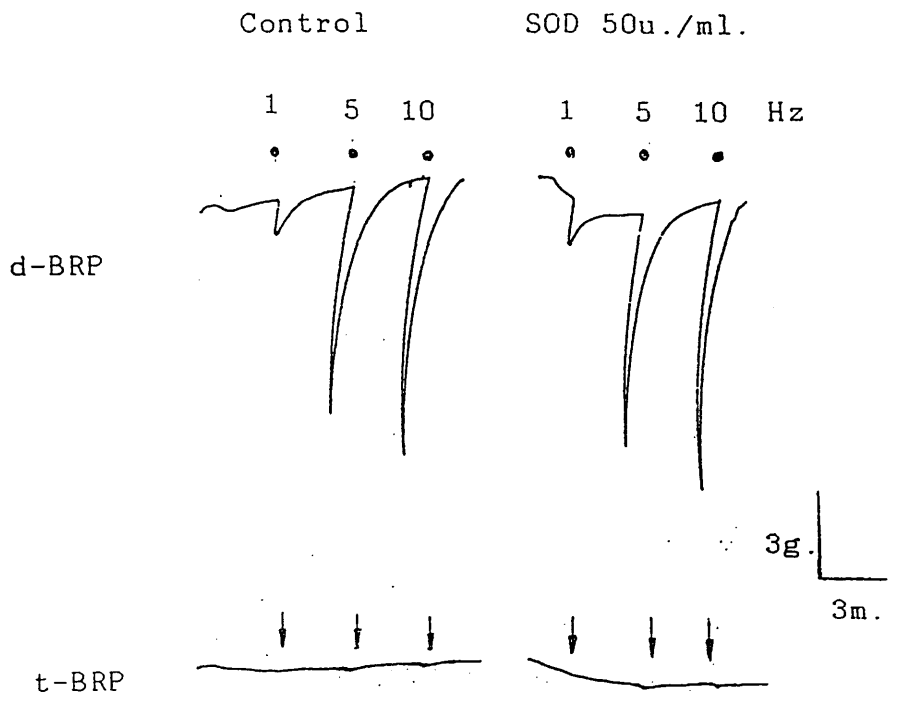


Fig. 16 Superoxide dismutase (SOD) does not potentiate the response of the donor BRP preparation (d-BRP) to stimulation of its NANC nerves at the frequencies shown above each response with the failure to protect NANC neurotransmitter released from the d-BRP and acting on the test preparation (t-BRP) to show any response. Flow rate of 6 ml. / ml. per minute was used. Stimulation was by 0.5 ms. pulses at supramaximal voltage for 10 seconds. Tone was raised in both preparations by guanethidine 10^{-5}M in the Krebs' reservoir. Bioassay in a bath as in figure 4.

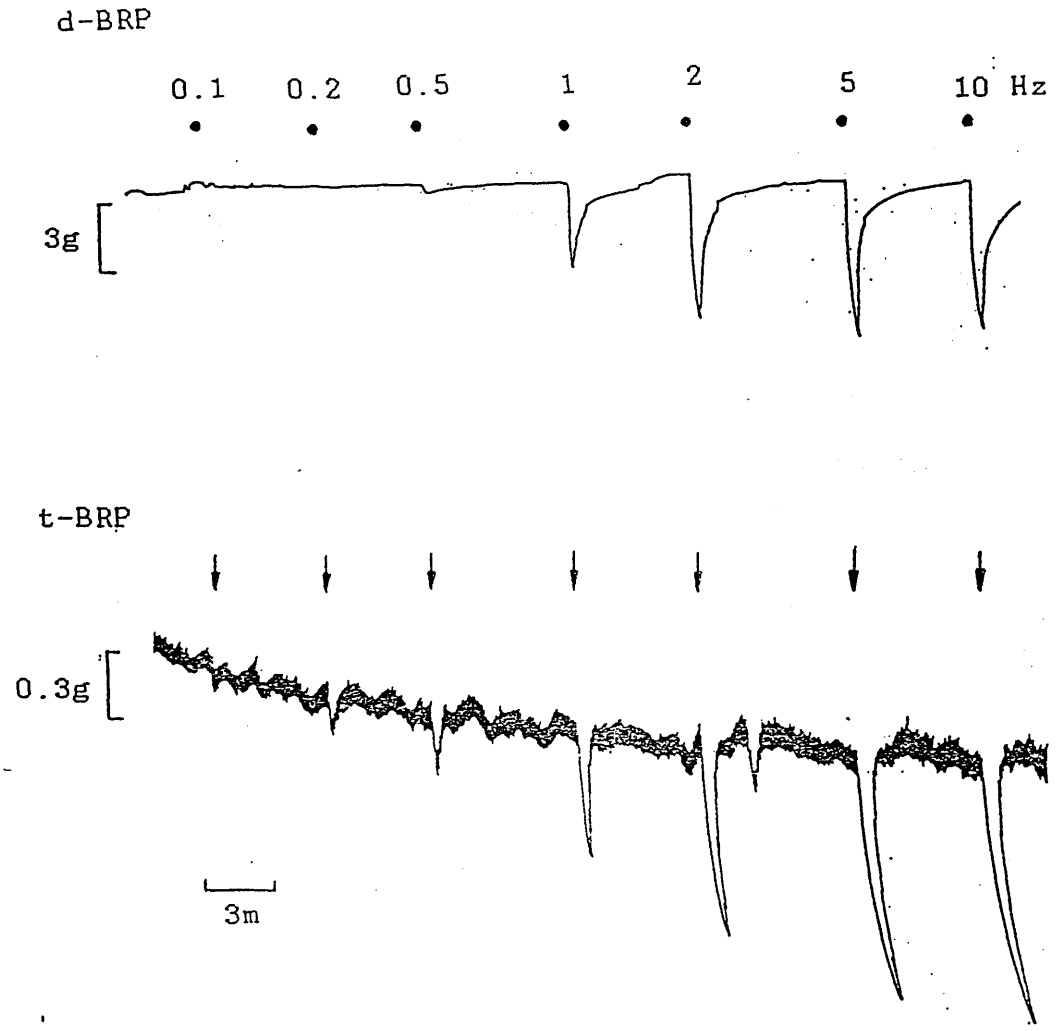


Fig. 17 The response of the donor BRP (d-BRP) and the test BRP (t-BRP) preparations to field stimulation (supramaximal voltage and 0.5 millisecond duration for 10 seconds at frequencies shown above) in a bath (Fig. 4). Tone was raised by 5-HT 10^{-5}M.

the test BRP muscle.

Therefore, experiments were designed to investigate this problem. First, if the relaxation of the test tissues was due to field stimulation of the NANC nerves of the test tissues, the rabbit aortic strip lacking such inhibitory nerves should show no response. If this inhibitory response in the test tissues was due to the action of the NANC inhibitory neurotransmitter, then assaying it also relaxes vascular smooth muscle, the test rabbit aortic strip should also relax. When the experiment was repeated with both the BRP and a rabbit aortic strip simultaneously present as the test preparations, the rabbit aortic strip exhibited no response while the donor BRP and the test tissues (BRP or rat anococcygeus muscle) produced inhibitory relaxation (Fig. 18). This result did not exclude the possibility that the relaxation of the test BRP or rat anococcygeus muscle was due to NANC neurotransmitter released from the donor BRP muscle since vascular muscle might not respond. A second experiment providing more direct evidence was therefore carried out. The donor BRP muscle was removed from the bath but leaving the electrodes, the other conditions were unchanged and the field stimulation was repeated. The test BRP and rat anococcygeus muscles still relaxed as they did when the donor preparation had been present. Clearly this response could not be due to the NANC neurotransmitter release but must be due to current escape acting directly on the NANC nerves in the test preparations as shown in Figure 19. Another explanation of these results was the release of some other substances as a result of the field stimulation, for example Cl_2 which was the product of electrolysis of the NaCl of the Krebs' solution. If this was so and stimulation of the NANC nerves of the test preparations was unnecessary then tetrodotoxin (3×10^{-6}M) or haemoglobin (3×10^{-6}M) which blocks these nerves on the effect of the neurotransmitter should have no effect. This was tested. In

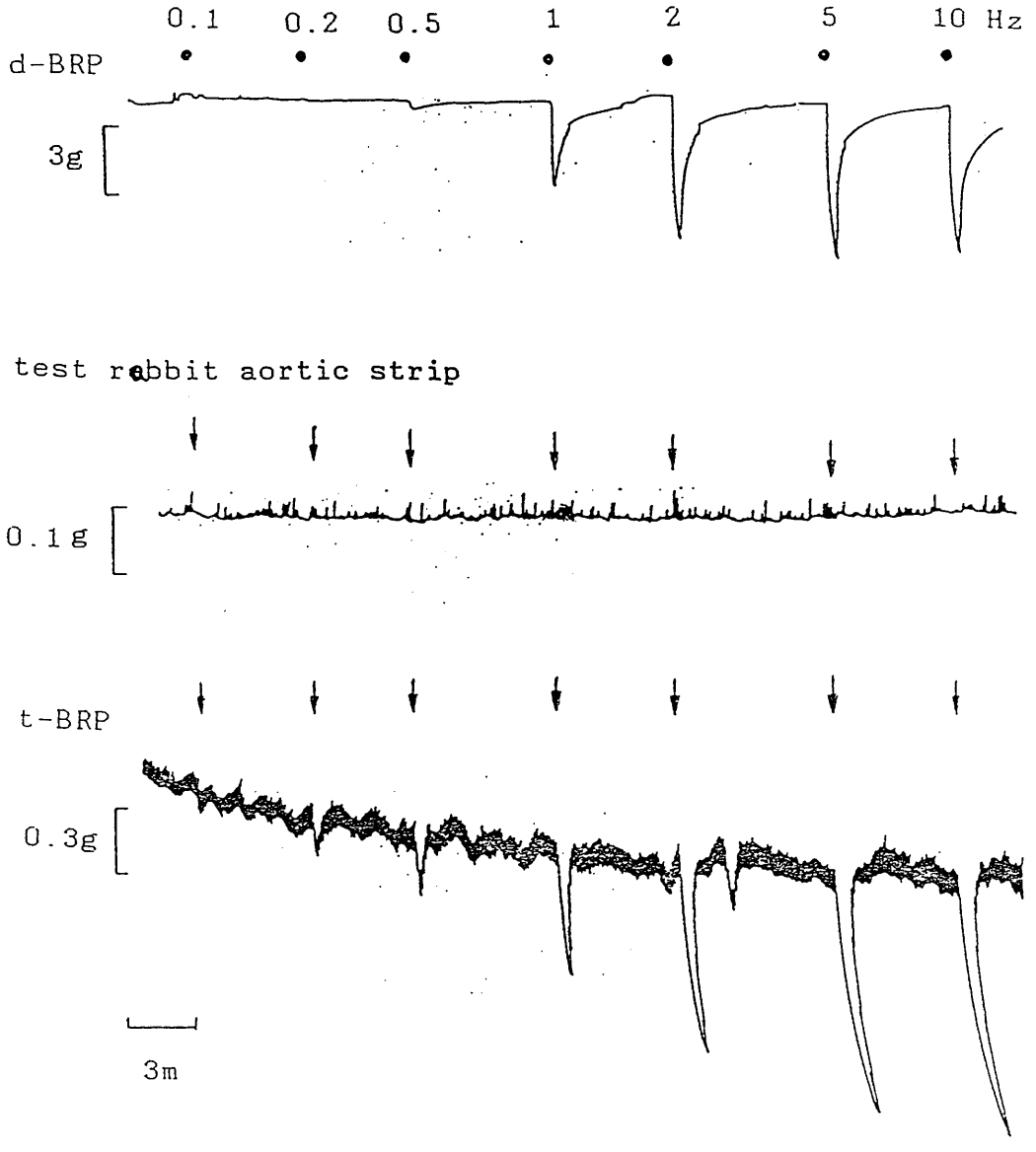


Fig. 18 The effect of field stimulation of the NANC nerves in a donor BRP (d-BRP) and two test preparations simultaneously present in the bath (Fig. 4). The test BRP (t-BRP) relaxes when the donor BRP is stimulated but there is no effect on the rabbit aortic strip. Stimulation was with 0.5 ms. pulses of supramaximal voltage for 10 seconds at the frequencies shown above each response. Tone was raised in all preparations with 5-HT (10^{-5}M.).

the presence of either tetrodotoxin or haemoglobin the inhibitory relaxation of the test BRP and rat anococcygeus muscles was abolished (Fig. 19). This result indicated the inhibitory relaxation of the test tissues was induced by stimulation of NANC nerves in the test preparations by the current escape.

The results of all of these experiments simply conform previous efforts that it is not possible with bioassay even using superoxide dismutase to protect the putative NANC neurotransmitter to demonstrate the neurotransmitter release into the bath fluid and superfusate.

THE EFFECT OF SOME DRUGS ON THE NANC INHIBITORY RELAXATION IN THE BRP AND THE RAT ANOCOCCYGEUS MUSCLE

An alternative approach to the identification of the NANC neurotransmitter is to study those drugs which mimic or abolish the inhibitory response. This method has been used with other traditional neurotransmitters, for example atropine in identifying cholinergic neurotransmission and ergot in adrenergic neurotransmission. The drugs already known to affect NANC neurotransmitter are haemoglobin (Bowman & Gillespie 1981; 1982a; 1982b; Bowman et al. 1982;), methylene blue (Bowman & Drummond 1984) and ethonal (Gillespie et al. 1981). None have identified the NANC neurotransmitter but haemoglobin has suggested there could be a relationship with EDRF. Recently there is evidence that EDRF is nitric oxide and may be synthesized from L-arginine by porcine aortic endothelial cells in culture (Palmer et al. 1988a). L-arginine is a physiological precursor of endothelium-derived nitric oxide (Palmer et al. 1988a;

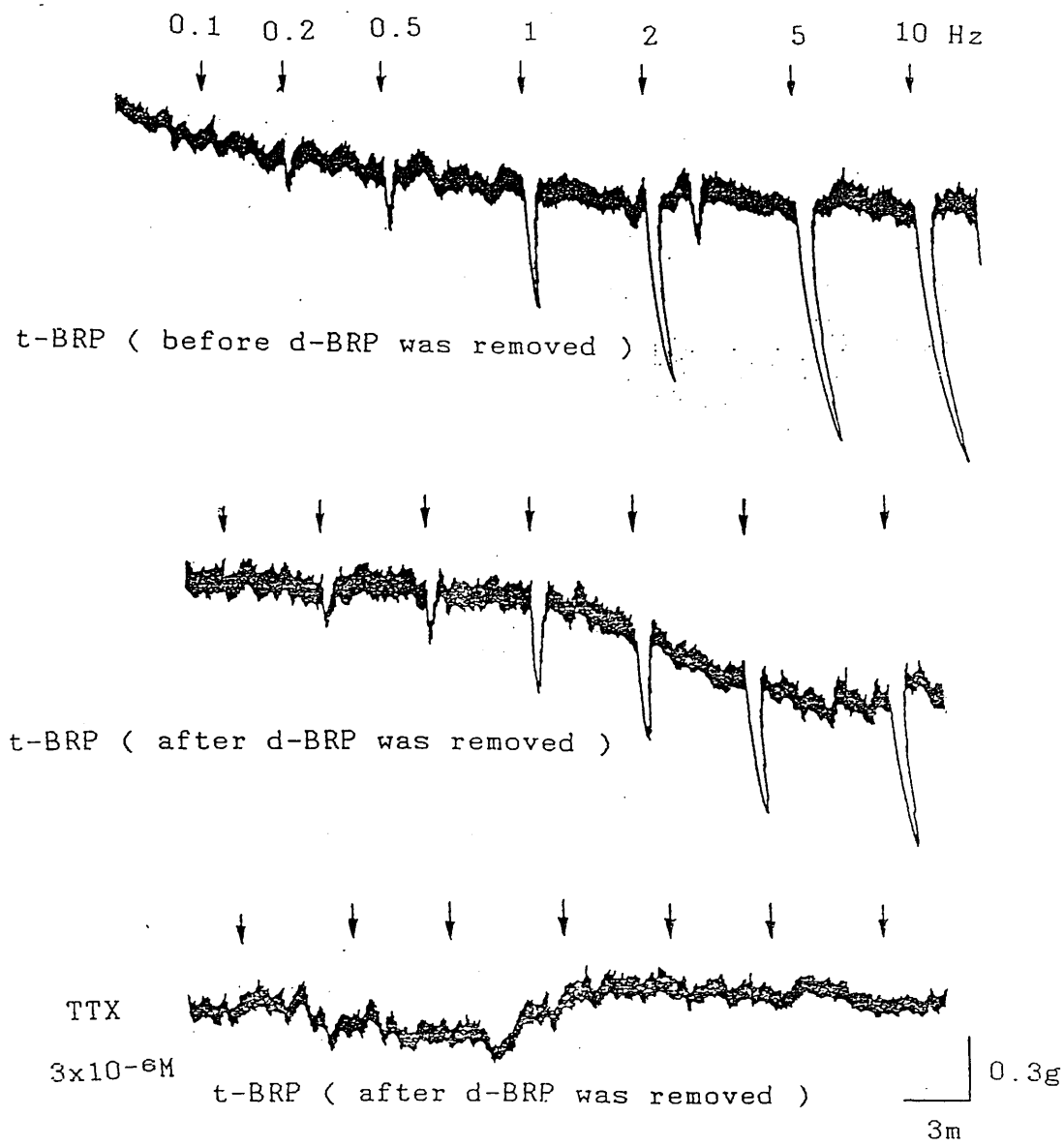


Fig. 19 The effect of field stimulation in the presence (upper record) and after removal of the donor BRP preparation. The test BRP relaxes to field stimulation whether the donor BRP is presence in the electrodes or not. The lower record shows the effect of adding tetrodotoxin (TTX) 3 x 10^{-6}M. to block the NANC nerves in the test preparation. Field stimulation in the presence of TTX is no longer effective. Tone was raised by 5-HT 10^{-5}M.

Palmer et al. 1988b; Schmidt et al. 1988a; Schmidt et al. 1988b).

THE EFFECT OF L-ARGININE ON THE NANC INHIBITORY RELAXATION IN THE FRESH BRP MUSCLE

The experiments were designed to investigate whether L-arginine could potentiate the NANC inhibitory response to field stimulation in the BRP muscles and therefore whether L-arginine is a physiological precursor of the NANC inhibitory neurotransmitter in the BRP muscles. If so this would strengthen the case for nitric oxide as the NANC inhibitory neurotransmitter.

Palmer et al. (1988a) reported the vascular endothelial cells synthesized nitric oxide from L-arginine (1 to 10 μM.) and the half-maximal effective concentration (EC_{50}) of L-arginine was 3.5 +/- 0.1 μM while D-arginine (10 to 100 μM.) was without effect in the cultured porcine aortic endothelial cells. Falling on these experiments, the effects of L-arginine in concentrations from 10^{-5}M. to 3×10^{-4}M. on the response of the BRP to NANC nerves stimulation have been examined.

Fresh BRP muscle strips were mounted in 10 ml. organ baths as shown in Fig. 3. The tone of the strips was raised by guanethidine (10^{-5}M.). The preparations produced NANC inhibitory relaxation induced by field stimulation at 0.1, 0.2, 0.5, 1, 2, 5 and 10 Hz frequencies of supramaximal voltage, 20 pulses and 0.5 milliseconds' pulse duration. The results for two individual experiments are shown in Fig. 20 and Fig. 21. Dose-response curves of L-arginine on the response were studied and the results are shown for L-arginine 3×10^{-5}M. and 3×10^{-4}M. in Figure 22. The NANC inhibitory response was unchanged by

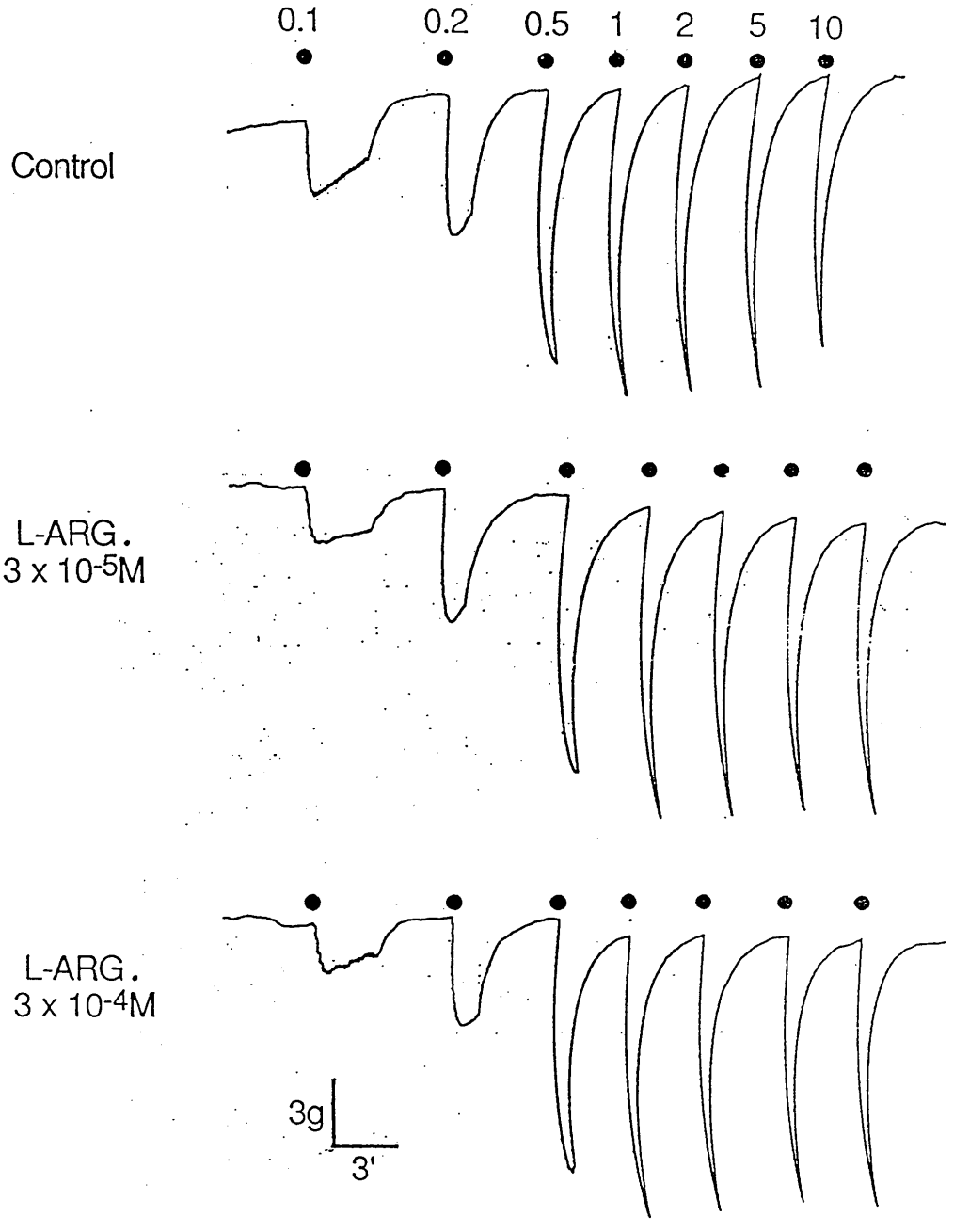


Fig. 20 The effect of L-arginine on the NANC inhibitory relaxation of fresh BRP preparations to field stimulation (supramaximal voltage, 20 pulses and 0.5 milliseconds duration at frequencies shown above each response). The upper records show the control response, the middle records are from the same experiment but after the addition of 3 x 10^{-5}M. L-arginine and the lower records again from the same experiment in the presence of 3 x 10^{-4}M. L-arginine. There is no potentiation of the response. Tone was raised by guanethidine 10^{-5}M.

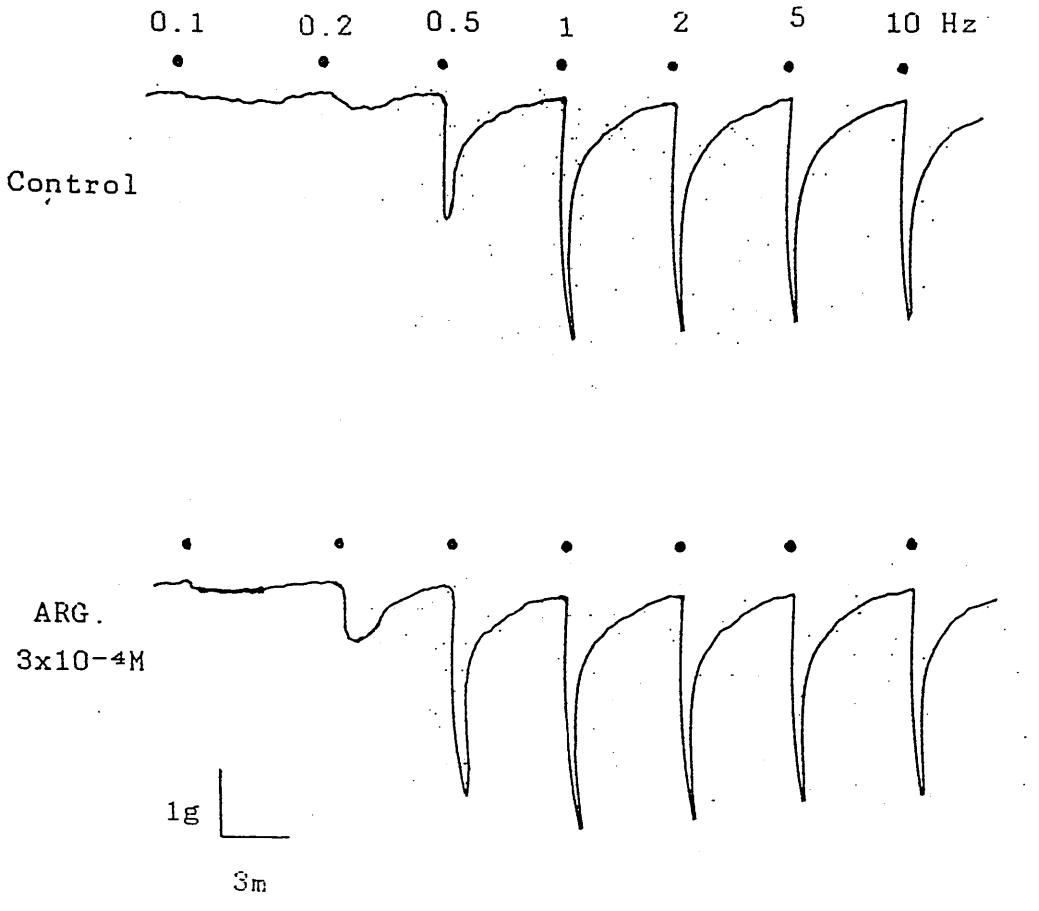


Fig. 21 The effect of L-arginine on the NANC inhibitory relaxation of fresh BRP muscles to field stimulation (supramaximal voltage and 0.5 milliseconds pulse duration for 20 pulses at frequencies shown above the response). The upper records are the control, the lower from the same experiment but after the addition of 3 x 10^{-4}M. L-arginine to the bath. In this experiment arginine increased the response at the three lower frequencies of stimulation. Tone was raised by guanethidine 10^{-5}M. in the bath.

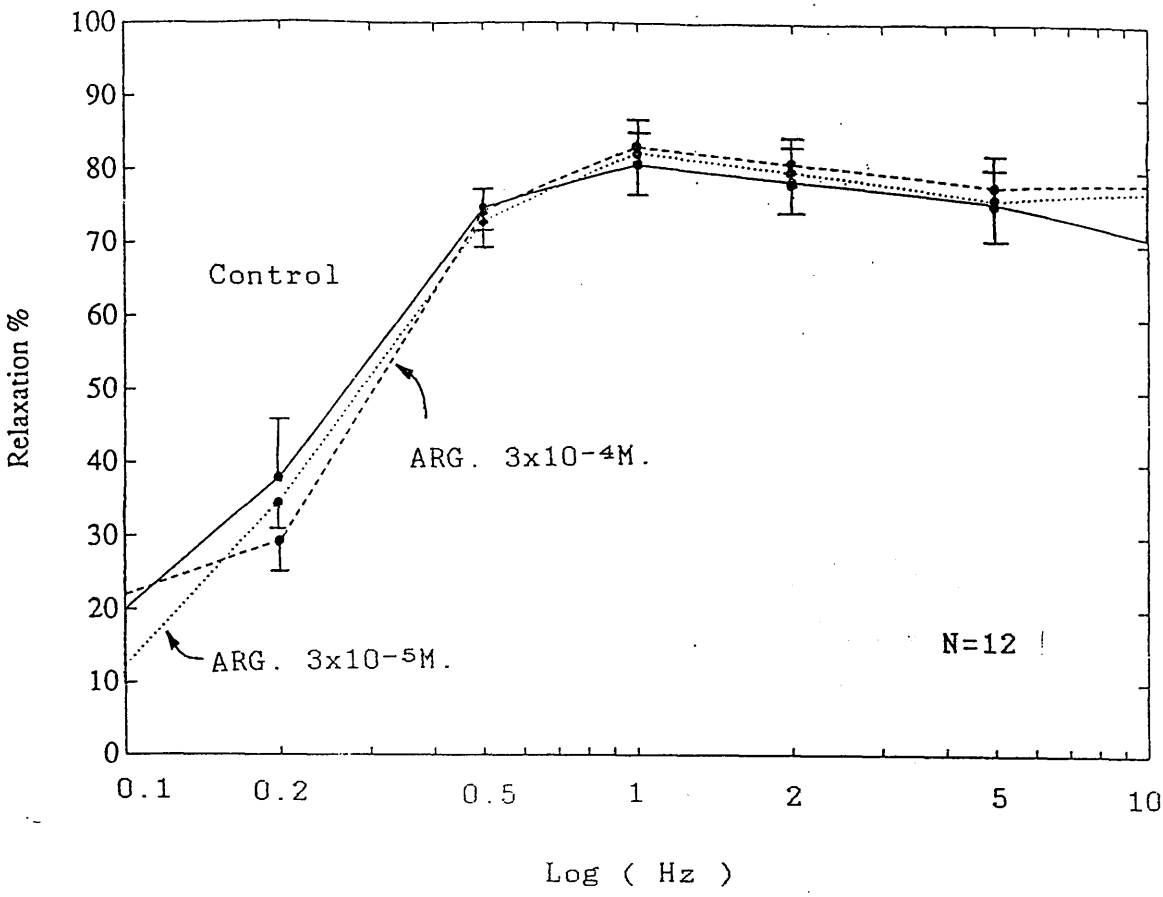


Fig. 22 Shows the combined results of all experiments without effects of L-arginine 3 x 10^{-5}M. and 3 x 10^{-4}M. on the response of fresh BRP to NANC nerve stimulation at the frequencies shown on the X-axis. The preparations were stimulated by field stimulation using 20 pulses at supramaximal voltages of 0.5 ms. pulse duration. Tone was raised with guanethidine 10^{-5}M. added to the bath. L-arginine had no effect on the response to field stimulation. Probability values are more than 0.05.

addition of L-arginine 3×10^{-5}M, and 3×10^{-4}M. (Fig. 20). Occasionally L-arginine potentiated the inhibitory relaxation to field stimulation at low frequencies, such an experiment is in Fig. 21 where L-arginine 3×10^{-4}M. potentiated the response at 0.1, 0.2 and 0.5 Hz. However when all the results with L-arginine were combined, no statistically significant effect on the NANC nerve stimulation in the BRP muscle was observed (Fig. 22). At all concentrations of L-arginine the probability values are more than 0.05 on the frequency-response curves. Nevertheless though no single concentration produced a statistically significant effect, it was noticed that at all concentrations the frequency-response curves in the presence of L-arginine were somewhat shifted to the left compared with the control (Fig. 22).

In the experiments described by Palmer et al. (1988a) L-arginine (1 to 10 μM.), but not D-arginine (10 to 100 μM.), perfused through a column of porcine endothelial cells for 5 minutes before and continuing during a 5 minutes exposure of the cells to bradykinin, increased the nitric oxide released from the cells. The cells were grown on culture on microcarrier beads and only if these cultured cells were washed three times and cultured for a further 24 hours in fresh culture medium without L-arginine, there was an enhancement of nitric oxide release observed. If the cultured cells were washed three times and cultured for a further 24 hours in fresh culture medium containing L-arginine, the infusion of L-arginine had no effect on the release of nitric oxide in 11 out 16 experiments. The results indicated that only if endogenous L-arginine is deficient in the cultured porcine endothelial cells will exogenous L-arginine be able to enhance the release of nitric oxide from the cells stimulated by bradykinin.

In the experiments with fresh BRP muscle preparations just dissected, it is

unlikely that endogenous L-arginine is depleted or deficient in anyway. Therefore, the likely explanation of failure of L-arginine to potentiate the NANC response to field stimulation of the BRP muscle was the presence of a sufficient supply of endogenous L-arginine for the nerves to synthesize the NANC inhibitory neurotransmitter. If this store of L-arginine could be deplete then exogenous L-arginine might indeed potentiate the NANC response. Two methods were used on an attempt to produce this depletion. First continuous field stimulation was applied at high frequencies. The BRP muscle strips exhibited complete NANC inhibitory relaxation and for prolonged period up to one hour. This inhibitory relaxation of the guanethidine-induced tone during this stimulation at 5 Hz was maintained throughout the period of stimulation. Increasing the frequency from 5 Hz up to 10, 20 or 40 Hz did not increase or decrease the inhibitory relaxation as shown in the upper records of Fig. 23. The results suggested that NANC inhibitory nerves had a considerable resource in their synthetic capability and were not as easily depleted as some other autonomic nerves, for example adrenergic nerves.

Nevertheless it was possible that some decrease in the NANC neurotransmitter release did take place, but the quantity of the neurotransmitter was still sufficient to induce and maintain complete relaxation of guanethidine-induced tone. If the tone was raised by the addition of agonist, it might then be possible to see the effect on the neurotransmitter release by adding L-arginine. Such an attempt is illustrated in the lower records of Figure 23. Noradrenaline 10-6M raised tone but still L-arginine when added subsequently was without effect on the inhibitory response to NANC nerve stimulation. That the nerves remained effective is shown by the rapid restoration of the original tone when field stimulation was stopped.

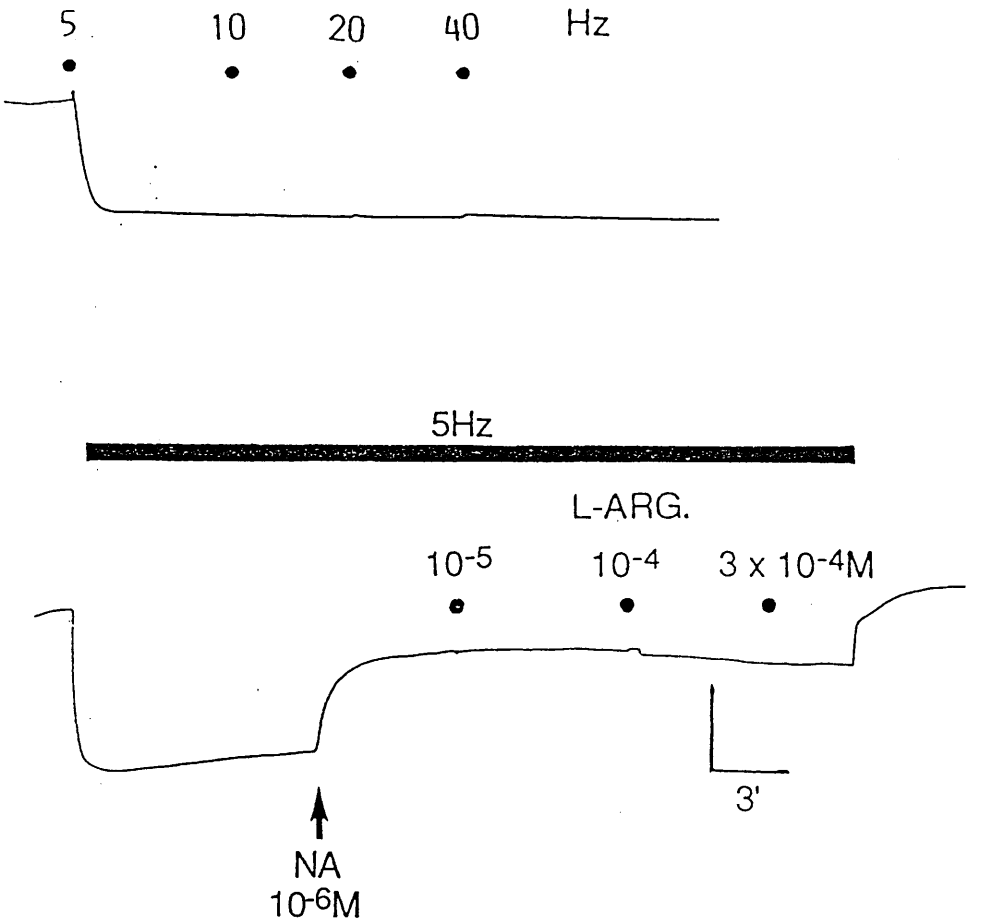


Fig. 23 The upper penal shows the response of a preparation of fresh BRP to continuous stimulation at 5 Hz. At the close circle the frequency was progressively raised to 10, 20 and 40 Hz. There was no evidence of ` escape ' of the inhibitory response. The lower records show the effect of continuous stimulation at 5 Hz during the period indicated by the bar. Noradrenaline (NA) 10^{-6}M. was added to raise tone. The subsequent addition of L-arginine 10^{-5}M. 10^{-4}M. and 3 x 10^{-4}M. did not increase the response. Tone was raised with guanethidine 10^{-5}M. added to the bath.

These experiments suggest the NANC nerves have a considerable safety margin for neurotransmitter release and that either arginine is not an essential precursor for the neurotransmitter or that it is not possible sufficiently to deplete it to cause a reduction in neurotransmitter release below that safety margin.

This property of the NANC inhibitory nerves, their ability to induce and maintain complete relaxation of the retractor penis muscles for long periods, might be very important physiologically for the reproductive function since relaxation of these muscles is necessary for the erection of penis.

THE EFFECT OF L-ARGININE ON THE NANC INHIBITORY RELAXATION OF THE COLD STORED BRP MUSCLE

Potentiation of the release of nitric oxide from endothelial cells can be shown only in cells cultured in the absence of arginine. An exhausted duplication of this experiment is not possible but it is possible to keep preparations alone and responding to nerve stimulation for several days by storing them at 4°C. At this temperature active uptake is abolished and it might be possible if the storage fluid was changed repeatedly to deplete the tissue of arginine. Such experiments were carried out with preparations of the BRP muscle. Tyrodes solution was used in these experiments to avoid the problem of calcium precipitation. BRP muscles were cut into ten preparations and each put into sterile Tyrodes solution in tissue cultured vessels and kept at 4°C in a refrigerator for periods of 1 to 10 days. Four BRP muscles were similarly treated so that each culture vessel contained four preparations each from a different animal. When each vessel was eventually used these gave a sufficient number of preparations for the four channel polygraph pen recorder. The solution in each vessel was changed daily for

fresh sterile Tyrodes to remove any L-arginine which had leaked from the preparations. The Tyrodes was sterilised by filtration through an 0.45 μm. filtration membrane. No antibiotics were applied because of the known effect of streptomycin on some nerve-muscle preparations. The NANC inhibitory responses of the preparations were studied at daily intervals from day 1 to day 10. Details of the methods and arrangements were in METHODS AND MATERIALS and Fig. 2. The tone of the preparations was raised by guanethidine (10^{-5}M). The parameters of the field stimulation were 20 pulses, supramaximal voltage and 0.5 milliseconds at 0.1, 0.2, 0.5, 1, 2, 5 and 10 Hz frequencies.

The cold stored BRP preparations were mounted as shown in Fig. 3 and allowed to equilibrate for 60 to 90 minutes before experiments were begun. The addition of exogenous L-arginine (3×10^{-5}M) had no effect on the NANC inhibitory relaxation at any time from day 1 to day 10. Further increase in the concentrations of exogenous L-arginine to 10^{-4}M and 3×10^{-4}M did not enhance the relaxation. None of these concentrations of L-arginine had any effect on the tone of the BRP preparations. The results are illustrated in Figure 24 to Figure 33. Occasionally in individual experiments some enhancement of the relaxation was observed (Fig. 34). However when all results were combined neither potentiation nor inhibition of the NANC inhibitory relaxation was visible nor was there any statistically significant effect though the dose-response curves of L-arginine group were often shifted to the left (Fig. 35 to Fig. 38).

Cold storage of preparations of the BRP muscle produced a steady decline in the response to NANC nerve stimulation and the differences between control and any time after day 4 was statistically significant (Fig. 39). The decay in the

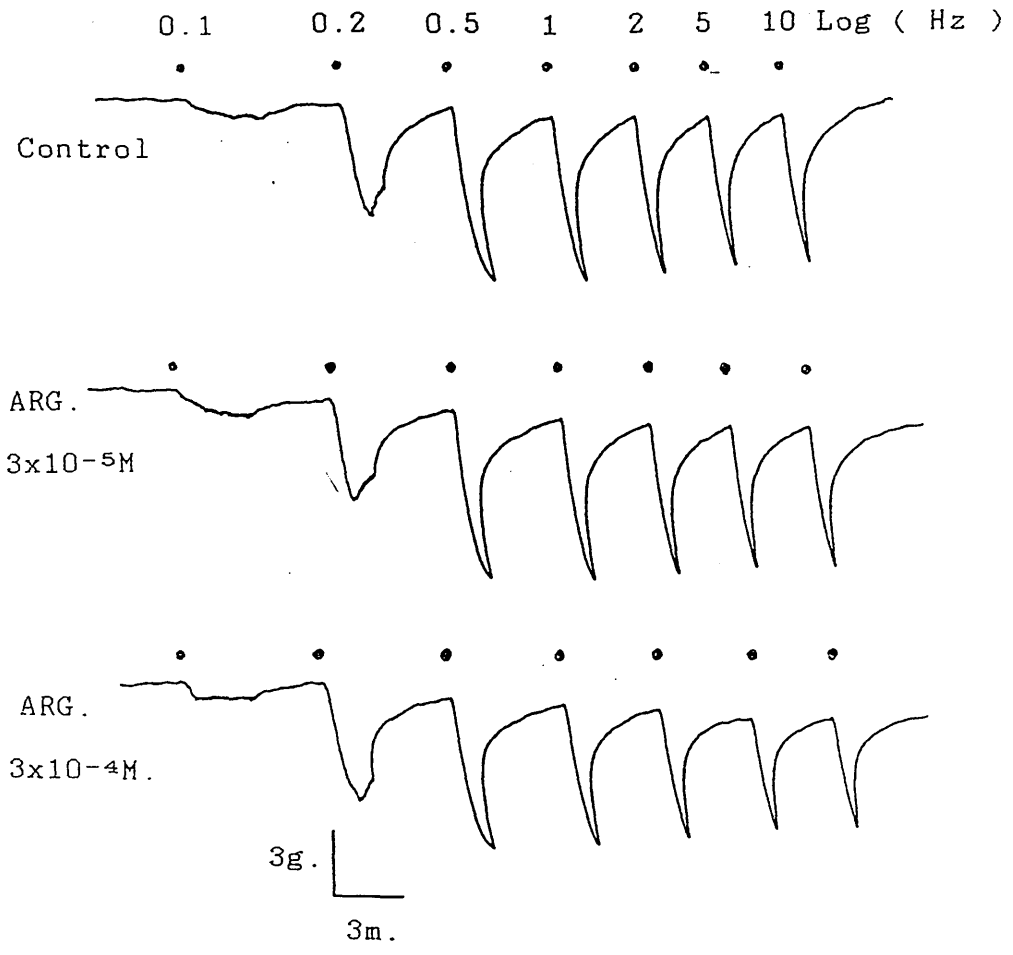


Fig. 24 The effect of L-arginine on the response of BRP stored for one days to field stimulation of its NANC nerves. The upper records are the control, the lower two records are from the same experiment but after the addition of L-arginine 3 x 10^{-5}M. and 3 x 10^{-4}M. respectively. The NANC nerves were stimulated with 20 pulses at supramaximal voltage at 0.5 ms. pulse duration. Neither concentration of L-arginine increases the response to field stimulation. Tone was raised by guanethidine 10^{-5}M. added to the bath.

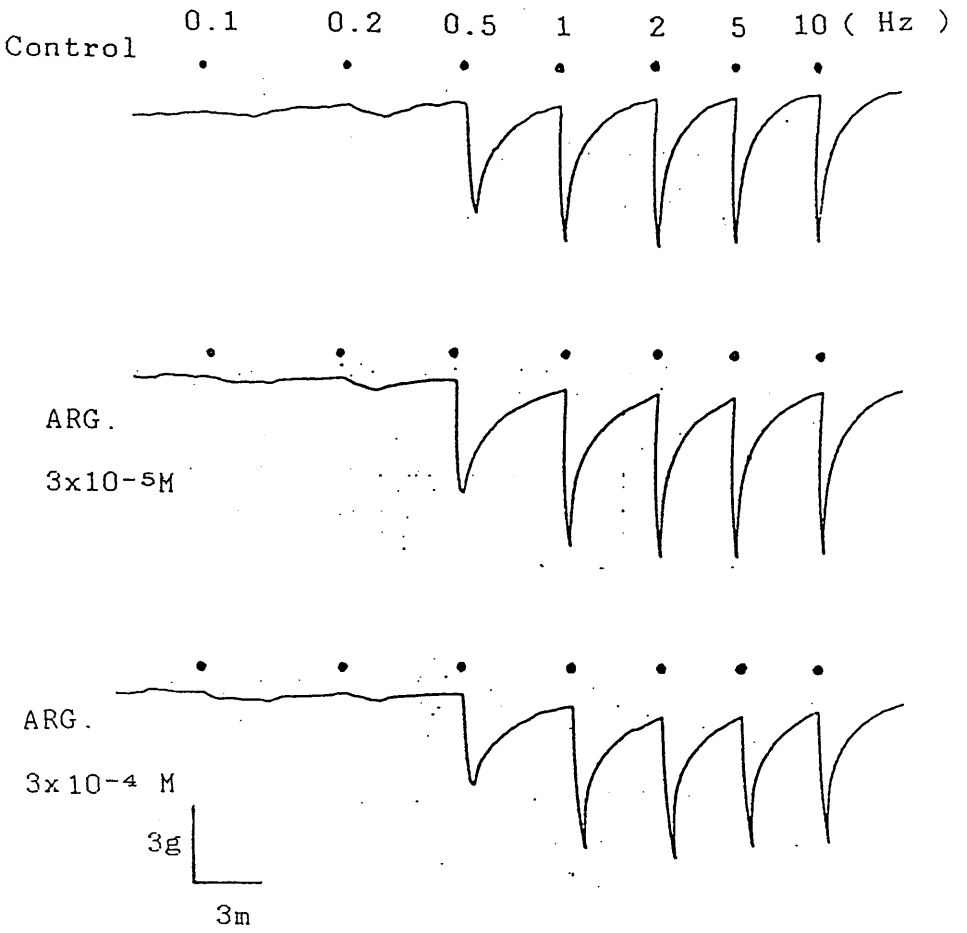


Fig. 25 The effect of L-arginine on the response of BRP stored for two days to field stimulation of its NANC nerves. The upper records are the control, the lower two records are from the same experiment but after the addition of L-arginine 3 x 10^{-5}M. and 3 x 10^{-4}M. respectively. The NANC nerves were stimulated with 20 pulses at supramaximal voltage at 0.5 ms. pulse duration. Neither concentration of L-arginine increases the response to field stimulation. Tone was raised by guanethidine 10^{-5}M. added to the bath.

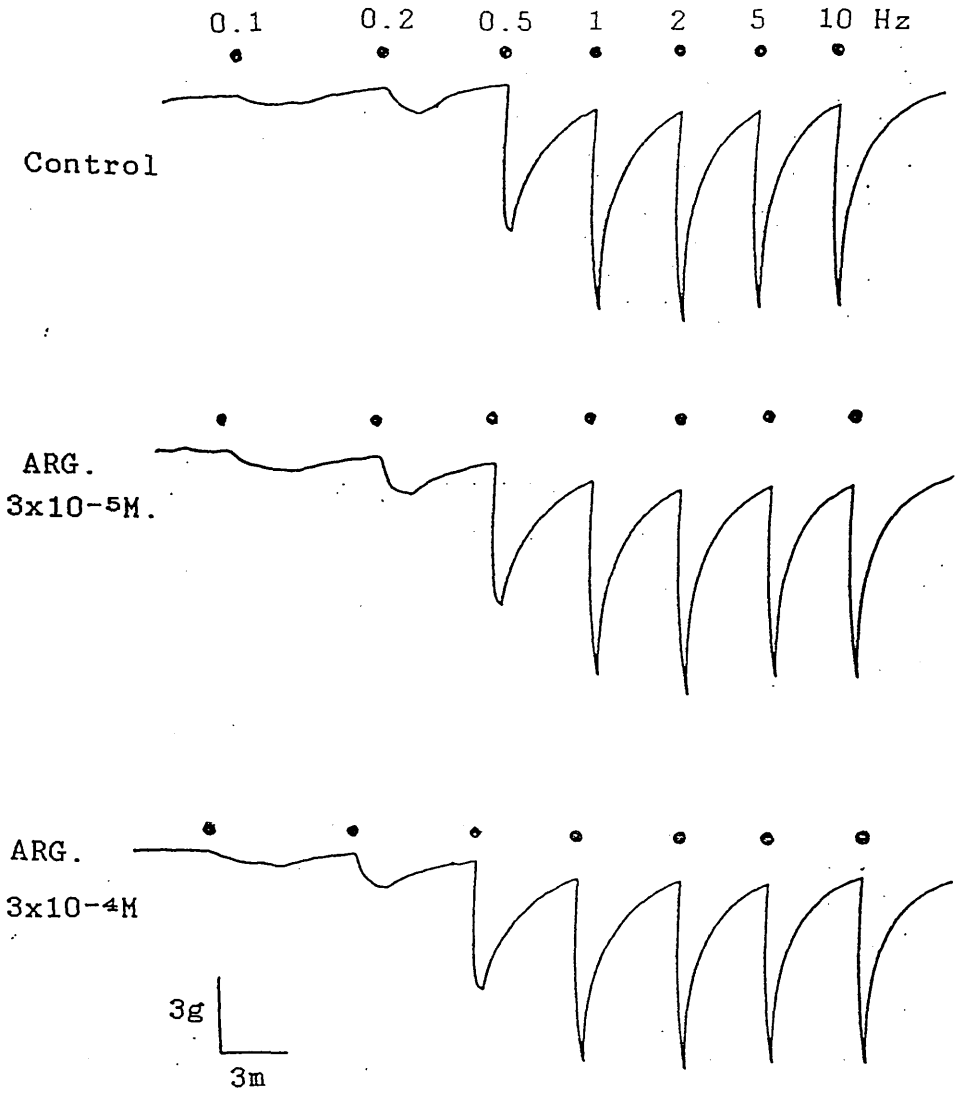


Fig. 26 The effect of L-arginine on the response of BRP stored for three days to field stimulation of its NANC nerves. The upper records are the control, the lower two records are from the same experiment but after the addition of L-arginine 3 x 10^{-5}M. and 3 x 10^{-4}M. respectively. The NANC nerves were stimulated with 20 pulses at supramaximal voltage at 0.5 ms. pulse duration. Neither concentration of L-arginine increases the response to field stimulation. Tone was raised by guanethidine 10^{-5}M. added to the bath.

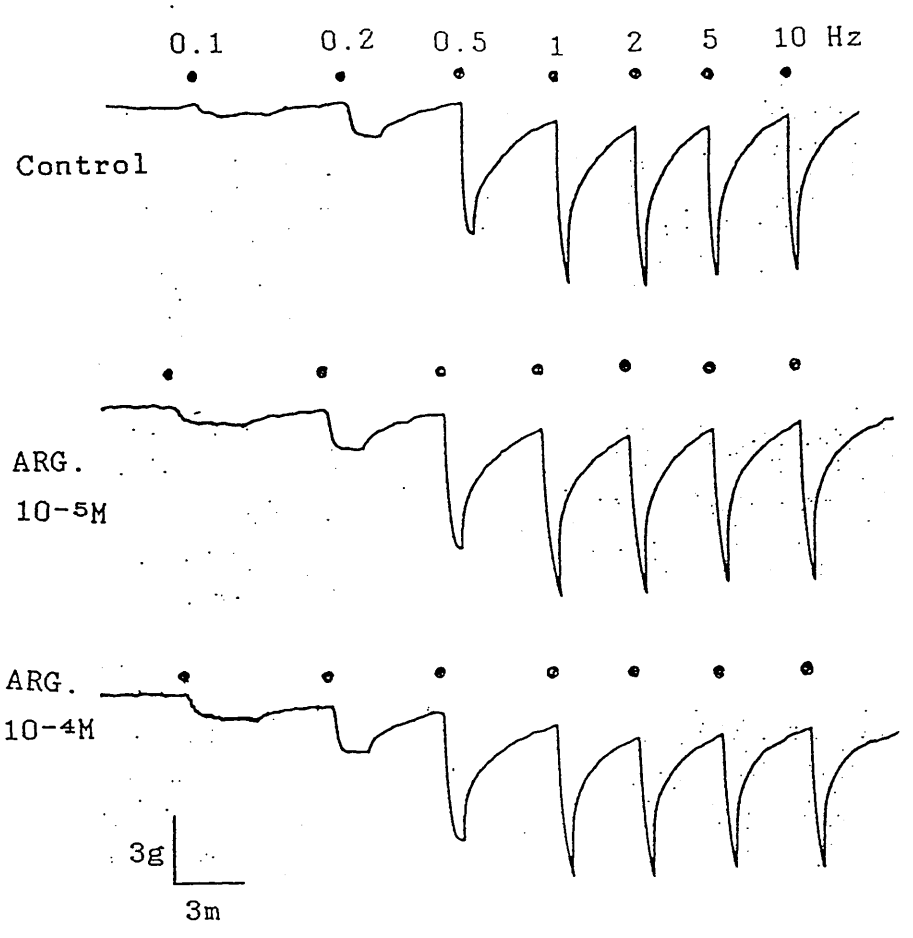


Fig. 27 The effect of L-arginine on the response of BRP stored for four days to field stimulation of its NANC nerves. The upper records are the control, the lower two records are from the same experiment but after the addition of L-arginine 10^{-5}M. and 10^{-4}M. respectively. The NANC nerves were stimulated with 20 pulses at supramaximal voltage at 0.5 ms. pulse duration. Neither concentration of L-arginine increases the response to field stimulation. Tone was raised by guanethidine 10^{-5}M. added to the bath.

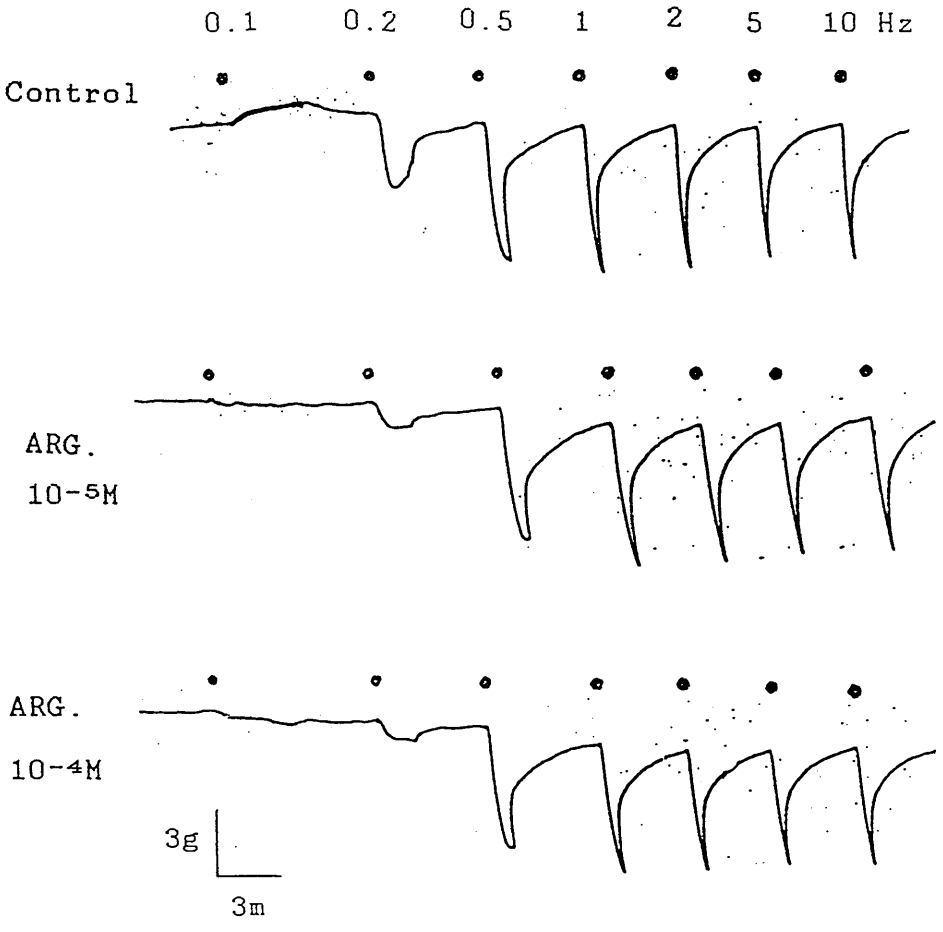


Fig. 28 The effect of L-arginine on the response of BRP stored for five days to field stimulation of its NANC nerves. The upper records are the control, the lower two records are from the same experiment but after the addition of L-arginine 10^{-5}M. and 10^{-4}M. respectively. The NANC nerves were stimulated with 20 pulses at supramaximal voltage at 0.5 ms. pulse duration. Neither concentration of L-arginine increases the response to field stimulation. Tone was raised by guanethidine 10^{-5}M. added to the bath.

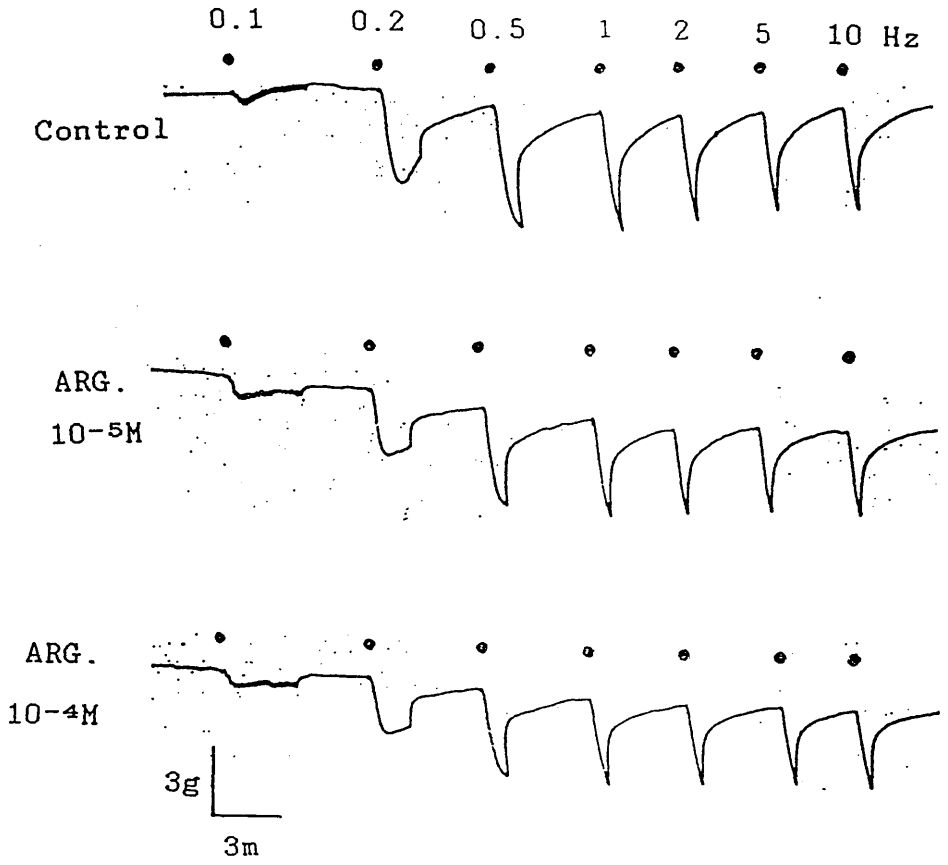


Fig. 29 The effect of L-arginine on the response of BRP stored for six days to field stimulation of its NANC nerves. The upper records are the control, the lower two records are from the same experiment but after the addition of L-arginine 10^{-5}M. and 10^{-4}M. respectively. The NANC nerves were stimulated with 20 pulses at supramaximal voltage at 0.5 ms. pulse duration. Neither concentration of L-arginine increases the response to field stimulation. Tone was raised by guanethidine 10^{-5}M. added to the bath.

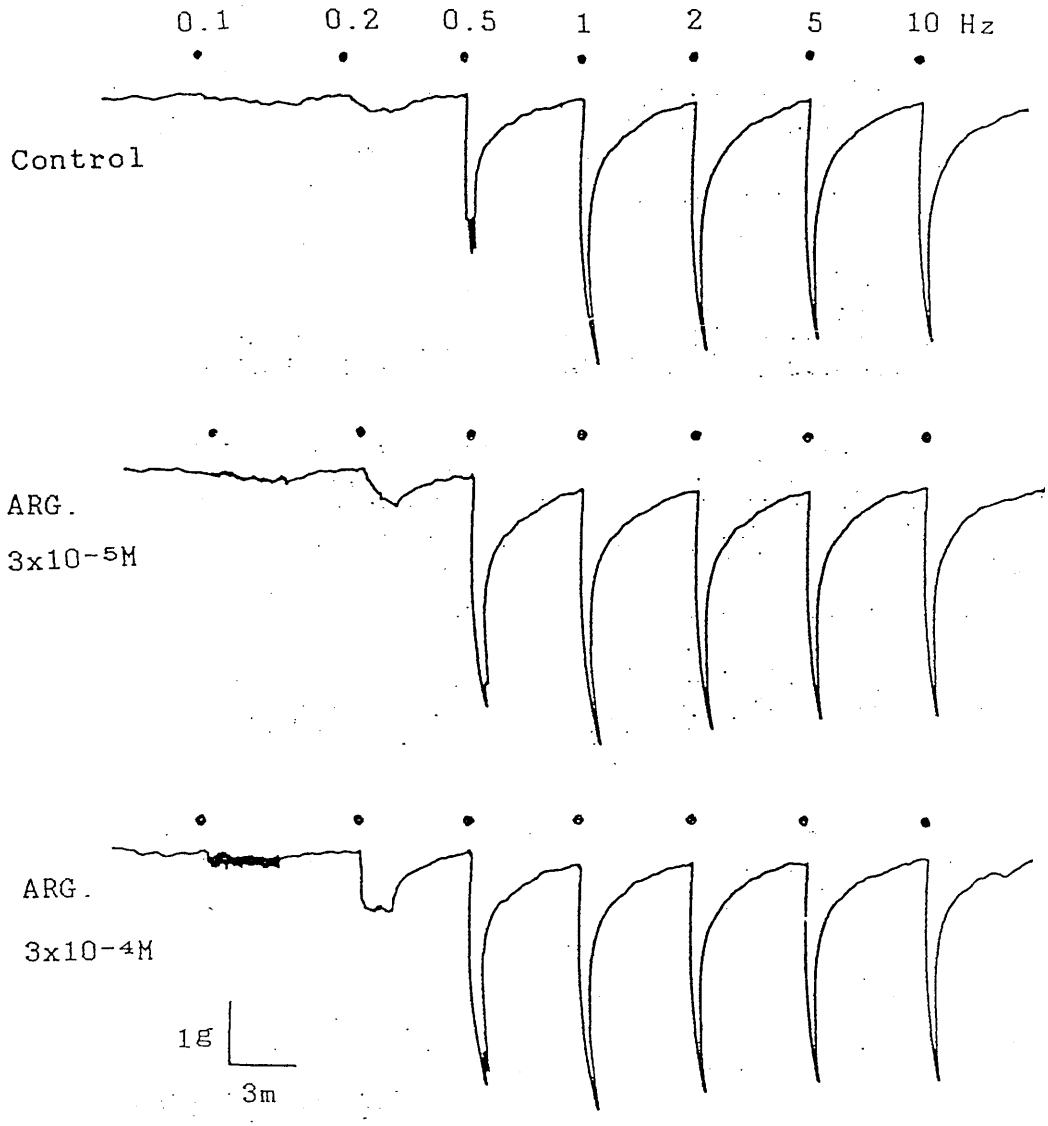


Fig. 31 The effect of L-arginine on the response of BRP stored for eight days to field stimulation of its NANC nerves. The upper records are the control, the lower two records are from the same experiment but after the addition of L-arginine 3 x 10^{-5}M. and 3 x 10^{-4}M. respectively. The NANC nerves were stimulated with 20 pulses at supramaximal voltage at 0.5 ms. pulse duration. Neither concentration of L-arginine increases the response to field stimulation. Tone was raised by guanethidi-ne 10^{-5}M. added to the bath.

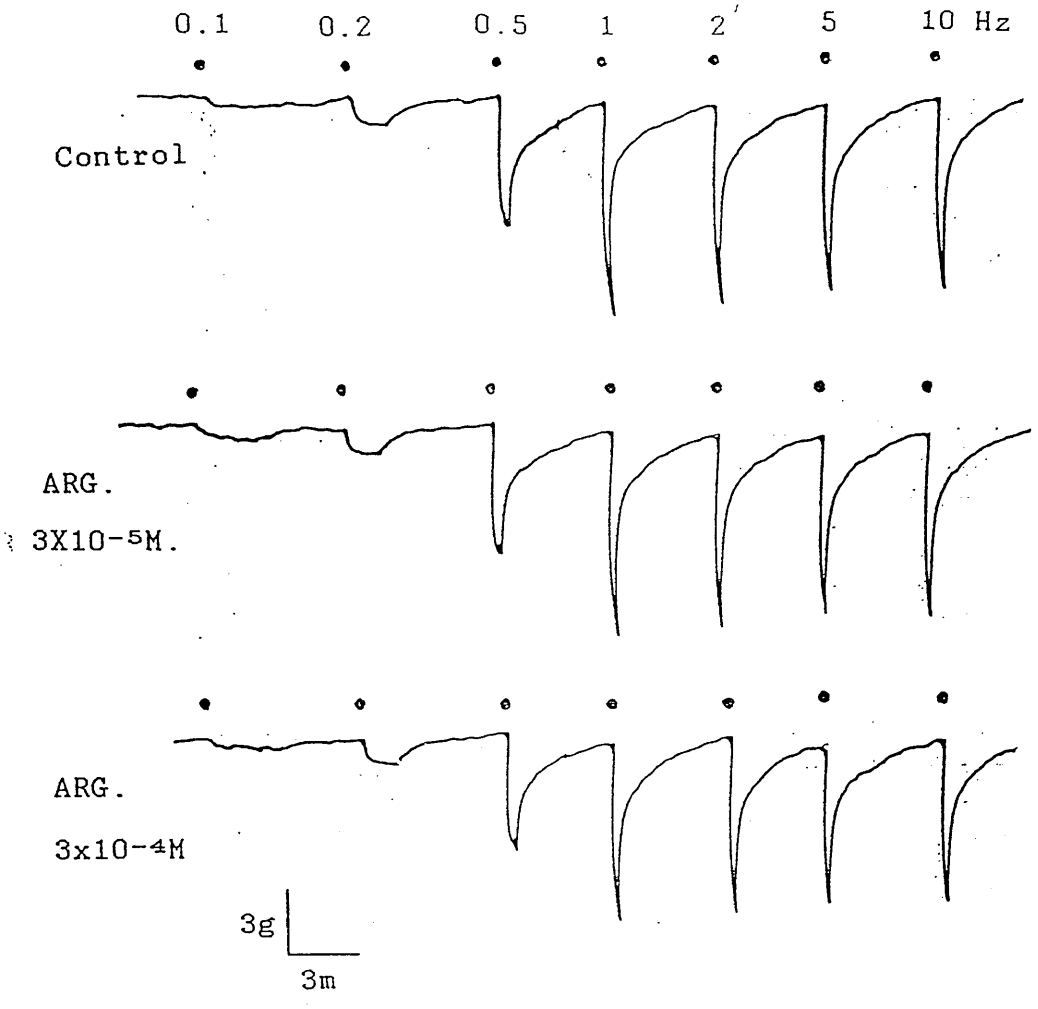


Fig. 32 The effect of L-arginine on the response of BRP stored for ninedays to field stimulation of its NANC nerves. The upper records are the control, the lower two records are from the same experiment but after the addition of L-arginine 3 x 10^{-5}M. and 3 x 10^{-4}M. respectively. The NANC nerves were stimulated with 20 pulses at supramaximal voltage at 0.5 ms. pulse duration. Neither concentration of L-arginine increases the response to field stimulation. Tone was raised by guanethidine 10^{-5}M. added to the bath.

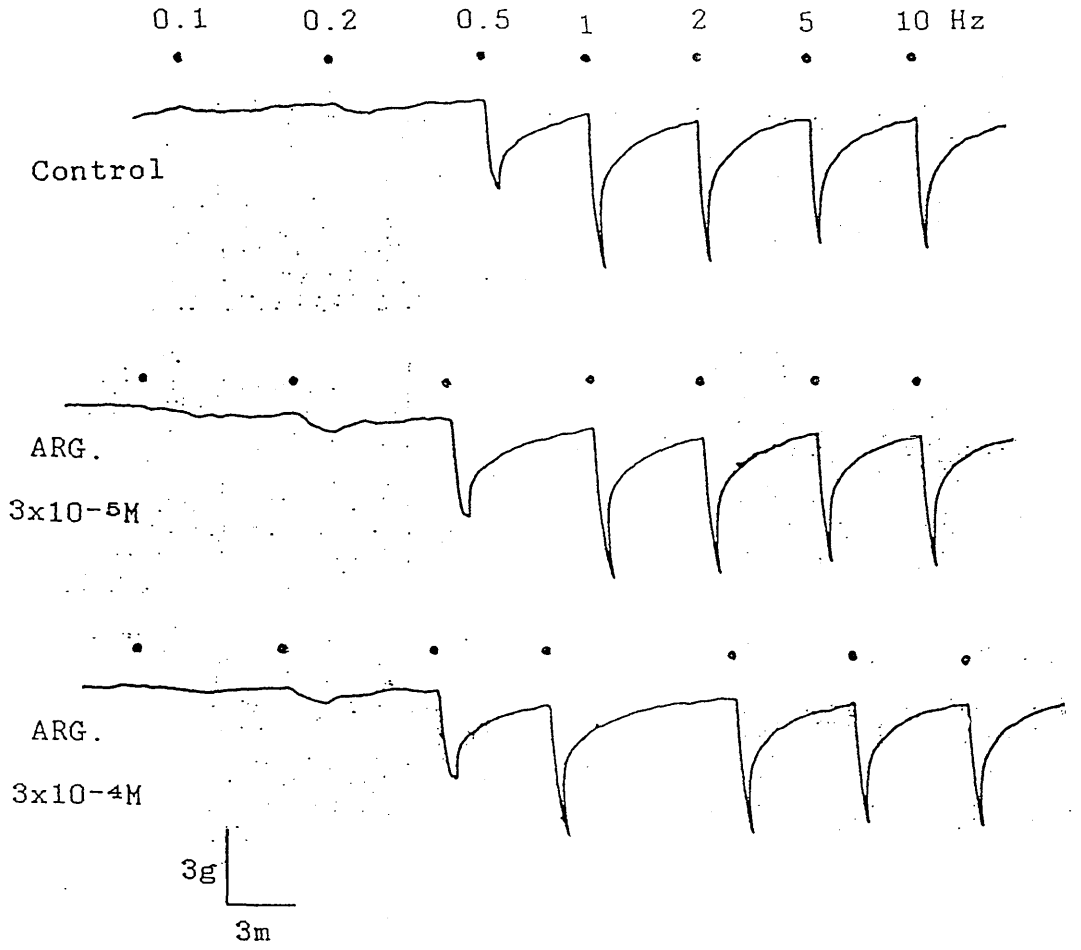


Fig. 33 The effect of L-arginine on the response of BRP stored for ten days to field stimulation of its NANC nerves. The upper records are the control, the lower two records are from the same experiment but after the addition of L-arginine 3 x 10^{-5}M. and 3 x 10^{-4}M. respectively. The NANC nerves were stimulated with 20 pulses at supramaximal voltage at 0.5 ms. pulse duration. Neither concentration of L-arginine increases the response to field stimulation. Tone was raised by guanethidine 10^{-5}M. added to the bath.

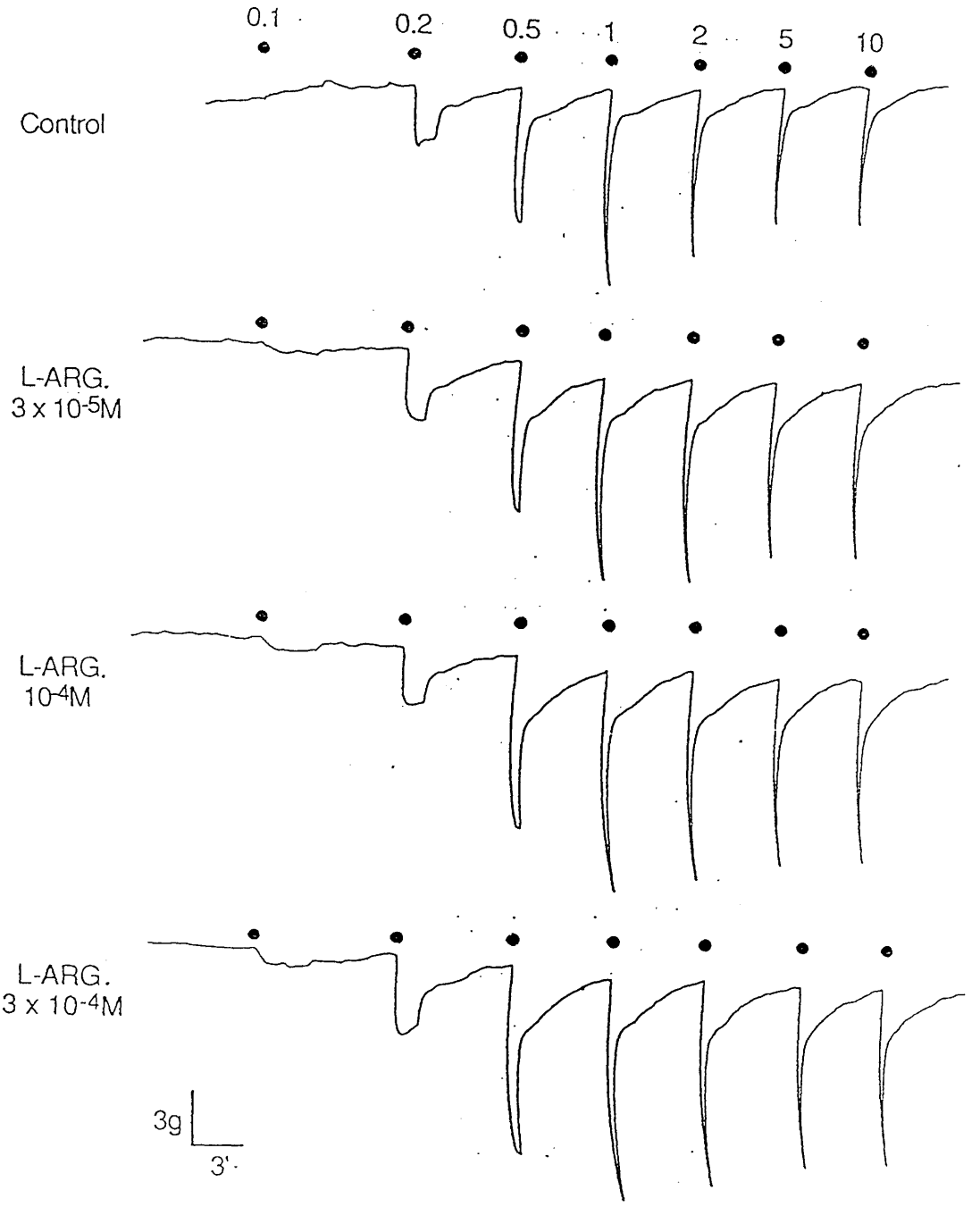


Fig. 34 The effect of L-arginine on the response of BRP stored for eight days to field stimulation of its NANC nerves. The upper records are the control, the lower three records are from the same experiment but after the addition of L-arginine 3 x 10^{-5}M. 10^{-4}M. and 3 x 10^{-4}M. respectively. The NANC nerves were stimulated with 20 pulses at supramaximal voltage at 0.5 ms. pulse duration. Either concentration of L-arginine increases the response to field stimulation. Tone was raised by guanethidine 10^{-5}M. added to the bath.

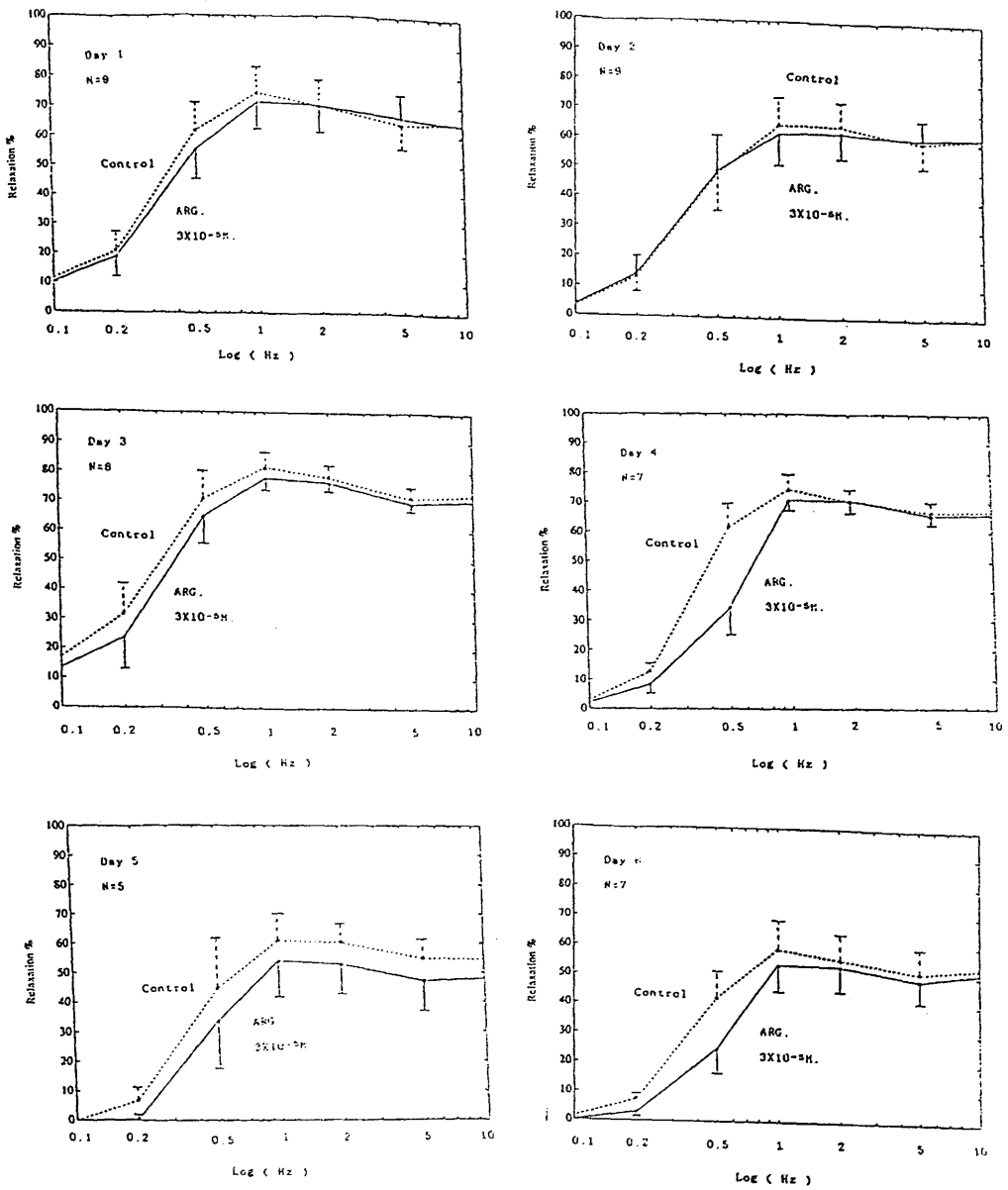


Fig. 35 Shows the combined results of all experiments on the effect of L-arginine 3 x 10^{-5}M. on the response of the BRP to NANC nerve stimulation. All of these preparations had been cold stored for one day up to six days shown above. The preparations were stimulated by field stimulation using 20 pulses at supramaximal voltages of 0.5 ms. duration and frequencies shown above. Tone was raised with guanethidine 10^{-5}M. added to the bath. L-arginine has no effect on the response to field stimulation. All probability values are more than 0.05.

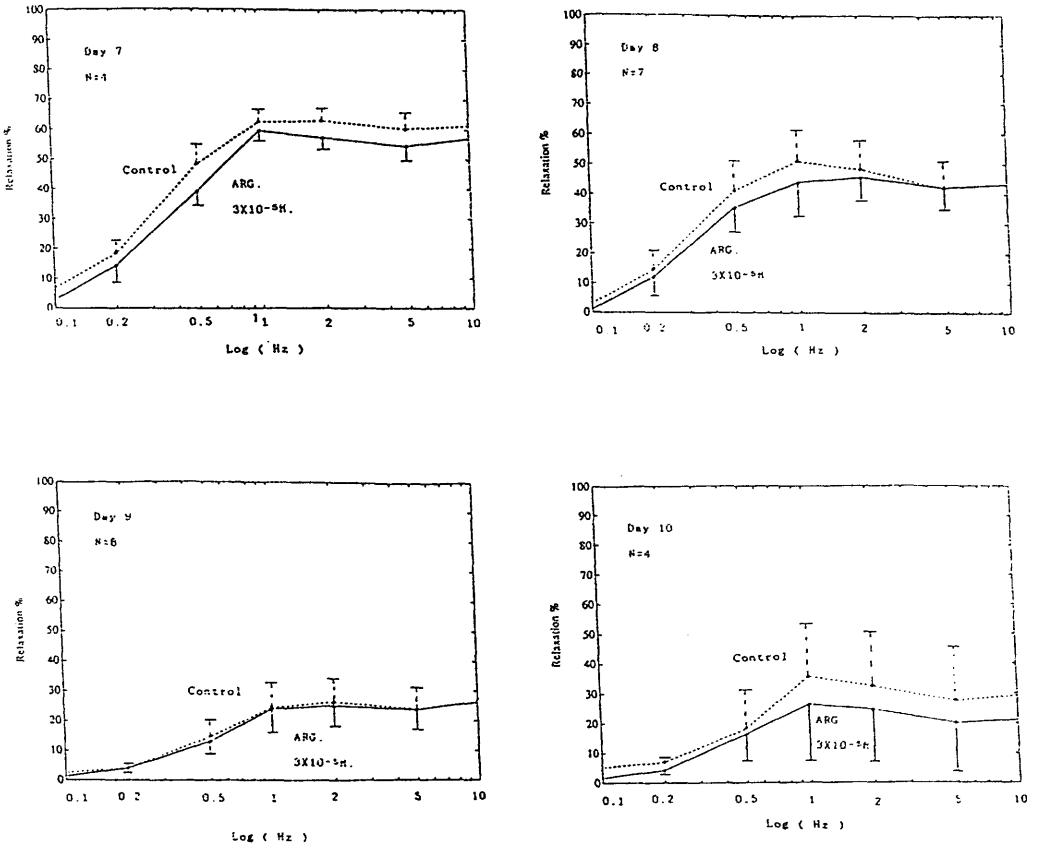
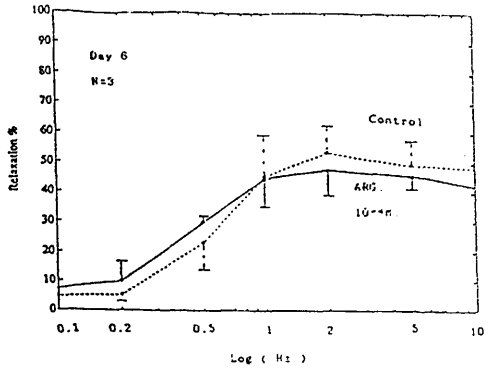
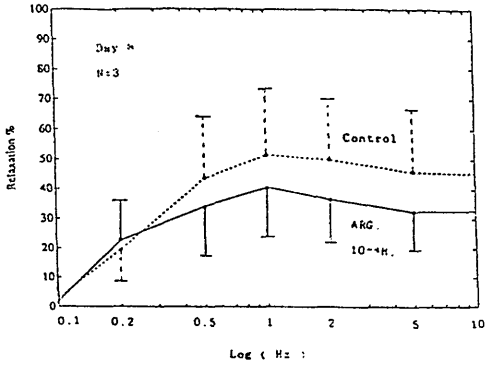


Fig. 36 Shows the combined results of all experiments on the effect of L-arginine 3 x 10^{-5}M. on the response of the BRP to NANC nerve stimulation. All of these preparations had been cold stored for seven days up to ten days shown above. The preparations were stimulated by field stimulation using 20 pulses at supramaximal voltages of 0.5 ms. duration and frequencies shown above. Tone was raised with guanethidine 10^{-5}M. added to the bath. L-arginine has no effect on the response to field stimulation. All probability values are more than 0.05.





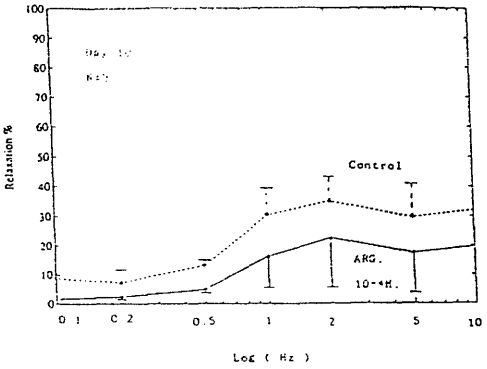
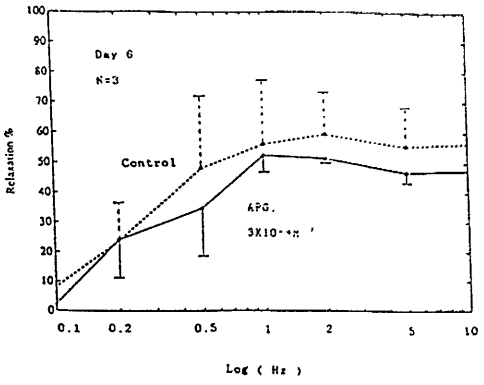
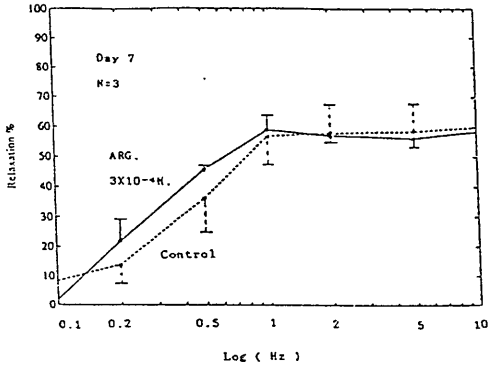


Fig. 37 Shows the combined results of the experiments on the effect of L-arginine 10^{-4}M. on the response of the BRP to NANC nerve stimulation. All of these preparations had been cold stored for 6, 8 and 10 days respectively shown above. The preparations were stimulated by field stimulation using 20 pulses at supramaximal voltages of 0.5 ms. duration and frequencies shown above. Tone was raised with guanethidine 10^{-5}M. added to the bath. L-arginine has no effect on the response to field stimulation. All probability values are more than 0.05.





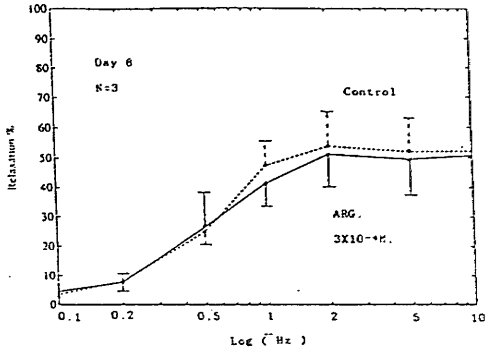


Fig. 38 Shows the combined results of all experiments on the effect of L-arginine 3 x 10^{-4}M. on the response of the BRP to NANC nerve stimulation. All of these preparations had been cold stored for 6, 7 and 8 days respectively shown above. The preparations were stimulated by field stimulation using 20 pulses at supramaximal voltages of 0.5 ms. duration and frequencies shown above. Tone was raised with guanethidine 10^{-5}M. added to the bath. L-arginine has no effect on the response to field stimulation. All probability values are more than 0.05.

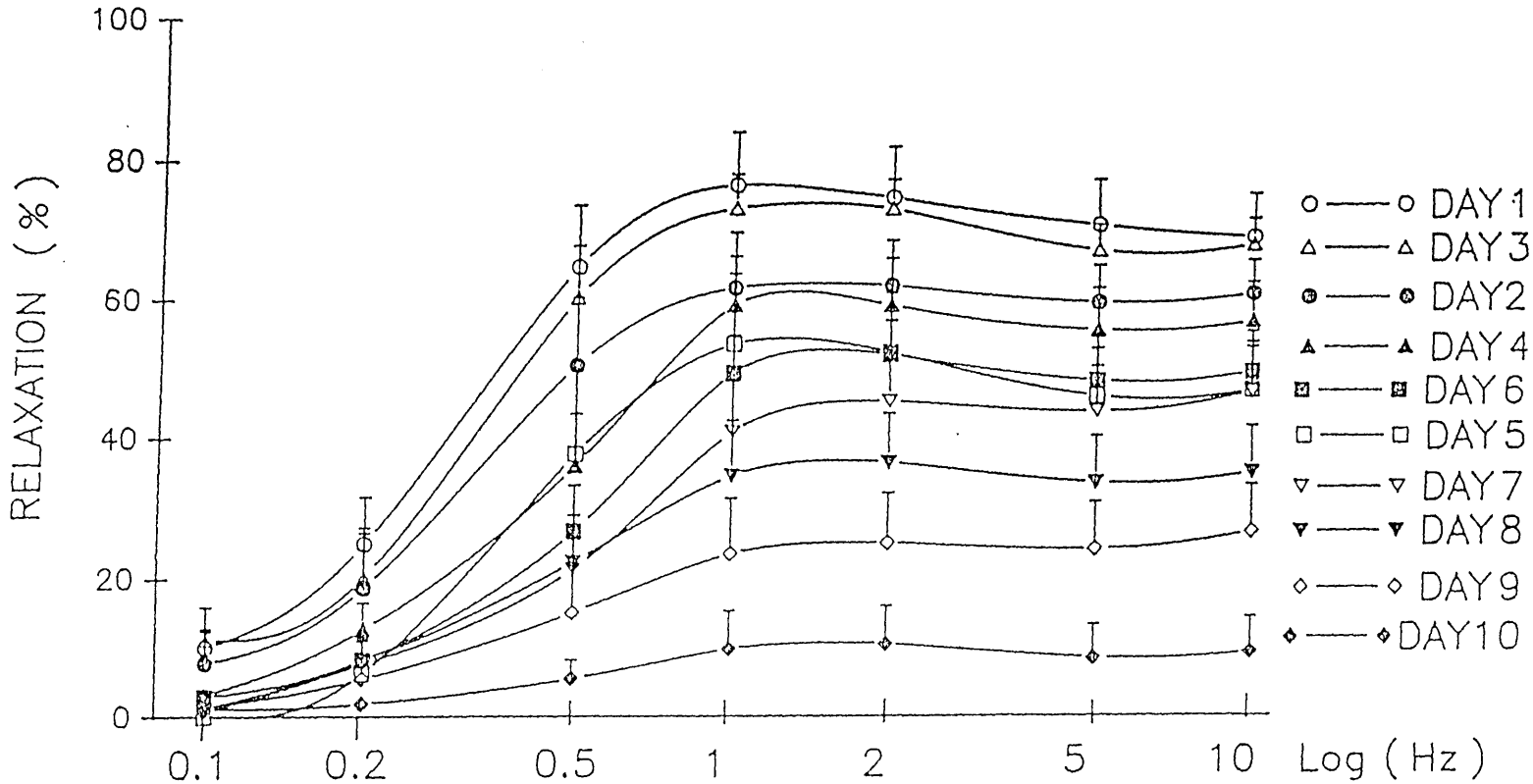


Fig. 39 Frequency-response curves of the NANC inhibitory relaxation to BRP nerve stimulation by supramaximal voltage and 0.5 ms. duration at frequencies shown above for 20 seconds from day 1 up to day 10. The probability value is significant difference ($P < 0.05$) between the curve on day 1 and that on any day after day 4. The numbers of observation are 12 in each curve.

response is unlikely to be the result of a diminished sensitivity of the smooth muscle cells since there was no corresponding decline in sensitivity to sodium nitrite (Fig. 40). If the decline was due to reduced NANC neurotransmitter release and this in turn due to a deficiency of precursor L-arginine then the later days of cold storage with the greatest decline in response should be the most suitable for testing the effect of exogenous L-arginine. An exception to this is day 10 where half the tissues tested no longer responded to field stimulation (Table 1),

Table 1. The Effect of Cold Storage on the Proportion of Preparations Responding to Field Stimulation

Day of cold storage	1	2	3	4	5	6	7	8	9	10
Preparations tested	12	12	12	12	12	12	12	12	12	12
Preparations responding	12	12	12	12	12	12	12	12	12	6

presumably because of anatomical disintegration of the NANC terminal nerve network. The effect of exogenous L-arginine 3×10^{-5}M was tested on a group of number for each day of cold storage over the ten day period (Fig. 35 and Fig. 36). In addition the higher concentration of 10^{-4}M was tested on a group of number for days 6, 8 and 10 (Fig. 37) and a still higher concentration of 3×10^{-4}M L-arginine on days 6, 7 and 8 (Fig. 38). None of these studies showed any significant effect of L-arginine on the NANC response.

The steady decline in the mean response to NANC nerve stimulation with increasing time of cold storage followed by a sudden failure of half the preparations to respond between the ninth day and the tenth day of storage prompted on

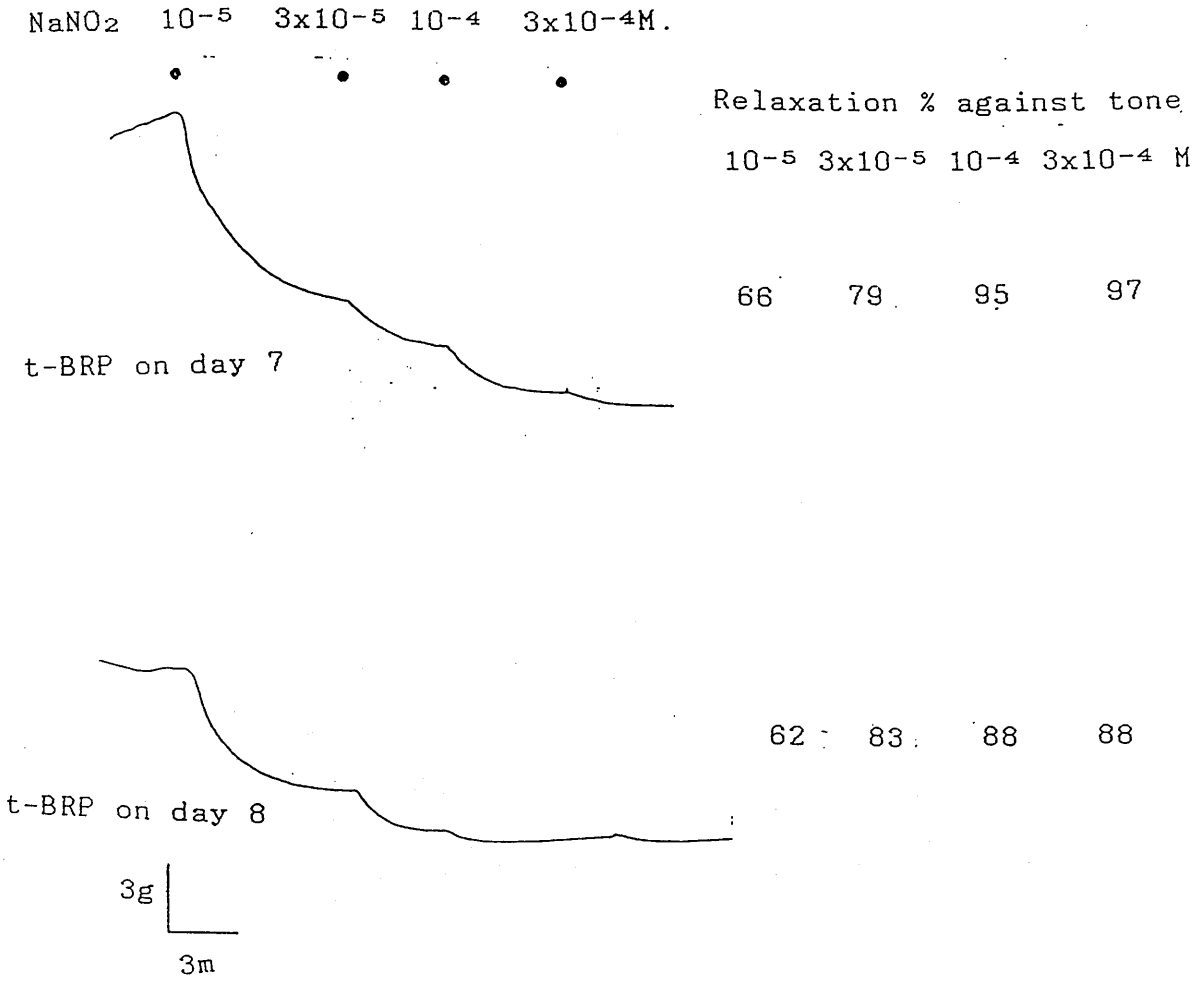


Fig. 40 Shows the good responsibility of the cold stored BRP muscles to sodium nitrite on day 7 and day 8 in a bath as shown in Fig. 4.

investigation into the effect of cold storage on the motor adrenergic response and on the ability of guanethidine to induce an indirect sympathomimetic rise in tone. In the some ten day experiments the maximum motor response to field stimulation at 10 Hz was tested followed by the addition of guanethidine to block these nerves and at the same time to raise tone by an indirect sympathomimetic action. The results are shown in Figure 41 and are similar to those for NANC nerve stimulation. For the first three day there was relatedly little change in response to adrenergic nerve stimulation, there was then a decline on day 4 and day 5 to a new level which was maintained with little further change up to day 9 and the response to motor nerve stimulation on day 10 disappeared completely in all preparations. The rise in tone caused by guanethidine 10^{-5}M was greater than the maximum response to adrenergic motor nerve stimulation averaging 15.6 +/- 1.5 g. compared with 4.6 +/- 1.1 g. for nerve stimulation in fresh tissues. Nevertheless there was a similar pattern of change in the response to guanethidine as was seen with motor nerve stimulation. Little change over the first three day, a decline between day 4 and day 5 to a level which was fairly well maintained until day 9 followed by a substantial decline between the ninth day and the tenth day. Unlike nerve stimulation some response to guanethidine was however still obtained on the tenth day. These results are consistent with the previous suggestion for NANC nerves that after the first three or four days there is a decline in NANC neurotransmitter output and quite suddenly between the ninth day and the tenth day there is anatomical disintegration of the nerve network. The continuing ability of guanethidine to produce a response on the tenth day suggests some stores of noradrenaline still exist in nerves in the tissue. The inability of nerve stimulation at this time to produce a response suggests the nerve action potential can no longer propagate into these store, a situation consistent with breaks appearing in the physical connection of the terminal nerve network.

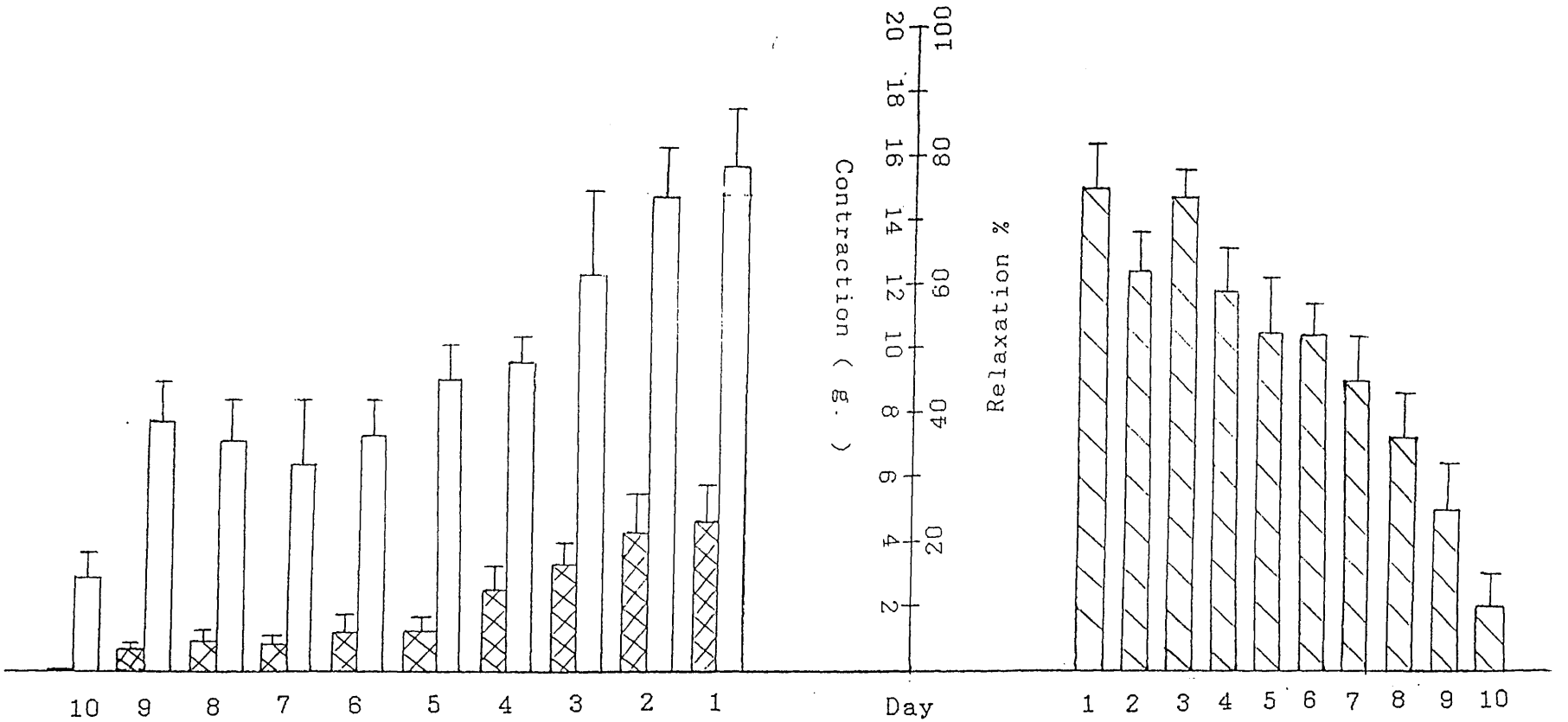


Fig. 41 The responses of cold stored BRP muscle fromday 1 to day 10. Tone (g.) of the BRP preparations induced by guanethidine 10^{-5}M. Contraction (g.) of the BRP preparations to field stimulation of supramaximal voltage and 0.5 ms. pulse duration for 10 seconds at 10 Hz. NANC inhibitory relaxation (%) of the BRP preparations induced by field stimulation of supramaximal voltage and 0.5 ms. pulse duration for 10 seconds at 2 Hz.

THE EFFECT OF L-NMMA AND D-NMMA ON THE NANC INHIBITORY RELAXATION OF THE BRP MUSCLE AND THE RAT ANOCOCCYGEUS MUSCLE

Endothelium-derived relaxing factor (EDRF) is almost certainly nitric oxide (Palmer et al. 1987) and L-arginine appears to be its physiological precursor (Schmidt et al. 1988a; Schmidt et al. 1988b; Palmer et al. 1988a; Palmer et al. 1988b). Furthermore the substrate specificity for nitric oxide synthesis appears to be restricted to L-arginine (Palmer et al. 1987; Palmer et al. 1988a; Schmidt et al. 1988a). Nitric oxide is synthesized by L-arginine-utilizing enzymes from the terminal guanidino nitrogen atom(s) of L-arginine (Palmer et al. 1988a; Palmer et al. 1988b; Schmidt et al. 1988b). L-monomethyl-N-arginine (L-NMMA), a competitive inhibitor of L-arginine-utilizing enzymes, but not D-monomethyl-N-arginine (D-NMMA), inhibited both the generation of nitric oxide by endothelial cells in culture and the endothelium-dependent relaxation of rabbit aortic rings. These effects were dose-dependently competitively reversed by L-arginine. The inhibitory effect of L-NMMA (0.3 to 10.0 μM.), but not D-NMMA (10.0 to 100.0 μM.), on nitric oxide release from the cultured endothelial cells was concentration-dependent manner (IC_{50} 3.0 +/- 0.7 μM.) and was reversed by L-arginine (30 μM.). Also the endothelium-dependent relaxation of rabbit aortic rings was inhibited by L-NMMA (3 to 300 μM.) again in a concentration-dependent manner with 50 % inhibition by L-NMMA 30 μM. and 64 % by L-NMMA 300 μM. of the relaxation induced by 1 μM. acetylcholine (Palmer et al. 1988b). These results suggested that inhibition by L-NMMA or its reversal by L-arginine on nitric oxide release from cultured endothelial cells or endothelium-derived relaxation in rabbit aortic rings did not need high concentrations of L-NMMA or L-arginine respectively.

The NANC inhibitory neurotransmitter in the BRP muscle and the rat anococcygeus muscle, and the inhibitory factor extracted from the BRP muscle have many properties in common with EDRF and nitric oxide, for example, they induced inhibitory relaxation in many smooth muscles by stimulation of guanylate cyclase and increase of cGMP content on these tissues, and their effects could be abolished by haemoglobin (Gillespie et al. 1981; Bowman et al. 1982; Bowman & Drummond 1984; Griffith et al. 1984). If the NANC inhibitory neurotransmitter in these tissues is nitric oxide and its precursor is L-arginine, L-NMMA but not D-NMMA should inhibit the NANC inhibitory relaxation and L-arginine should reverse this effect in both the BRP muscle and the rat anococcygeus muscle.

The experiments reported here were intended to investigate the effect of L-NMMA and D-NMMA in the BRP muscle and the rat anococcygeus muscle and particularly to see whether L-NMMA specifically blocked the NANC inhibitory relaxation and if so, could L-arginine reverse this effect. A positive finding would suggest L-arginine as a physiological precursor of the NANC inhibitory neurotransmitter and reinforce the possibility of nitric oxide as the NANC inhibitory neurotransmitter.

THE EFFECT OF L-NMMA AND D-NMMA ON THE BRP MUSCLE

Fresh BRP muscle preparations, 10 to 20 mm. in length and 1.5 to 2 mm. in width, were mounted as Fig. 3. The investigation was to study the effect of L-NMMA on the tone of the BRP muscle induced by guanethidine (10^{-5}M) and on the NANC inhibitory relaxation induced by field stimulation (supramaximal voltage, 0.5 milliseconds' pulse duration at 0.1, 0.2, 0.5, 1, 2, 5 and 10 Hz

frequencies for 20 pulses). In each experiment control NANC inhibitory frequency-response curves were constructed before treatment with L-NMMA and repeated after 10 minutes' exposure and in the continuing presence of L-NMMA. Concentrations from 3×10^{-5}M up to 10^{-3}M L-NMMA were studied.

At the final concentrations of 3×10^{-5}M, 10^{-4}M and 3×10^{-4}M, L-NMMA was without any significant effect on the tone induced by guanethidine or the NANC inhibitory relaxation induced by field stimulation. The results for a single experiment are illustrated in Fig. 42. When all the results with these three concentrations of L-NMMA were combined, there was no significant difference between the control and the drug-treated frequency-response curves as shown in the left of the Fig. 43. Since these concentrations of L-NMMA were greater than those used to inhibited nitric oxide production from cultured endothelial cells, these results were interpreted as negative and it was assumed L-NMMA had no inhibitory effect on NANC inhibitory relaxation in the BRP muscle. After the experiments with the rat anococcygeus muscle in which it was quite clear that L-NMMA did inhibit NANC inhibitory relaxation, the BRP was re-examined with the higher concentration of 10^{-3}M. L-NMMA. The results from a single illustration are shown in Fig. 44 and for all experiments shown in the right graph of the Fig. 43. The higher dose of L-NMMA had two effects, first it caused a further rise in tone and secondly it reduced the response to NANC nerve stimulation. Both effects are visible in the experiment illustrated in Fig. 44. The increase in tone arranged 24.5 % of that originally induced by guanethidine and the inhibition of the NANC response, which was maximal at 16.7 +/- 4.6 % to 5.6 +/- 2.1 % reduction at 0.2 Hz. Both effects of L-NMMA were partially reversed by 10^{-3}M. L-arginine and the subsequent magnitude of the response at 0.2 Hz, 12.1 +/- 3.3 % was not significantly difference from the control. These results show

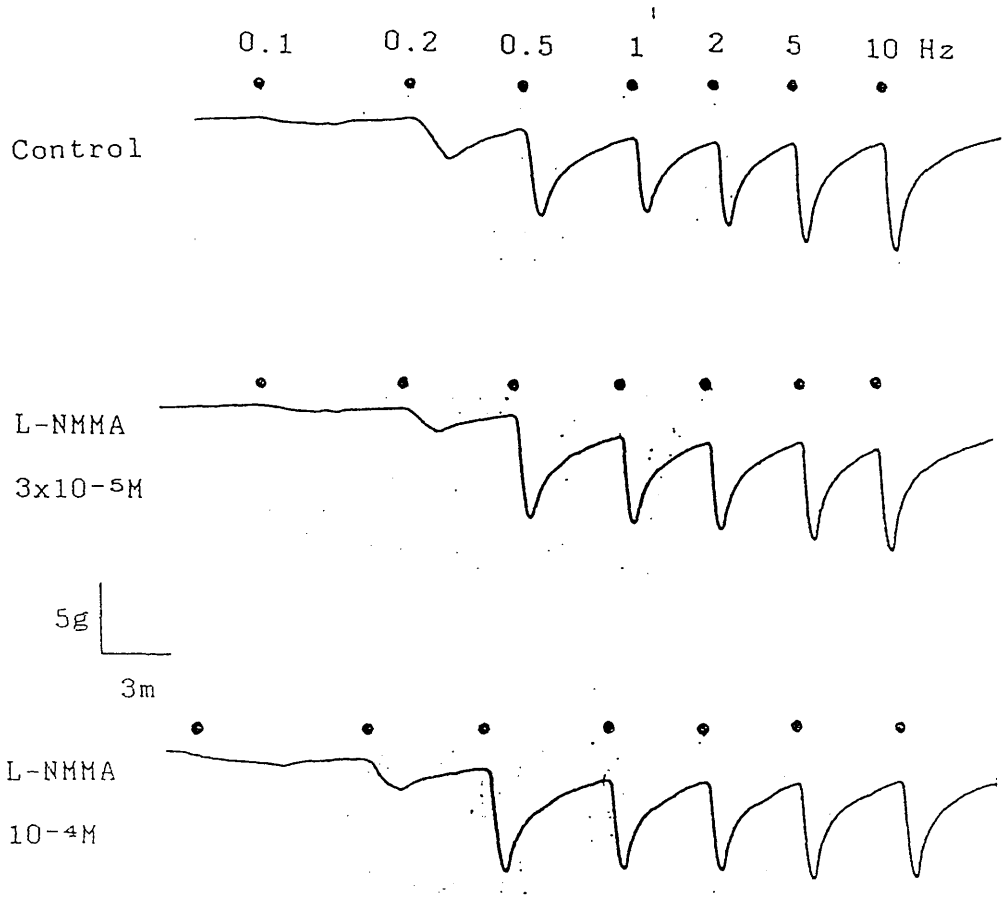


Fig. 42 Shows at concentrations of 3 x 10^{-5}M. and 10^{-4}M. L-monomethyl-N-arginine (L-NMMA) had no effect on inhibitory relaxation of the BRP muscle to NANC nerve stimulation by supramaximal voltage and 0.5 ms. pulse duration at frequencies shown above each response for 20 seconds.

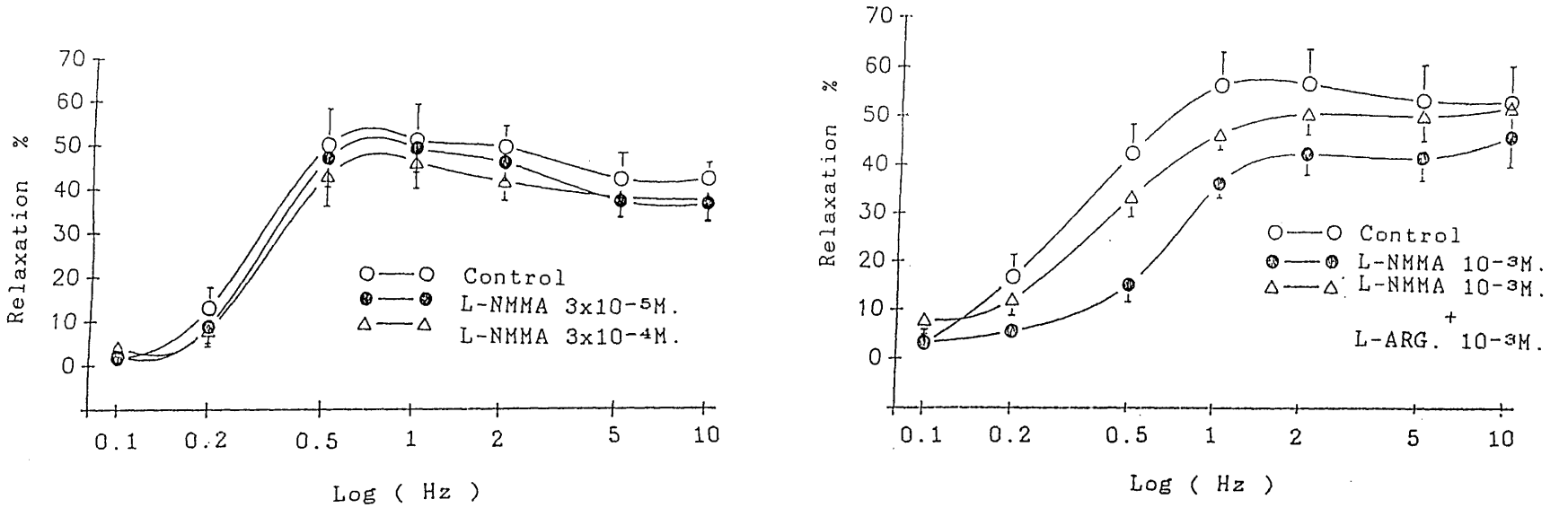


Fig. 43 Shows L-monomethyl-N-arginine (L-NMMA) had no effect on inhibitory relaxation of the BRP muscles to NANC nerve stimulation at concentrations from 3 x 10^{-5}M. up to 3 x 10^{-4}M. as in the left ($P > 0.05$; N=6). But increasing concentration to 10^{-3}M. L-NMMA did inhibit the relaxation ($P < 0.05$; N=6), an effect partially reserved by 10^{-3}M. L-arginine (L-ARG.) as in the right graph above. Field stimulation was by 0.5 ms. pulse duration and supramaximal voltage for 20 pulses at frequencies shown above.

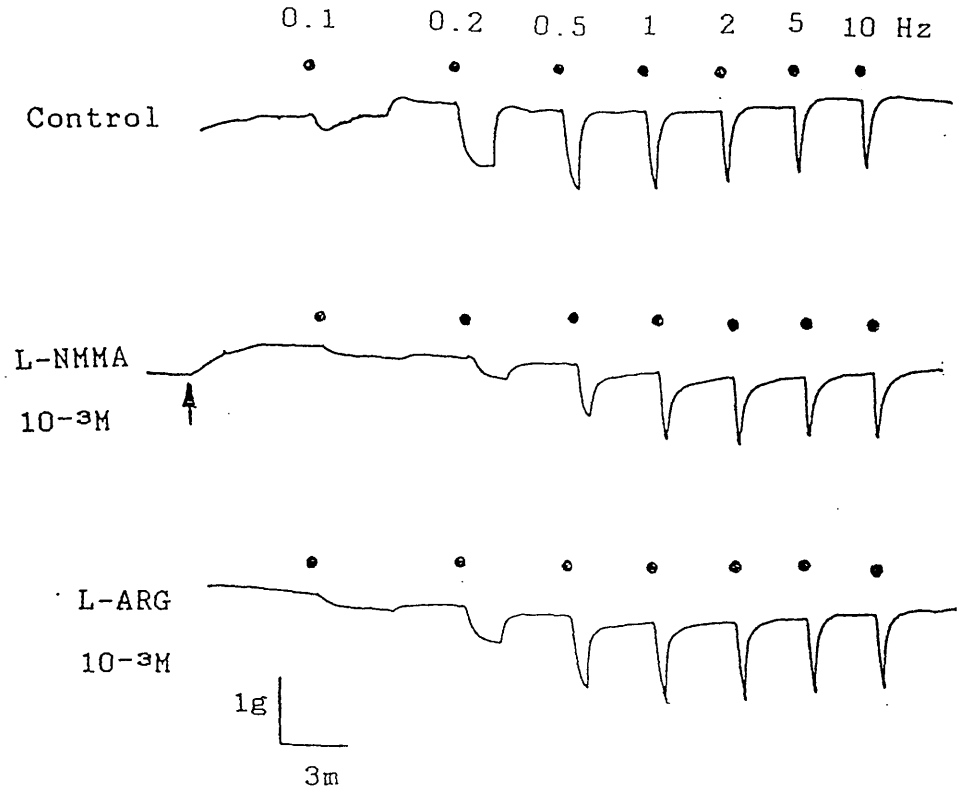


Fig. 44 The effects of a high concentration of 10^{-3}M. L-monomethyl-N-arginine (L-NMMA) on the tone of and the NANC inhibitory relaxation of the BRP muscle induced by field stimulation (supramaximal voltage and 0.5 ms. pulse duration for 20 pulses at frequencies shown above each response). L-NMMA produces a small inhibitory effect on NANC nerve response specially at lower frequencies of 0.2 and 0.5 Hz. L-NMMA 10^{-3}M. causes a partial reversal of this inhibition.

that while L-NMMA is not as effective in inhibiting NANC inhibitory relaxation in the BRP muscle as it is in the rat anococcygeus muscle (compare Fig. 42, Fig. 43 and Fig. 44 with Fig. 45 and Fig. 46), it can at higher concentrations cause both the rise in tone and inhibiting the NANC response especially at low frequencies.

THE EFFECT OF L-NMMA ON THE NANC INHIBITORY RELAXATION OF THE RAT ANOCOCCYGEUS MUSCLE

The rat anococcygeus muscle was first discovered and introduced as a very good example of an NANC innervated muscle preparation by Gillespie (1972) and has many properties on the NANC inhibitory relaxation in common with the BRP muscle (Gillespie 1972; Gillespie 1980; Gillespie & Martin 1980; Bowman & Gillespie 1982). The retractor penis muscle is a continuation of the anococcygeus muscle (Gillespie & Martin 1980; Gillespie 1987). It would be expected therefore that the innervation and neurotransmitter in the both muscles would be the same. To check this the effect of L-arginine and L-NMMA on NANC inhibitory neurotransmission in the rat anococcygeus was examined.

Fresh rat anococcygeus muscles were mounted as Fig. 3. The effect of L-NMMA, D-NMMA and the reversion of the effect of L-NMMA by L-arginine on the response to NANC nerve stimulation were studied. Similar frequencies of stimulation and stimulation conditions were used as were used for the BRP and tone was raised with guanethidine 10^{-5}M. Control frequency-response curves of NANC relaxation were constructed and compared with similar curves in the presence of L-NMMA or D-NMMA at final concentrations at 10^{-5}M, 3×10^{-5}M, 10^{-4}M and 3×10^{-4}M, and finally the ability of L-arginine to reversed the effect of

L-NMMA was examined.

L-NMMA had two effects on the rat anococcygeus; first it caused an immediate rise in tone and secondly it produced a dose-related inhibition of the response to NANC nerve stimulation. An experiment illustrating the inhibition of the nerve response is showed in Fig. 45. L-NMMA 10^{-5}M. inhibits the response at all frequencies of stimulation and this effect increases with dose up to 3×10^{-4}M. The results for all the experiments at four dose levels of L-NMMA are shown in Fig. 46. The inhibitory response is significantly inhibited at the probability < 0.01 level or better at all doses (except the responses at 0.1 Hz). The effect of 3×10^{-4}M. L-NMMA is not significantly different from that of 10^{-4}M L-NMMA presumably represents the maximum inhibitory effect. This inhibitory action was rapidly reversed by washing (Fig. 47). This inhibitory property of L-NMMA was not possessed by its D isomer which consistently and in even higher doses had no effect on the response to NANC nerve stimulation. An experiment illustrating this point is shown in Fig. 48 and the Fig. 49 summarises all the results with D-NMMA in concentrations up to 3×10^{-4}M. The effect of L-NMMA was therefore stereoselective. If the mode of action was as a competitive inhibitor of endogenous L-arginine then high exogenous concentrations of L-arginine might be expected to reverse the inhibition. This was tested and illustrative results from a single experiment are shown in the upper three records of the Fig. 50 and a summary graph of all the results in Fig. 51. In Fig. 50 L-NMMA 10^{-4}M. causes an immediate rise in tone together with about 70 % inhibition of the response to NANC nerve stimulation. The addition of L-arginine 3×10^{-4}M. reverses the rise in tone and at some time produces a partial restoration of the nerve stimulation. The lower records in Fig. 50 show that these effects are not due to an enhanced sensitivity to nitric oxide which in the presence of even 3×10^{-4}M. L-NMMA is

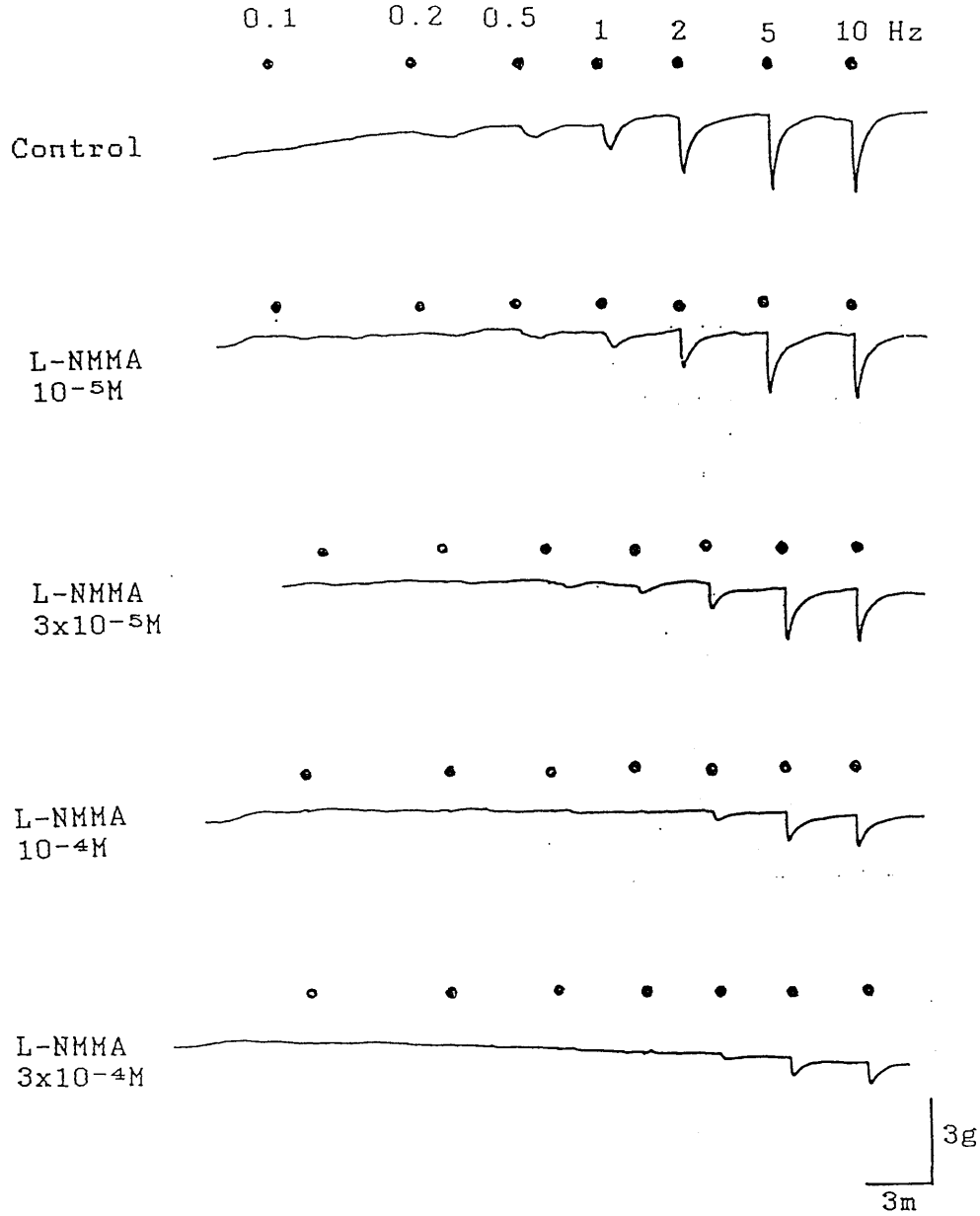


Fig. 45 L-monomethyl-N-arginine (L-NMMA) inhibits the inhibitory relaxation of the rat anococcygeus muscle to NANC nerve stimulation in a dose-dependent manner at concentrations of 10^{-5}M. up to 3 x 10^{-4}M. Stimulation was by supramaximal voltage and 0.5 ms. pulse duration for 20 pulses at frequencies shown above each response.

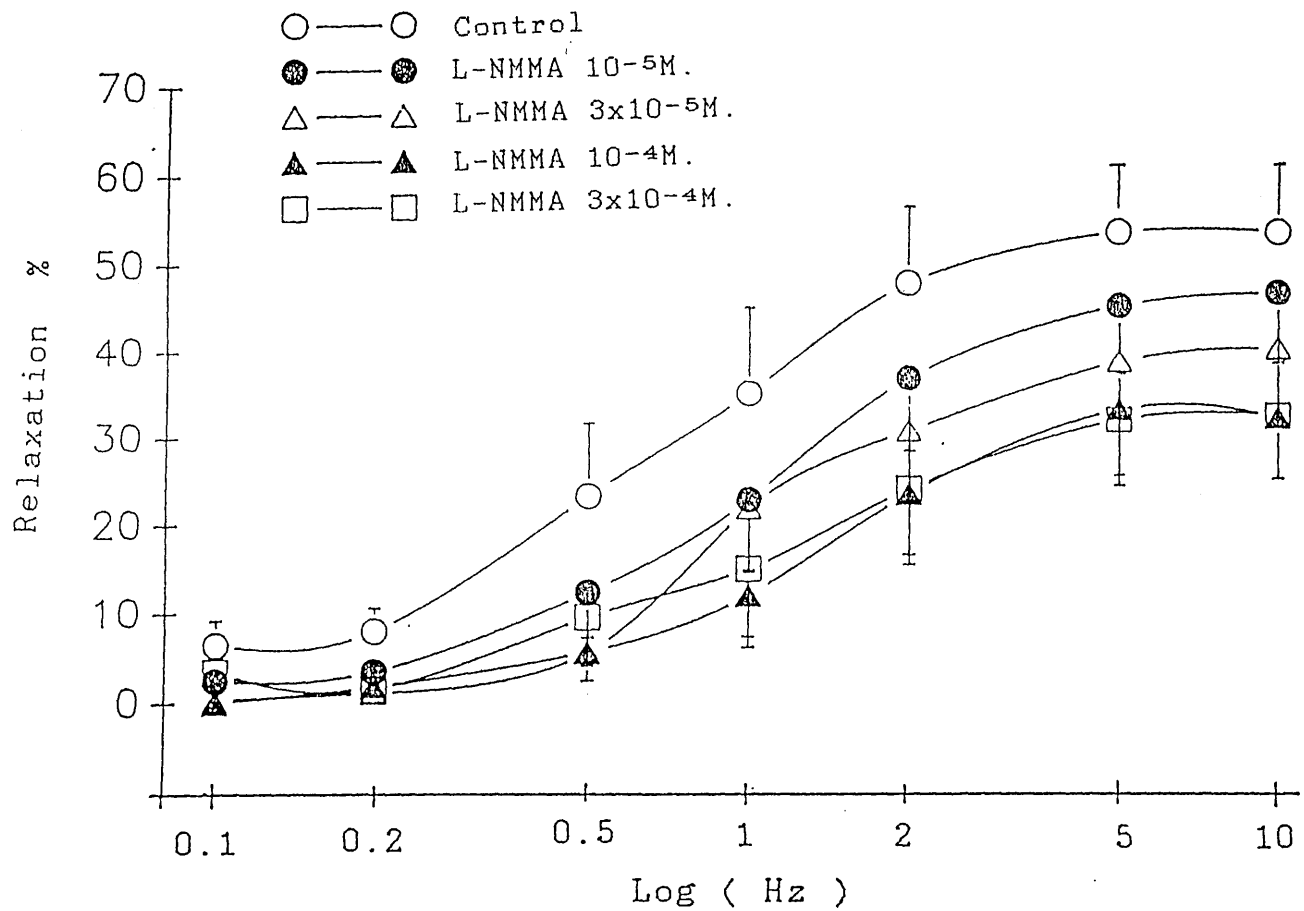


Fig. 46 The frequency-response curves show the concentration-dependent inhibition of L-NMMA (L-monomethyl-N-arginine) on the NANC inhibitory relaxation of the rat anococcygeus muscle to the nerve stimulation. Stimulation was by supramaximal voltage and 0.5 ms. pulse duration for 20 pulses at frequencies shown on X-axis. The probability value is less than 0.01 (except at 0.1 Hz) between the control and any one of the L-NMMA group. There is no significant difference between the effects of L-NMMA 10^{-4}M. and L-NMMA 3 x 10^{-4}M. ($P > 0.05$). The numbers of the observations were 8.

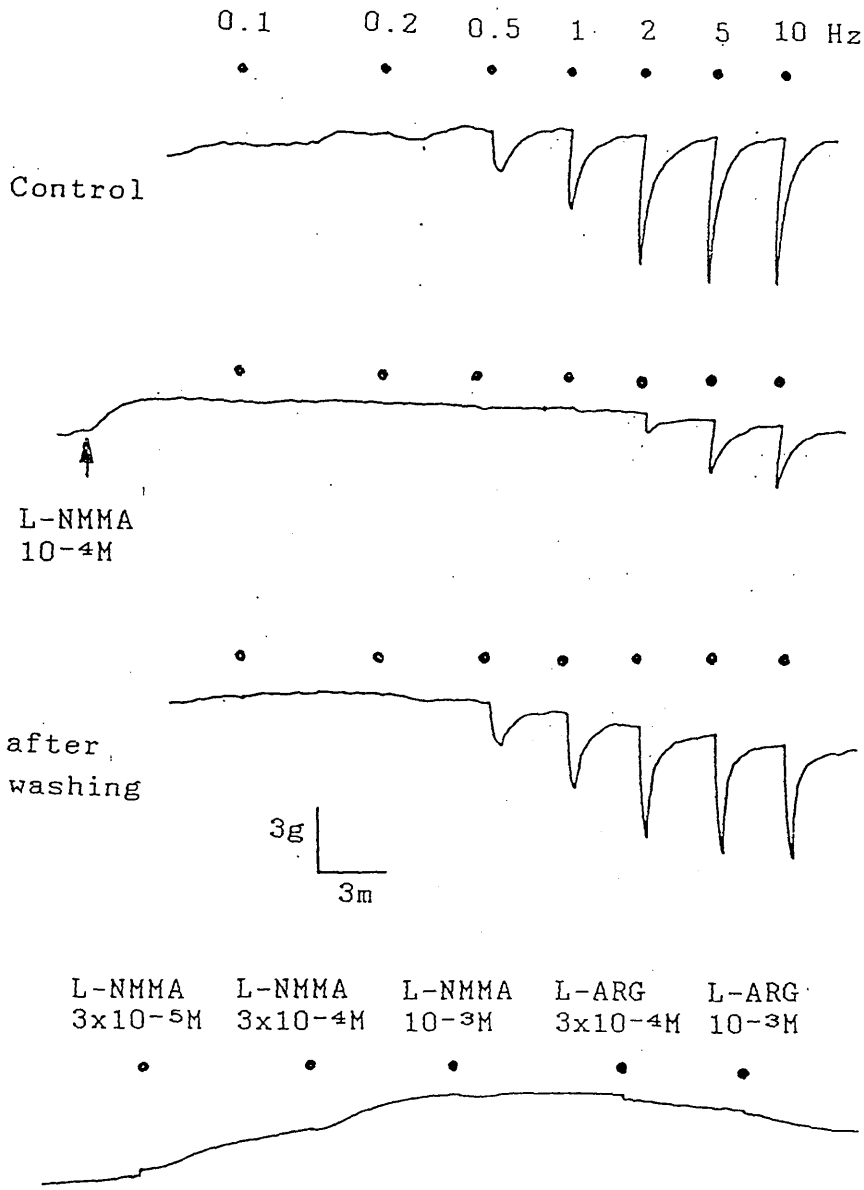


Fig. 47 L-monomethyl-N-arginine (L-NMMA) raises the tone of and inhibits the inhibitory relaxation of the rat anococcygeus muscle to NANC nerve stimulation at concentration of 10^{-4}M. and these effects are reversed by washing out L-NMMA as shown in the upper three records. Stimulation was by supramaximal voltage and 0.5 ms. pulse duration for 20 pulses at frequencies shown above each response. The lowest records also show L-NMMA dose-dependently raises the tone of the rat anococcygeus muscle before the tone raised by agonist, an effect reversed by L-arginine.

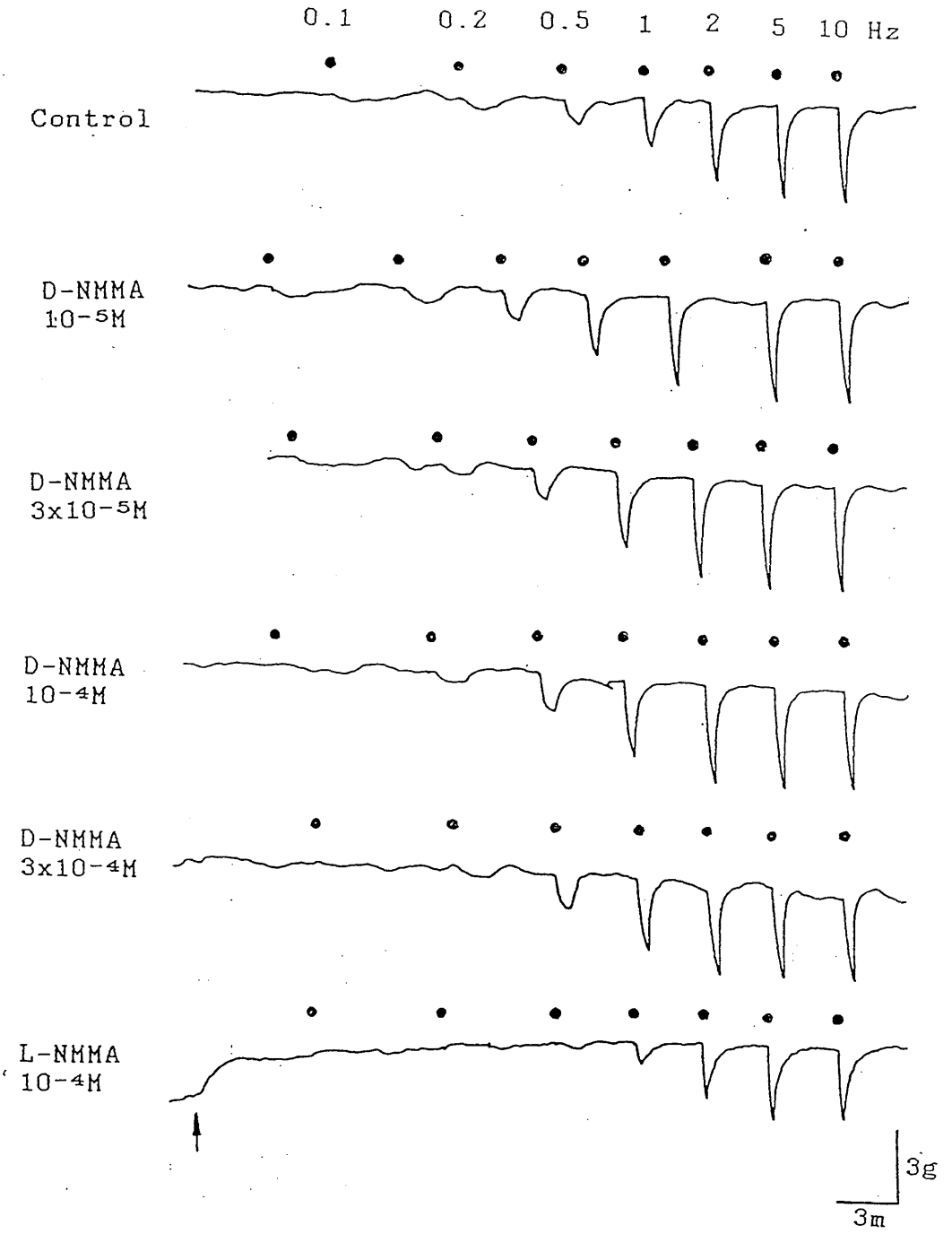


Fig. 48 Shows D-monomethyl-N-arginine (D-NMMA) is without effect on inhibitory relaxation of the rat anococcygeus muscle to NANC nerve stimulation at concentrations from 10^{-5}M. up to 3×10^{-4}M. compared with L-NMMA 10^{-4}M. does inhibit the relaxation shown in the lowest records. Field stimulation was by supramaximal voltage and 0.5 ms. pulse duration for 20 pulses at frequencies shown above each response.

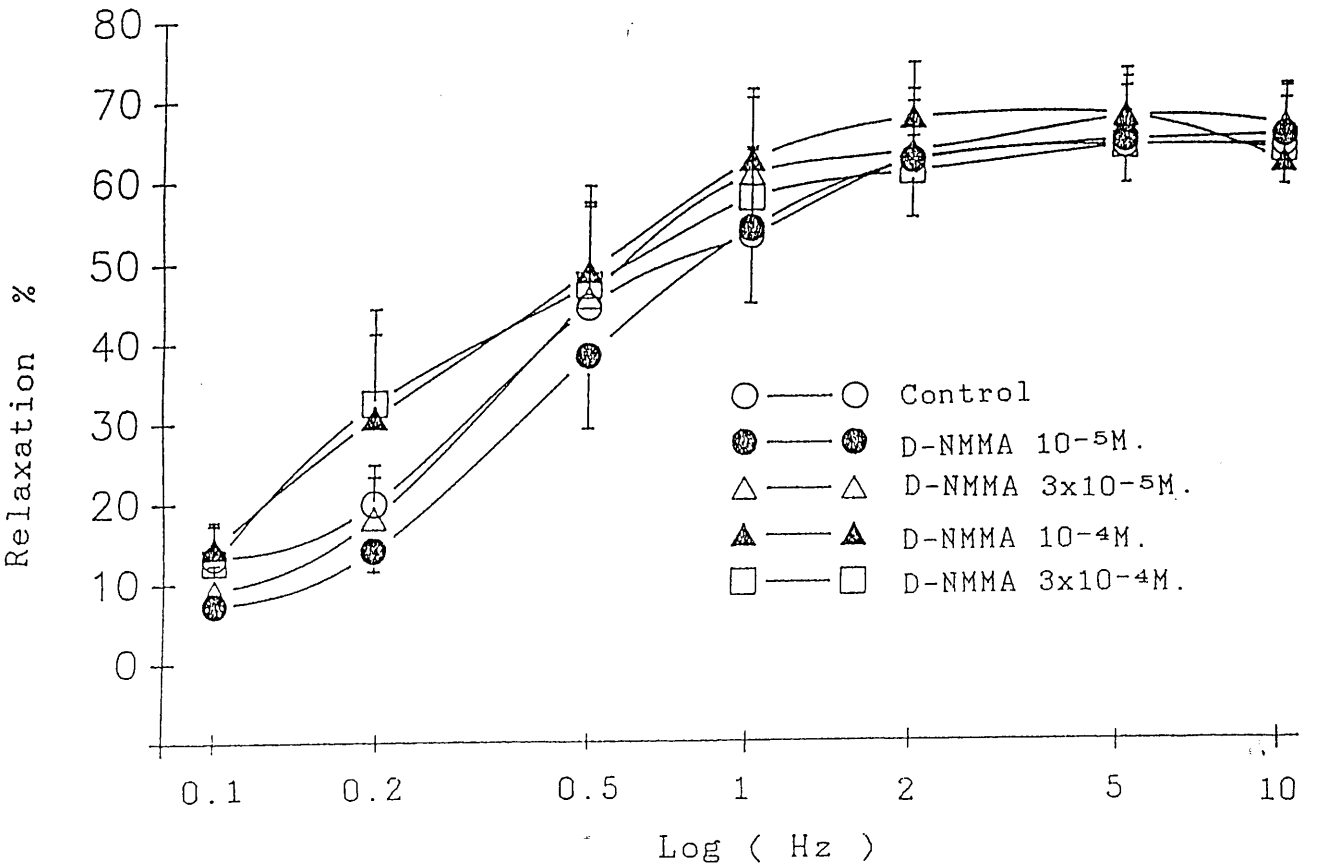


Fig. 49 The frequency-response curves show there is no effect of D-monomethyl-N-arginine (D-NMMA) on the inhibitory response of the rat anococcygeus muscle to NANC nerve stimulation at concentrations from 10^{-5}M. up to 3 x 10^{-4}M. Probability value is more than 0.05 between the curve of control and any curve of the D-NMMA group. The numbers of the observations were 8. Field stimulation was by supramaximal voltage and 0.5 ms. pulse duration for 20 seconds at frequencies shown on X-axis.

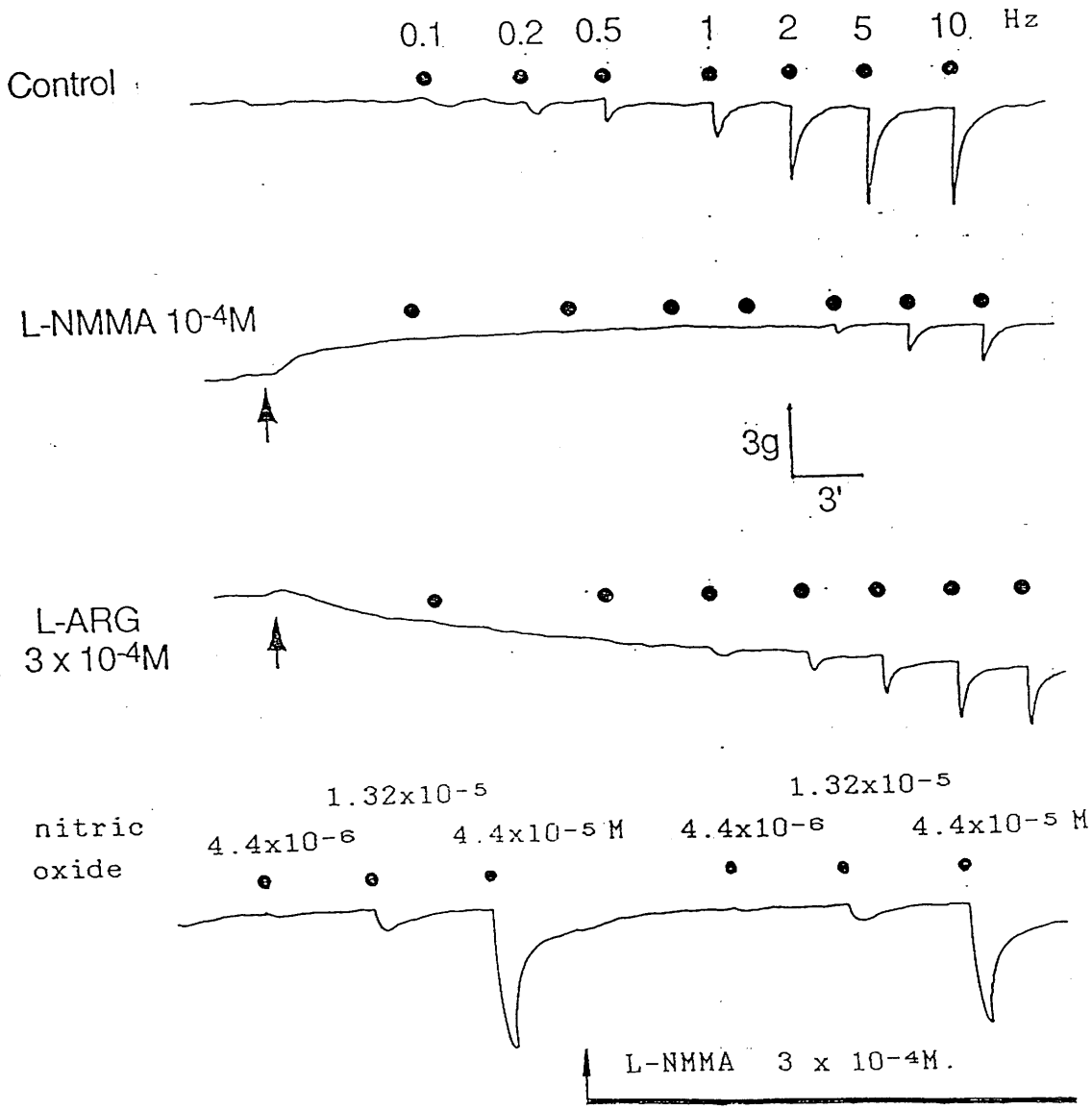


Fig. 50 L-monomethyl-N-arginine (L-NMMA) raises the tone of and inhibits the inhibitory relaxation of the rat anococcygeus muscle to NANC nerve stimulation at concentration of 10^{-4}M. and these effects are partially reversed by 3 x 10^{-4}M. L-arginine as shown in the upper three records. Stimulation was by supramaximal voltage and 0.5 ms. pulse duration for 20 pulses at frequencies shown above each response. The lowest records show 3 x 10^{-4}M. L-NMMA does not inhibit the dose-dependent relaxation induced by nitric oxide in the rat anococcygeus muscle.

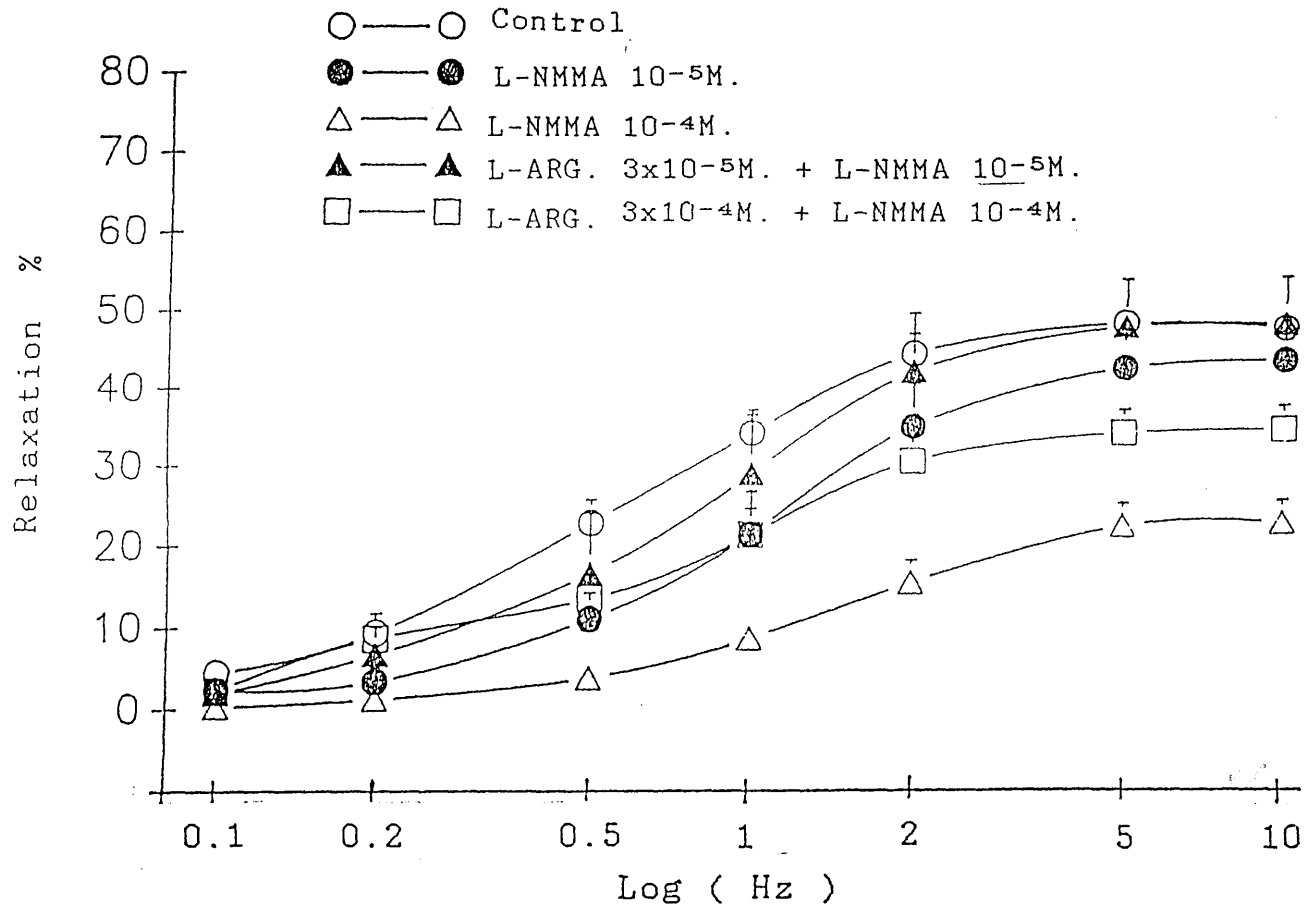


Fig. 51 The frequency-response curves show L-monomethyl-N-arginine (L-NMMA) dose-dependently inhibits the NANC inhibitory relaxation of the rat anococcygeus muscle to the nerve stimulation and this effect of L-NMMA is reserved by L-arginine competitively ($P < 0.01$; N=12). Stimulation was by supramaximal voltage and 0.5 ms. pulse duration for 20 pulses at frequencies shown on X-axis.

unchanged. The frequency-response curve in Fig. 51 show the inhibitory effect of 10^{-5}M. L-NMMA is almost competitively reversed by L-arginine 3×10^{-5}M. and the greater inhibition by 10^{-4}M. L-NMMA is reduced by about 50 % by L-arginine 3×10^{-4}M. Some additional experiments were performed adding mixtures of L-arginine and L-NMMA. The results of these are illustrated in Fig. 52 and Fig. 53. At both doses of 10^{-5}M. and 10^{-4}M. L-NMMA an appropriate competitive concentration of L-arginine completely prevented the inhibitory effect on the response to NANC nerve stimulation.

The second effect of L-NMMA on the rat anococcygeus muscle was to cause a further rise in tone. This is illustrated in Fig. 47, Fig. 48 and Fig. 50. The effect begins after a short talent period of less than a minute and develops quite rapidly to a new and well maintained plateau of tone. This effect was dose dependent from 1 g (10 %) at 10^{-5}M; 1.4 g (14 %) at 3×10^{-5}M; 1.7 g (17 %) at 10^{-4}M and 2 g (20 %) at 3×10^{-4}M and was easily reversed by washing out the L-NMMA. The rise in tone was also reversed by L-arginine (Fig. 50). These results are consistent with a basal release of the NANC inhibitory neurotransmitter and at least some similar relaxant with a similar origin in L-arginine as shown in lower records in Fig. 47.

The effects of L-NMMA, D-NMMA and the reversal of L-NMMA by L-arginine were also studied on the release of EDRF from rabbit aortic rings with their endothelium intact. These experiments were original intended as controls to demonstrate that L-NMMA did indeed inhibit an arginine-dependent process. Such controls were required after the first series of the experiments on the BRP where L-NMMA had no effect. The need for such controls diminished after successfully demonstrating the action of L-NMMA on the rat anococcygeus.

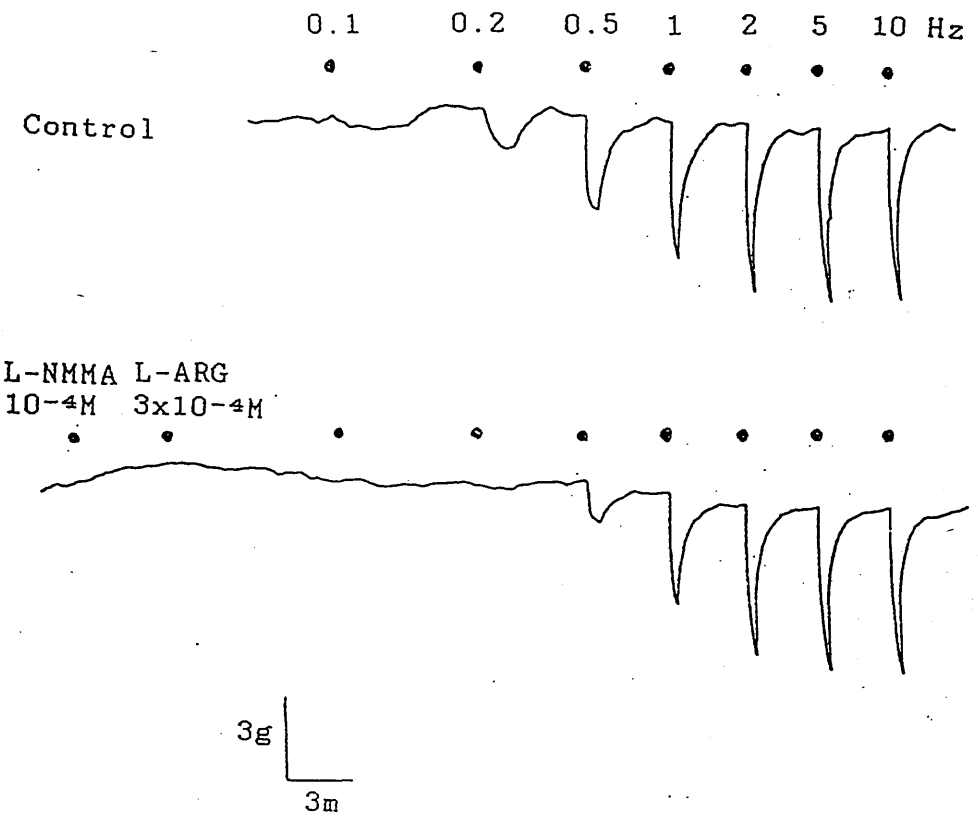


Fig . 52 The inhibition of L-monomethyl-N-arginine (L-NMMA) 10^{-4}M. on rat anococcygeus NANC inhibitory relaxation is prevented by 3 x 10^{-4}M. L-arginine (L-ARG.) added simultaneously with L-NMMA. Stimulation was by supramaximal voltage and 0.5 ms. pulse duration for 20 pulses at frequencies shown on X-axis.

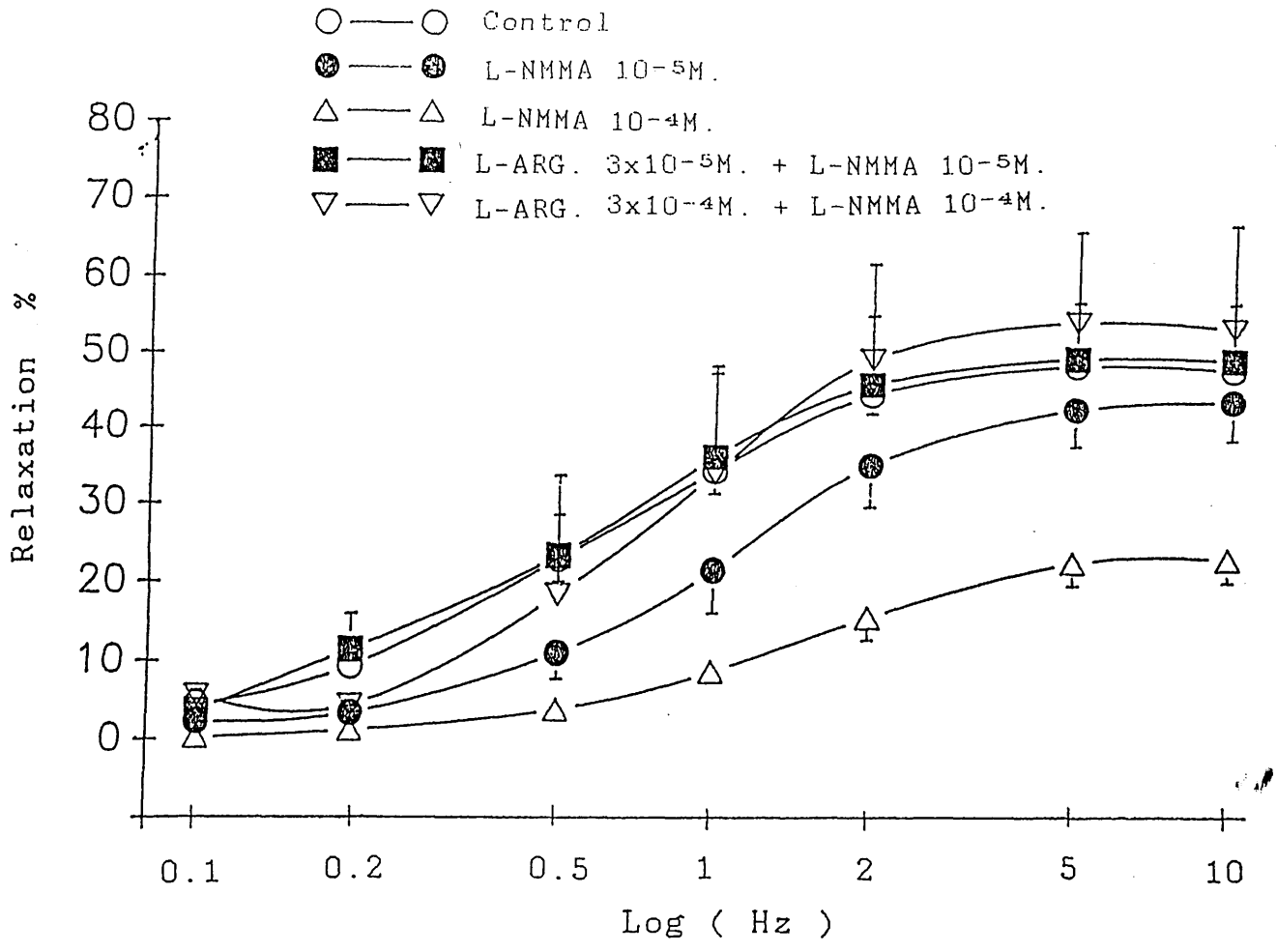


Fig. 53 The frequency-response curves show L-monomethyl-N-arginine (L-NMMA) dose-dependently inhibits the NANC inhibitory relaxation of the rat anococcygeus muscle to the nerve stimulation and this effect of L-NMMA is prevented by L-arginine (L-ARG.) added simultaneously with L-NMMA ($P < 0.01$; N=4). Stimulation was by supramaximal voltage and 0.5 ms. pulse duration for 20 pulses at frequencies shown on X-axis.

Rings of about 2 mm. width were cut from rabbit thoracic or abdominal aortae, then each ring cut through to produce an open strip which was then mounted on a hook in a 10 ml. organ bath. Tone was raised by adding phenylephrine 7.5×10^{-6}M. and once the raised tone had stabilised, EDRF was released by adding acetylcholine to the bath. As Fig. 54 illustrates, D-NMMA in concentration up to 3×10^{-4}M. had no effect on the response whereas L-NMMA in concentrations from 3×10^{-5}M. to 10^{-4}M. caused a dose related inhibition of the response to EDRF. The effect of L-NMMA 10^{-4}M. was easily reversed by washing out L-NMMA or by L-arginine 3×10^{-4}M. When L-NMMA and L-arginine in appropriate doses were added simultaneously, the effect of L-NMMA was prevented (Fig. 54). Just as in the rat anococcygeus this effect of L-NMMA was not due to a reduced effectiveness of EDRF since the dose-response relationship for nitric oxide induced relaxation was unaltered by the presence of L-NMMA 10^{-4}M. (Fig. 54).

THE EFFECT OF L-CANAVANINE ON THE NANC INHIBITORY RELAXATION OF THE BRP MUSCLE AND THE RAT ANOCOCCYGEUS MUSCLE

The rat anococcygeus muscle is much more sensitive to L-monomethyl-N-arginine than the bovine retractor penis muscle. There are three possible explanations. First and most likely assumes the NANC inhibitory neurotransmitter in both tissues is the same but its physiological precursor is either not L-arginine or not exclusively L-arginine. Secondly, the NANC inhibitory neurotransmitter and its precursor are the same in both tissues but the enzymes which synthesize the

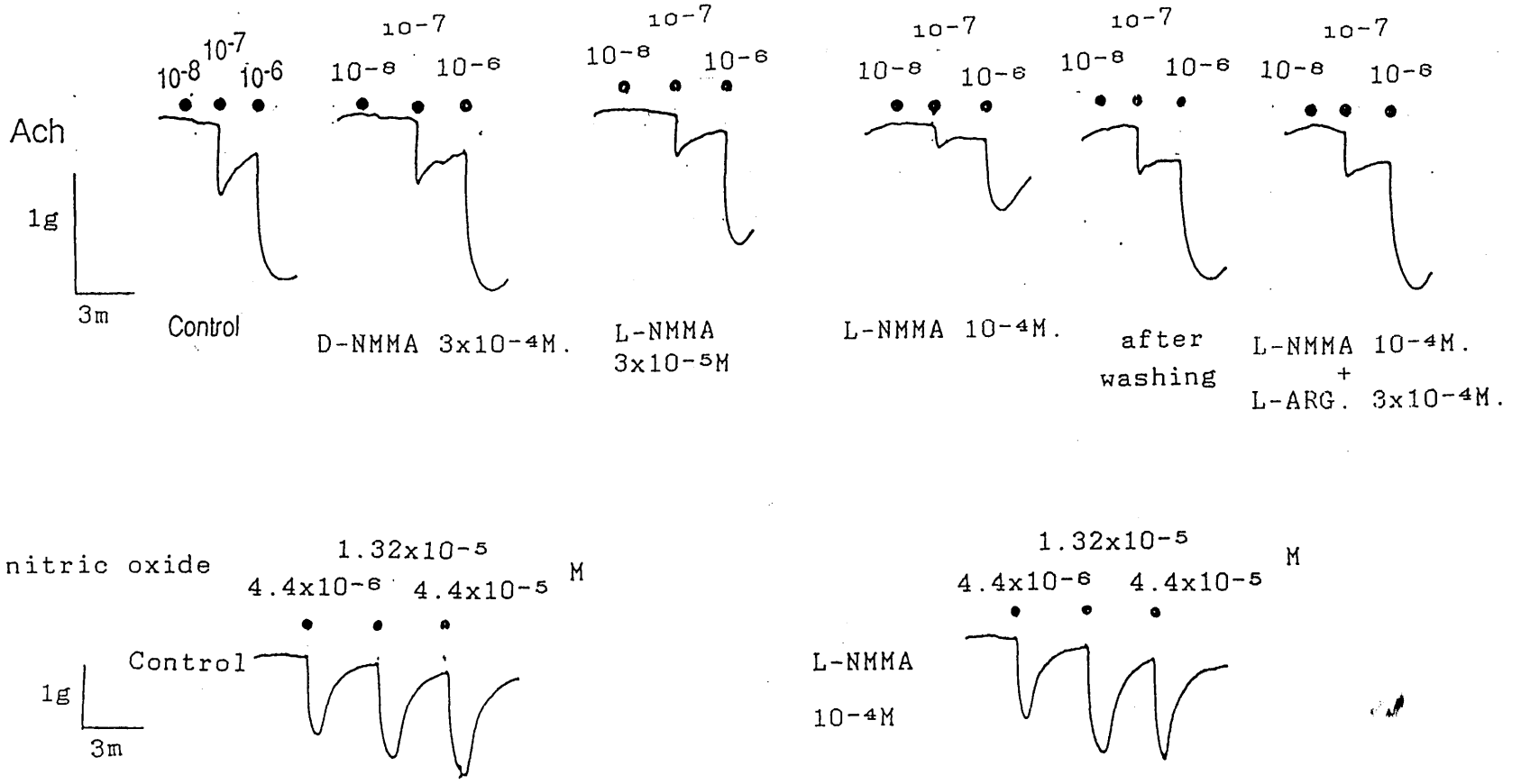


Fig. 54 Compared D-monomethyl-N-arginine (D-NMMA) 3 x 10^{-4}M. is without effect on endothelium-dependent relaxation of the rabbit aortic preparation induced by acetylcholine, L-monomethyl-N-arginine (L-NMMA) inhibits the relaxation at concentrations of 3 x 10^{-5}M. and 10^{-4}M. and this effect of L-NMMA is reversed by washing out L-NMMA and prevented by L-arginine (L-ARG.) added simultaneously with L-NMMA as shown in the upper records. The lower records show the relaxation of the rabbit aortic preparation induced by nitric oxide is not inhibited by L-NMMA 10^{-4}M.

neurotransmitter are not and have different sensitivity to L-monomethyl-N-arginine. Thirdly, the NANC inhibitory neurotransmitter might be different in the two tissues.

L-canavanine, a guanidinooxy structural analogue of arginine and an inhibitor of various L-arginine-utilizing enzymes, was reported to inhibited the endothelium-dependent relaxation of rat thoracic aortic rings induced by ATP and acetylcholine (Schmidt et al. 1988a) and to abolish basal and ATP-induced nitric oxide formation and release (Schmidt et al. 1988b). Iyengar et al (1988 a) demonstrated an L-arginine-dependent biosythesis of nitrite, nitrate and, when morpholine was presented, N-nitrosomorpholine by cultured macrophages; the nitrogen of the nitric oxide moieties was derived from the terminal guanidino nitrogen of arginine. L-arginine inhibited the formation of these NO-containing compounds. Therefore L-canavanine might through some light on these possibilities so that its effect on the NANC inhibitory relaxation of the bovine retractor penis muscle and the rat anococcygeus muscle was examined.

Bovine retractor penis muscle preparations or rat anococcygeus muscle preparations were mounted as Fig. 3. The tone of the muscles was raised by guanethidine (10^{-5}M). The inhibitory relaxation was induced by field stimulation (supramaximal voltage and 0.5 milliseconds' pulse duration at 0.1, 0.2, 0.5, 1, 2, 5 and 10 Hz frequencies for 20 pulses).

In the BRP L-canavanine exhibited no inhibitory effect on the NANC inhibitory relaxation induced by field stimulation at concentrations of 3×10^{-5}M, 10^{-4}M up to 3×10^{-4}M. as shown in Fig. 55 from a single experiment and a summary of all the experiments shows in Fig. 56. But at very high concentration (10^{-3}M), L-

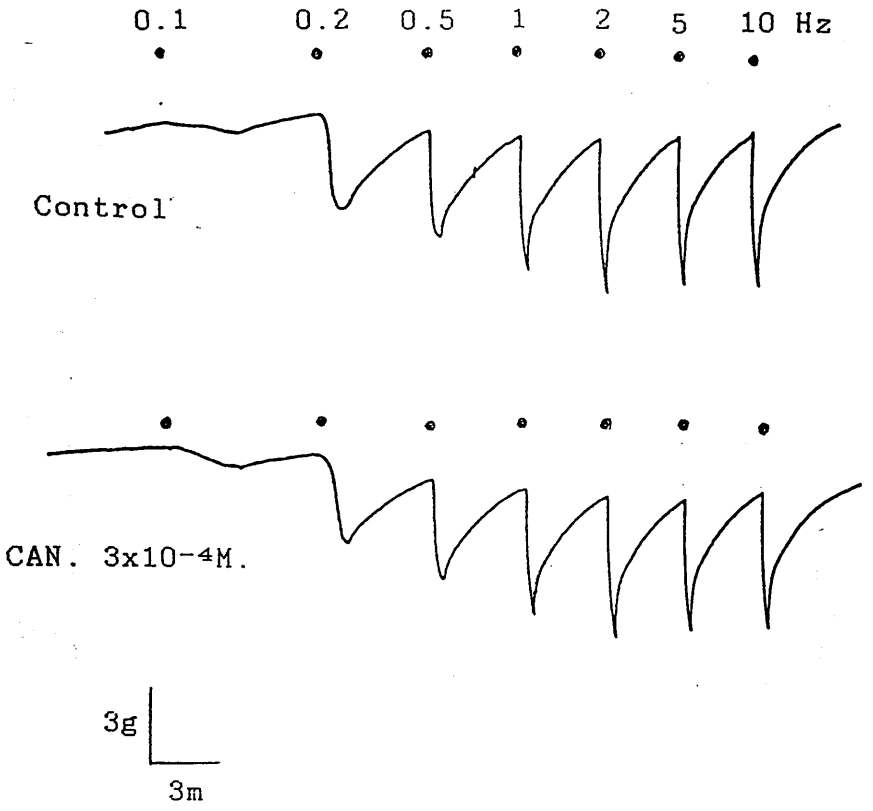


Fig. 55 L-canavanine (L-CAN.) does not inhibit the NANC inhibitory relaxation of the BRP muscle to the nerve stimulation at concentration of 3 x 10 $^{-4}$M. Stimulation was by supramaximal voltage and 0.5 ms. pulse duration for 20 pulses at frequencies shown above each response.

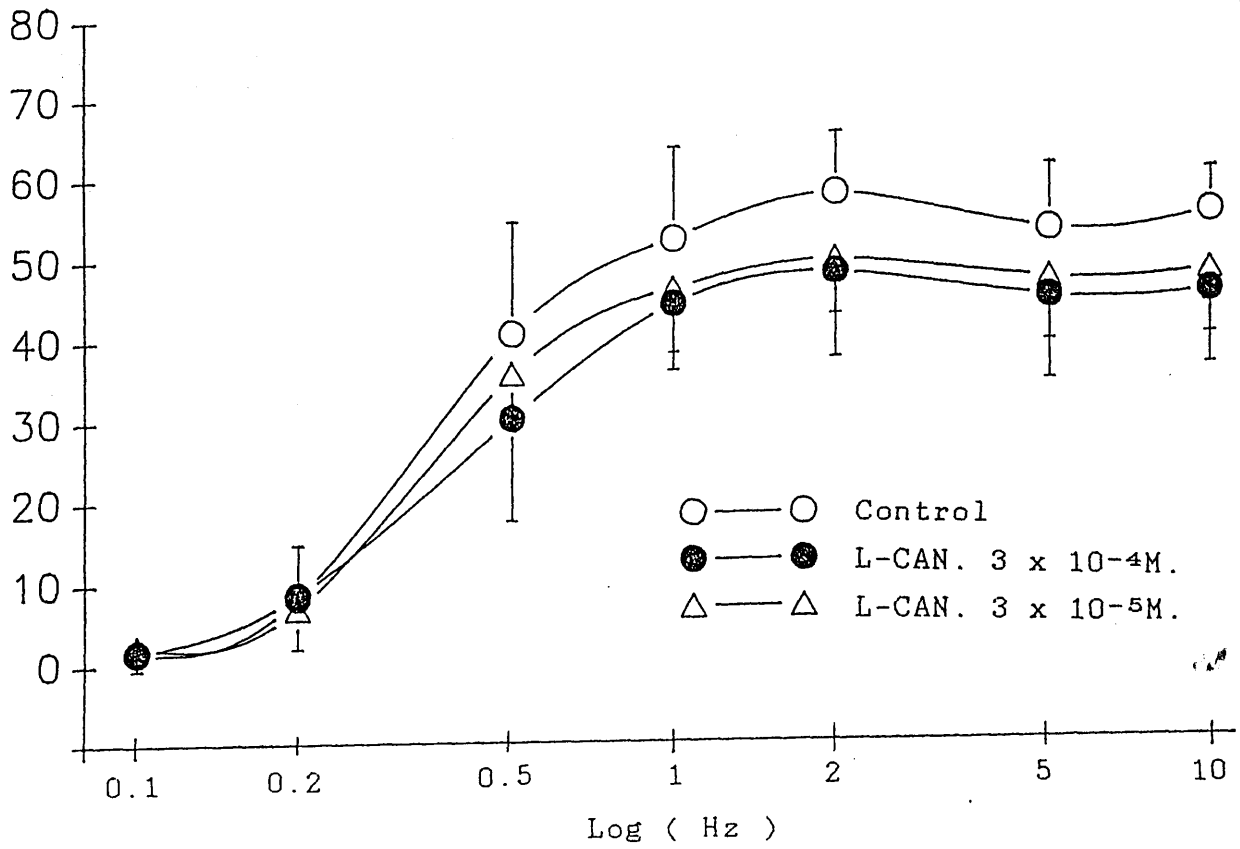


Fig. 56 The frequency-response curves show L-canavanine (L-CAN.) is without effect on the NANC inhibitory relaxation of the BRP muscle to the nerve stimulation at concentrations from 3 x 10^{-5}M. up to 3 x 10^{-4}M. (P > 0.05; N=6). Stimulation was by supramaximal voltage and 0.5 ms. pulse duration for 20 pulses at frequencies shown on X-axis.

canavanine inhibited the NANC inhibitory relaxation, an effect partially reversed by L-arginine 10^{-3}M. as shown in Fig. 57 from a single experiment and in Fig. 58 for the summary of these experiments.

In rat anococcygeus muscle, L-canavanine was without effect on the guanethidine-induced tone of the rat anococcygeus muscle and the NANC inhibitory relaxation induced by field stimulation at concentrations from 3×10^{-5}M. up to 2×10^{-3}M. and pretreatment even for two hours as shown in Fig. 59 from a single experiment and in Fig. 60 for the summary of all the experiments.

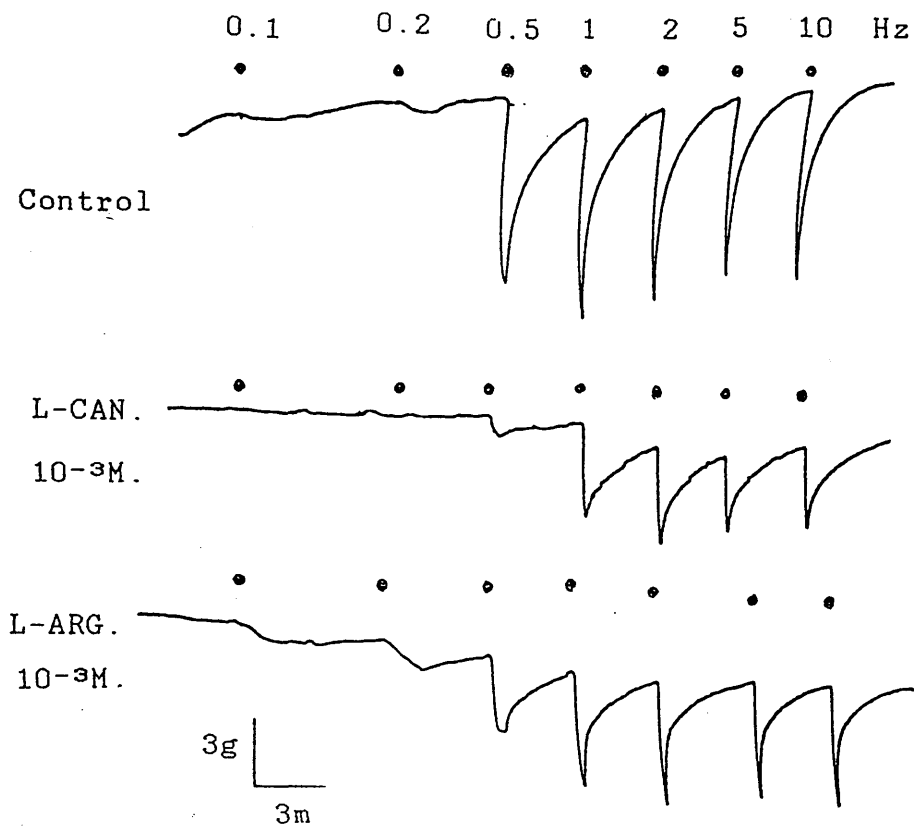


Fig. 57 High concentration of 10^{-3}M. L-canavanine (L-CAN.) inhibits the inhibitory relaxation of the BRP muscle to NANC nerve stimulation, an effect partially reversed by 10^{-3}M. L-arginine (L-ARG.). Stimulation was by supramaximal voltage and 0.5 ms. pulse duration for 20 pulses at frequencies shown above each response.

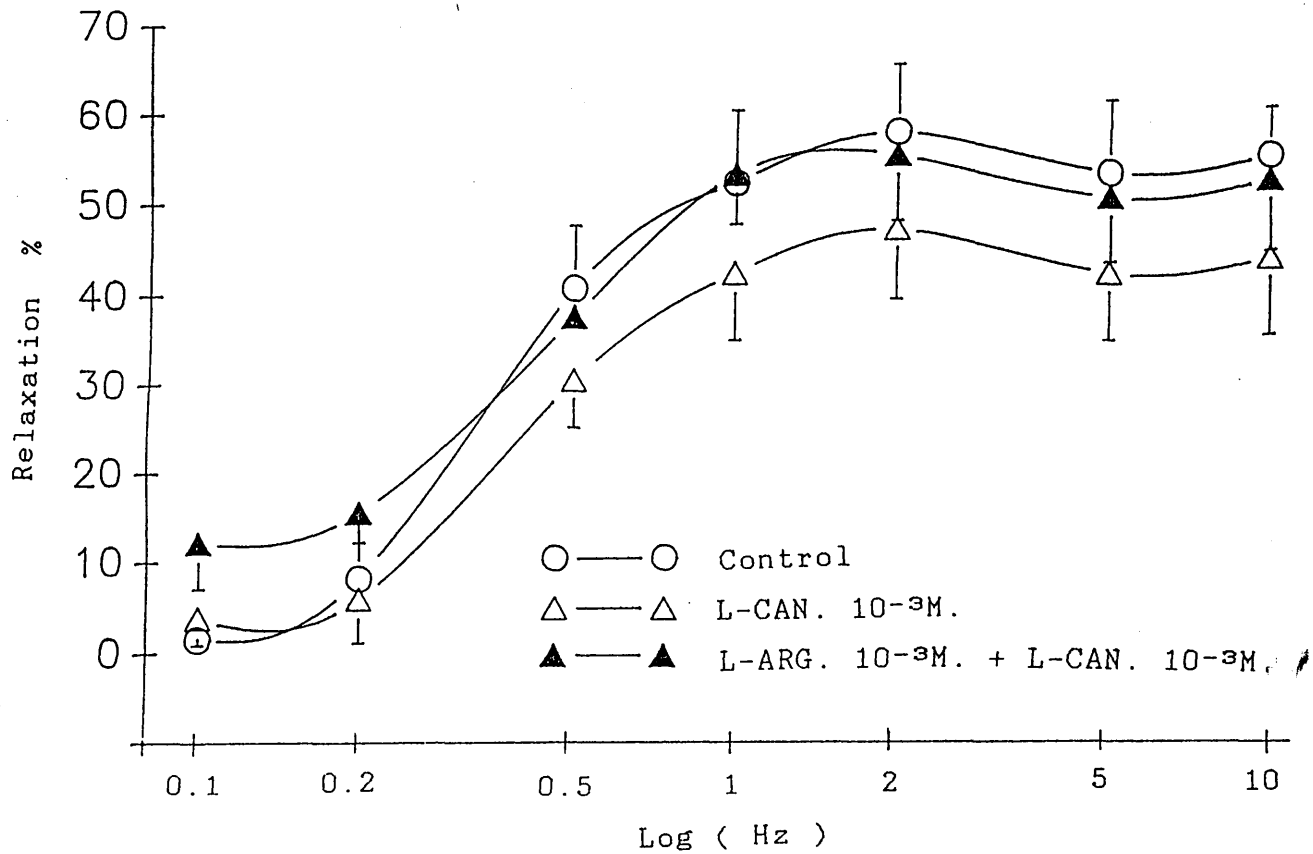


Fig. 58 The frequency-response curves show L-canavanine (L-CAN.) inhibits the NANC inhibitory relaxation of the BRP muscle to the nerve stimulation at high concentration 10^{-3}M. ($P < 0.05$; N=6), an effect reversed by 10^{-3}M. L-arginine (L-ARG.). Stimulation was by supramaximal voltage and 0.5 ms. pulse duration for 20 pulses at frequencies shown on X-axis.

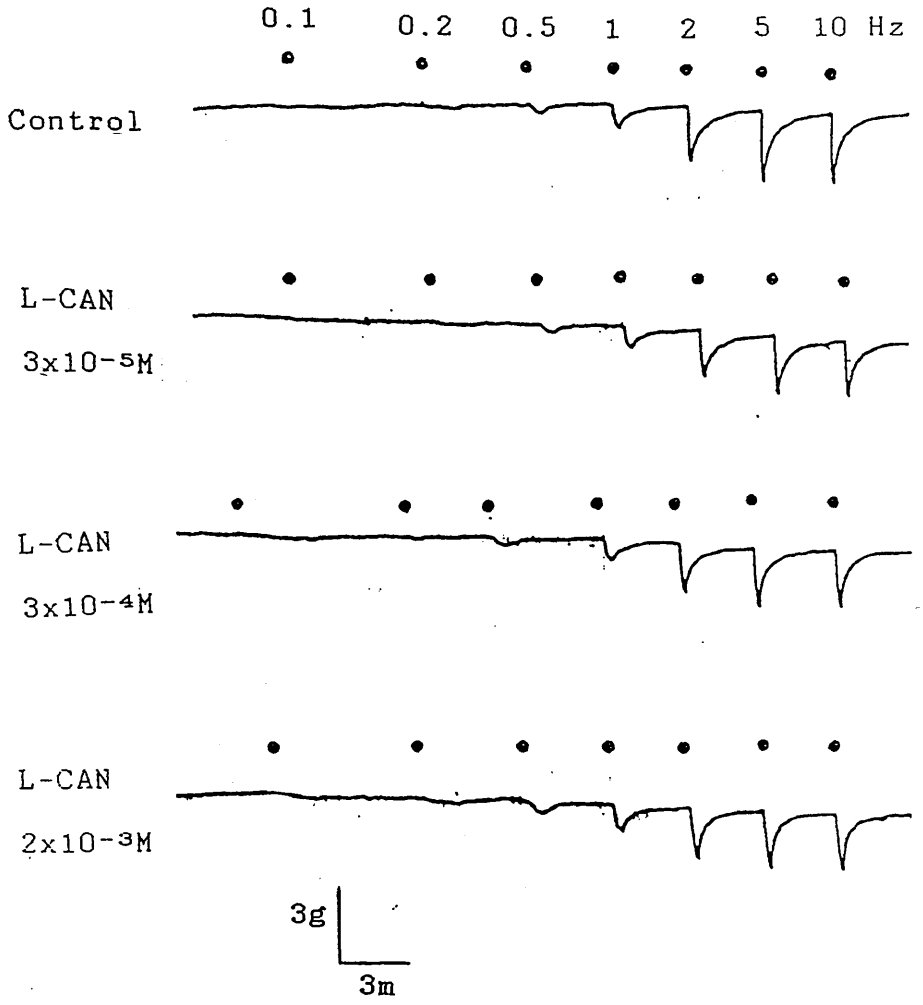


Fig. 59 L-canavanine (L-CAN.) does not inhibit NANC inhibitory relaxation of the rat anococcygeus muscle to the nerve stimulation at concentrations from 3 x 10^{-5}M. up to 2 x 10^{-3}M. Field stimulation was by supramaximal voltage and 0.5 ms. pulse duration for 20 pulses at frequencies shown above each response.

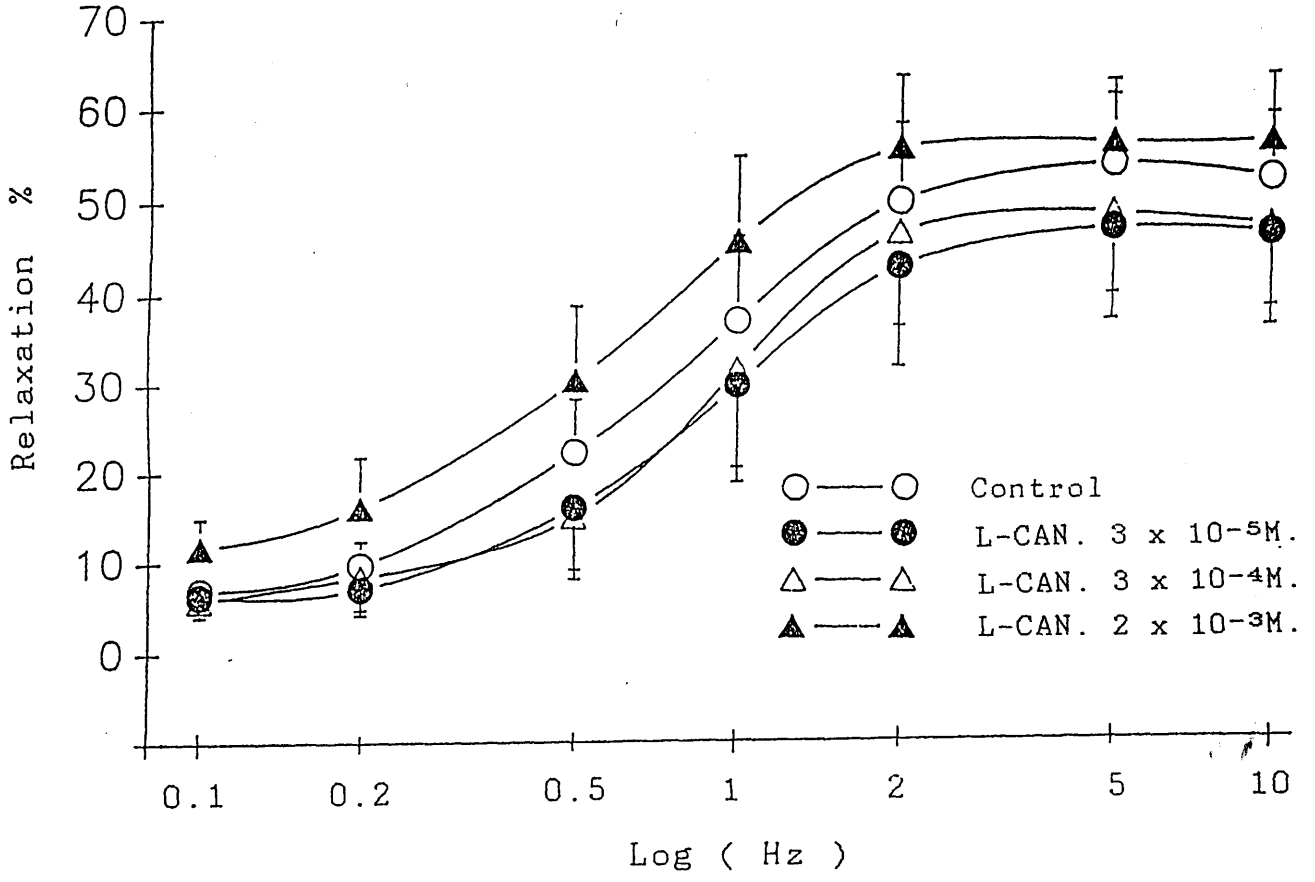


Fig. 60 The frequency-response curves show L-canavanine (L-CAN.) is without effect to NANC inhibitory relaxation of the rat anococcygeus muscle to the nerve stimulation at concentrations from 3 x 10^{-5}M up to 2 x 10^{-3}M. (P > 0.05; N=6). Stimulation was by supramaximal voltage and 0.5 ms. pulse duration for 20 pulses at frequencies shown on X-axis.

DISCUSSION

The initial objective of this project was to try to detect by bioassay the non-adrenergic non-cholinergic inhibitory neurotransmitter released from the bovine retractor penis muscle by field stimulation. The BRP has a dense NANC innervation and many properties of neurotransmission by these nerves are known nevertheless the neurotransmitter remains a mystery which has defied all attempts to identify chemically its nature (Klinge et al. 1970; Klinge & Sjostrand 1974; Ambache & Killick 1978; Bowman & Gillespie 1981; Gillespie et al. 1981; Bowman et al. 1982b; Bowman & Drummond 1984). If the NANC neurotransmitter could be detected biologically, then it might be possible to isolate, extract and purify it, which should make identification a relatively simple matter. Even if isolation was not possible a variety of experiments on its properties and interaction with drugs would be possible as was done with EDRF experiments which would through new light on the nature of the NANC neurotransmitter. Unfortunately this initial objective was without success. A second approach to the identification of the NANC neurotransmitter was to study the effect of drugs which are known to potentiate or block effects of potential neurotransmitters such as the inhibitory factor from the BRP and EDRF, both of which possess many properties in common with the NANC inhibitory neurotransmitter. This was the second objective of this project.

Like many smooth muscles the mechanical function of the BRP requires both an excitatory and inhibitory innervation. Since Sertoli (1883) first studied the retractor penis muscle of several species both in vitro and in vivo, many reports

have appeared on this subject (Langley & Anderson 1895; Oppenheimer 1938; Goldenberg 1965; Luduena & Grigas 1966; 1972; Klinge 1970; 1972a). The main points to emerge from this work are first to confirm the existence of a powerful excitatory and inhibitory innervation. The excitatory innervation appeared to be classically adrenergic in so far as the nerves had their origins from the lumbar sympathetic spinal cord. Most of the preganglionic fibres travel to the paravertebral sympathetic chains, synapse in the sacral ganglia of the sympathetic chain and then the postganglionic fibres are distributed via the pudic nerve to the muscle. Some of the fibres reach the target via the hypogastric nerve and a few follow the pelvic parasympathetic nerve. The excitatory neurotransmitter of these sympathetic fibres is noradrenaline. The inhibitory innervation in contrast has its origin in the sacral spinal cord and the neurotransmitter is unknown. While readily blocked by TTX, adrenergic or cholinergic blocking agents were without effects on the NANC inhibitory response. In 1974 Klinge and Sjostrand systematically studied the physiology and pharmacology of the BRP. They verified and extended these results. The excitatory contraction was induced by noradrenaline and mediating by excitatory α-adrenoceptors. The contraction evoked by transmural stimulation were abolished or profoundly depressed by adrenergic neuron blocking agents or α-adrenoceptor blocking agents and augmented by inhibitors of neuronal noradrenaline uptake. Also the retractor penis was induced to contract by the indirectly acting sympathomimetic amine tyramine.

While tetrodotoxin and local anaesthetics abolished the inhibitory response to field stimulation of the BRP confirming the neural nature of the response, acetylcholine and pilocarpine contracted rather than relaxed the BRP and these contractions were inhibited by atropine and scopolamine. The muscarinic receptors

accessible to exogenous cholinomimetics appeared therefore to be excitatory in the BRP. The inhibitory response to field stimulation of the BRP was not affected by physostigmine, atropine, scopolamine, ganglionic or neuromuscular blocking agents. The inhibition was also unimpaired by hemicholinium and botulium toxin. These suggest the inhibitory response to field stimulation of the BRP is not due to the release of acetylcholine from the inhibitory nerves.

Although PGE_1 relaxed the retractor penis at low concentration and was suggested as the inhibitory neurotransmitter (Luduena & Grigas 1972), the inhibitory response to field stimulation was uninfluenced by inhibitors of prostaglandin synthesis (Klinge & Sjostrand 1974).

ATP, ADP and AMP contracted the retractor penis. Many nucleosides and nucleotides were without effect or had a weak contractile effect on the retractor penis. These suggest the inhibitory neurotransmitter is unlikely purinergic (Klinge & Sjostrand 1974).

Histamine, 5-HT, bradykinin and Substance P contracted the retractor penis. The inhibitory response to field stimulation was unaffected by antihistamines and 5-HT antagonists (Klinge & Sjostrand 1974).

Posterior pituitary hormones, GABA, glycine, glutamic acid, aspartic acid and several other amino acids were without effects on the retractor penis.

This inability to mimic the effect of NANC nerve stimulation excludes many substances as potential NANC inhibitory neurotransmitter.

THE BIOASSAY DETECTION OF THE NANC NEUROTRANSMITTER

One of the prime requirements for a substance as a neurotransmitter is its release by nerve stimulation. If the NANC neurotransmitter could be detected by a test tissue after liberation by field stimulation, this requirment would have been met. It would also provide a starting point for more extensive study of its properties. Several manipulations of the standard Loewi-type experiment were tried in the hope of detection of the NANC inhibitory neurotransmitter. For example having failed with donor preparations of standard size, large donor BRP's were used in the hope of increasing the amount of neurotransmitter released. When this failed, thinner donor BRP was used to improve the diffusion of the neurotransmitter into the bath fluid. On the assay preparation side both rat anococcygeus and rabbit aortic strip were used in the hope that one or other would be the most sensitive detection system. Also many changes in the parameters of field stimulation were used including frequencies from 0.1 Hz up to 128 Hz, different duration of field stimulation from 10 seconds up to 120 seconds, even continuous stimulation for 60 minutes at frequencies from 5 Hz up to 40 Hz. But all these attempts were without success (Fig. 8 & Fig. 9). In these experiments the donor BRP exhibited different degree of relaxation to stimulation from almost no relaxation at 0.1 Hz to complete relaxation maintained for the duration of stimulation at high frequencies. This graded response is presumably due to the graded release of neurotransmitter from the donor BRP. The most likely situation in which to detect neurotransmitter release would be while the donor BRP exhibited continuous complete relaxation. Nevertheless even in these circumstances the experiments failed. Possible explanations for this failure were first and most likely that

the half-life of the neurotransmitter is so short that it is all destroyed during diffusion to the bath fluid. Secondly it may reach the bath fluid but is so diluted as to be below the level of detection of the test tissues. The open cascade system (Fig. 5) which greatly reduces this dilution was used to try to overcome this last problem. The flow rates of superfusion varied from 0.5 up to 15 ml. per minute. These gave a wide range of dilutions but still the test tissues failed to detect the neurotransmitter although changes in frequencies and duration were also applied and in spite of donor BRP exhibiting very powerful relaxation to field stimulation.

In this laboratory Gillespie et al. (1988) successfully detected EDRF released from rabbit aortic endothelium by using an improved closed cascade as illustrated in Fig. 6 whereas the system illustrated in Fig. 4 was not successful. Since the response to EDRF and to NANC field stimulation share many properties such as mediation by stimulation of guanylate cyclase and increasing the level of cGMP and block by haemoglobin and methylene blue, a system successful for EDRF might also for NANC neurotransmitter. This was tried but again failed in spite of many variations in the size of the donor BRP, the frequency, the duration of stimulation and the rate of flow. Because Gillespie et al. (1988) successfully detected EDRF released from endothelium in the similar cascade using relatively high flow rates of 4 to 7 ml./min., it is unlikely that flow rates other than those tested would have made it possible to detect the NANC neurotransmitter. That it was not the conditions of the experiment but rather the nature of the NANC neurotransmitter which was the problem was suggested by the successful demonstration of the release of the neurotransmitter noradrenaline by field stimulation of the motor nerves. These results demonstrate the possibility of releasing neurotransmitter from this donor tissue and detecting it with suitable test tissues.

Another possible explanation for failure to detect the NANC neurotransmitter is the presence of a powerful inactivation system in the muscle. This could either be an uptake system or enzymatic destruction. In the detection of noradrenaline the Krebs' solution was always newly made up with deionised distilled water and sometime with addition of EDTA 10^{-4}M. and ascorbic acid 10^{-5}M. While these manoeuvres would very successfully protect noradrenaline from chemical destruction, no special measures were required either to block neuronal uptake or the enzymes MAO and COMT. Since both the chemical nature of the NANC neurotransmitter and its method of inactivation are still unknown, it is difficult to know how to protect against either chemical destruction or biological inactivation. Nevertheless as the NANC response to field stimulation has many properties in common with EDRF which is readily oxidized by the superoxide anion (Gryglewski et al. 1986; Rubanyi & Vanhoutte 1986), the possibility that superoxide dismutase would both potentiate the response to NANC nerve stimulation and made it possible to detect the protected neurotransmitter was investigated but again without success.

In general from all the above results, the most probable reason for the failure to detect the NANC inhibitory neurotransmitter by bioassay is the very short half-life of the neurotransmitter rather than a deficient amount of the neurotransmitter released from the donor BRP. The amount of the neurotransmitter released from the donor BRP at high frequencies of continuous stimulation which produced complete relaxation throughout the stimulation period with no evidence of " escape " should have been suitable for the detection of the neurotransmitter if the neurotransmitter was not rapidly destroyed.

THE EFFECT OF SUPEROXIDE DISMUTASE ON THE NANC INHIBITORY RESPONSE

EDRF is very unstable with a half-life of between 6 seconds (Griffith et al. 1984; Cocks et al. 1985) and 50 seconds (Forstermann 1985). Rubanyi & Vanhoutte (1986) investigated the effects of oxygen-derived free radicals on the production and biological activity of endothelium-derived relaxing factor released by acetylcholine. Rings of canine coronary arteries without endothelium (bioassay rings) were superfused with solution previously passed through a canine femoral artery with endothelium. They found superoxide dismutase caused maximal relaxation of the bioassay ring when injected beyond the donor femoral artery and doubled the half-life of the EDRF. This protective effect of the enzyme was augmented fivefold by lowering the oxygen content of the perfusate from 95 % to 10 % . They believed superoxide anion inactivated the relaxing factor released by acetylcholine from endothelial cells, an effect favoured by hyperoxia. About the same time Gryglewski et al. (1986) reported EDRF released from vascular endothelial cells cultured on microcarrier beads and bioassayed using a cascade of superfused aortic smooth muscle strips was protected from breakdown by superoxide dismutase. They concluded that the superoxide anion contributed to the instability of EDRF. Apps & Harnden (1985) indicated superoxide anion was degraded as follow,

$$2\,O_2^- + 2\,H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$2\,H_2O_2 \xrightarrow{catalase} 2\,H_2O + O_2$$

In spite of EDRF possessing many properties in common with the NANC response to nerve stimulation, superoxide dismutase did not potentiate the inhibitory response to NANC nerve stimulation in the BRP in concentrations from 10 u./ml. up to 400 u./ml. (Fig. 14, Fig. 15 & Fig. 16). It could be however the

neuromuscular junctions of the NANC nerves were very narrow so that the time available for superoxide anion to destroy the neurotransmitter was too short. Superoxide dismutase might still protect the neurotransmitter which was diffusing into the bath fluid and which was to be detected by the test tissues. If this was the case superoxide dismutase should result in the successful detection of the neurotransmitter release. Unfortunately there was neither an inhibitory response detected in the test tissues of the BRP, rat anococcygeus or rabbit aorta nor potentiation of the NANC response in the donor BRP in the presence of superoxide dismutase at concentrations from 100 u./ml. up to 200 u./ml.

As well as representing failure in the first objective of the present research, these results suggest that inactivation of the NANC neurotransmitter is unlikely to be by superoxide anion or heavy metal ions and it is unlikely that EDRF is the NANC inhibitory neurotransmitter in the BRP.

In bioassay experiments at necessary high sensitivity it is easy to produce artefact relaxation of the test tissues (Fig. 17). For example the stimulating current applied to the donor tissue may escape and stimulate inhibitory nerves in the test tissues. Electrodes of a non-reversible type (platinum) may cause electrolysis of the saline with the release of chlorine which is a powerful relaxant. The mechanical movement of the donor tissue if in same bath as the test tissues may be transmitted as a component of the response in the test tissues, especially since the recording sensitivity of the later is very high. Examples of all these were seen during this work. Escape of current will produce a relaxation simultaneous with the response in the donor tissue whereas if it was due to the release of neurotransmitter we might expect a delay between the response in the donor and that in the test tissue. The problem can be avoided by using a test tissue

such as the rabbit aortic strip which does not possess NANC inhibitory nerves though this brings with it the problem of knowing whether its smooth muscle is sensitive to the NANC neurotransmitter. The problem can also be detected by removing the donor tissue but leaving the electrodes and repeating stimulation in the absence of the donor tissue. If the response is due to current escape, this will still take place and the test tissues respond. Reversible Ag/AgCl electrodes will eliminate the problem of electrolysis and a judicious rearrangement of the threads to the transducers can diminish or eliminate the transfer of mechanical movement from the donor to the test tissues. When all of these precautions were taken, no evidence of a humourally transferable neurotransmitter was found.

These experiments were carried out before the results of L-monomethyl-N-arginine (L-NMMA) and its reversal by L-arginine in the rat anococcygeus were known, results which suggest nitric oxide might be the neurotransmitter in the rat anococcygeus but not the BRP. Clearly it would have been better to use the anococcygeus, especially in the experiments with superoxide dismutase. If time had been available this would have been done .

THE EFFECT OF L-ARGININE ON THE NANC INHIBITORY RELAXATION IN THE BRP MUSCLE

The second approach to identify the NANC inhibitory neurotransmitter was to study the effect of some drugs known to potentiate or block endothelium-derived relaxation in the hope of demonstrating some features in common between EDRF and NANC neurotransmitter. These substances were L-arginine, L-monomethyl-N-arginine and L-canavanine.

In the BRP many substances were rejected as the NANC neurotransmitter. These substances are acetylcholine, adrenalin, noradrenaline, isoprenaline, histamine, 5-HT, a prostaglandin or any of the nucleosides, nucleotides or amino acid (Klinge & Sjostrand 1974). Although the NANC inhibitory neurotransmitter is still unknown, many properties of this neurotransmitter have been reported such as its block by haemoglobin and methylene blue, and potentiation by M & B 22948, a selective inhibitor of cyclic GMP phosphodiesterase (Bowman & Drummond 1984; Bowman et al. 1986).

Palmer et al. (1987) reported experimental evidence that the EDRF was almost certainly nitric oxide. They studied the release of EDRF from the endothelial cells in culture and showed not only the ability to detect EDRF by bioassay but that it could also be detected by chemiluminescence technique which was relatively specific for nitric oxide. Furthermore the chemical measurement was sufficient to account for the biological activity i.e. bioassay and chemical assay were in agreement. Nitric oxide itself caused relaxation indistinguishable from that induced by EDRF. Both substances were equally unstable. Bradykinin caused concentration-dependent release of nitric oxide from the cells in amounts sufficient to account for the biological activity of EDRF. The relaxation induced by EDRF and nitric oxide were inhibited by haemoglobin and enhanced by superoxide dismutase to a similar degree. They believed EDRF and nitric oxide were identical. In a more recent report (Palmer et al. 1988a) they identified L-arginine as the precursor of the nitric oxide. The evidence for this was that the release of nitric oxide from the endothelial cells cultured in the absence of L-arginine by bradykinin or the calcium ionophore A23187 was reversibly enhanced by infusions of L-arginine but not by its stereo-isomer D-arginine. Mass spectrometry studies using ^{15}N-labelled L-arginine indicated this

enhancement was due to the formation of nitric oxide from the terminal guanidino nitrogen atom(s) of L-arginine. The strict substrate specificity of this reaction suggests that L-arginine is the precursor for nitric oxide synthesis in vascular endothelial cells.

Since the NANC inhibitory neurotransmitter like EDRF owes its relaxant action to stimulation of soluble guanylate cyclase and the consequent elevation of cyclic GMP (Diamond & Chu 1983; Furchgott & Jothianandon 1983; Rapoport & Muir 1983a; 1983b) and both actions are blocked by methylene blue and haemoglobin and potentiated by M & B 22948 (Gruetter et al. 1981a; 1981b; Bowman & Gillespie 1981; Martin et al. 1985; 1986), it seemed worthwhile to test the effect of L-arginine on the NANC inhibitory response.

In the fresh BRP L-arginine had no effect on the NANC inhibitory neurotransmission (Fig. 20 & Fig. 22) although concentrations of L-arginine up to 3×10^{-4}M. were used. These concentrations compare with an EC_{50} of 3.5 +/- 0.1 μM. for potentiation of the release of EDRF from vascular endothelial cells (Palmer et al. 1988a). It was possible, indeed likely, that the fresh BRP tissue contained sufficient stores of arginine to support many response to nerve stimulation especially using short trains of 20 pulses. In the experiments with cultured endothelial cells reported by Palmer et al. (1988a) the cells were washed three times and cultured in fresh culture medium without L-arginine for a further 24 hours. If the cells were cultured in a medium containing L-arginine, there was no enhancement on the nitric oxide release in 11 out 16 experiments. Nevertheless attempts to deplete the BRP of arginine either by long stimulation trains or by storing in arginine-free Tyrodes for up to ten days were ineffective in demonstrating a potentiation by L-arginine of the response even though by the sixth

day of storage this was significantly smaller than the response in fresh controls. There are various explanations for this failure. The obvious one and the one accepted at the time was that arginine was not a precursor of the neurotransmitter. This still remains a likely possibility. The later finding with L-NMMA in the rat anococcygeus suggested quite strongly that it was a precursor in that tissue and the two preparations are so comparable that it is unappealing to believe they use different neurotransmitter. Alternative possibilities are that none of the procedures aimed at lowering arginine level were sufficiently effective to lower it below the level for neurotransmitter synthesis or that in the BRP L-arginine is not the sole precursor of nitric oxide. In favour of either of these hypotheses was the observation that in cold stored preparations L-arginine did often shift the dose-response curve to the left i.e. potentiate though the degree of shift was not statistically significant (Fig. 35 to Fig. 38). Certainly the progressive decline in response to NANC nerve stimulation in cold stored tissue after the fifth day was not due to a fall in postsynaptic sensitivity to nitric oxide since the response to sodium nitrite was well maintained (Fig. 40).

In ability of the BRP muscle to maintain complete relaxation over long periods of stimulation without any exhaustion is consistent with its role in reproduction. In animals with a sheathed and retracted penis relaxation of the retractor penis is essential as well as engorgement of the erectile tissue. Also in these experiments many preparations produced about 80 -90 % relaxation of the raised tone to only one pulse of field stimulation further supporting the importance of the relaxation of the retractor penis in the productive function and the high capability of BRP to produce relaxation. When the stimulation of the BRP was stopped, the rapid restoration of the BRP original tone (Fig. 24) might be also very important for the reproductive function because the sudden withdraw of the penis by the

contraction of retractor penis is important to protect the penis from damage after ejaculation. These might be the special properties of retractor penis and its NANC innervation.

THE EFFECT OF L-NMMA AND L-CANAVANINE ON THE NANC INHIBITORY NEUROTRANSMISSION IN THE BRP AND THE RAT ANOCOCCYGEUS MUSCLE

The evidence for L-arginine as the precursor of nitric oxide in endothelial cells has been given. Macrophages are other cells which also possess the ability to synthesize nitric oxide which is converted to nitrite and nitrate (Iyengar et al. 1987). This biosynthesis is L-arginine-dependent and the nitrogen of the nitric oxide like the nitrogen of EDRF is derived from the terminal guanidino nitrogens of L-arginine. No other amino acid nor ammonia could substitute for L-arginine except L-arginyl-L-aspartate. L-Canavanine (2 mM.), a guanidinooxy structural analogue of arginine which inhibits the various L-arginine-utilizing enzymes, inhibited the formation of these NO-containing compounds when the macrophage was preincubated with L-canavanine for 12 hours.

If L-arginine is enzymatically converted to nitric oxide then inhibition of this conversion should block the formation and therefore the response. Two substances related chemically to arginine have been reported to do this. The first is L-monomethyl-N-arginine (L-NMMA). Moncada and his group (Palmer et al. 1988b) have reported that L-NMMA can inhibit the release of nitric oxide by bradykinin in cultured endothelial cells and the response to EDRF released by

acetylcholine in rabbit aortic strip preparations. These effects are not showed by its D-isomer and are therefore stereospecific and the inhibition by L-NMMA can be competitively reversed by L-arginine i.e. it is an example of substrate competition (Palmer et al. 1988b). In the present experiments we have confirmed this stereospecific inhibition of the EDRF response in rabbit aortic strips together with its reversion by L-arginine (Fig. 54). The second substance is L-canavanine. In 1988 Schmidt et al. (1988a; 1988b) also reported the attenuation of the endothelium-dependent relaxation of rat thoracic aorta rings induced by ATP and acetylcholine after the rings were pretreated with L-canavanine (2 mM.) for two hours. ATP dose-dependently stimulated the release of nitric oxide as detected by chemiluminescence. L-Canavanine abolished both basal and ATP-induced nitric oxide release.

If nitric oxide is the neurotransmitter of the NANC nerves and its origin is a similar enzymatic synthesis from L-arginine then L-NMMA and L-canavanine should inhibit the response. This was tested first in the BRP and in concentrations up to 3×10^{-4}M. did not inhibit the NANC inhibitory relaxation (Fig. 42 & Fig. 43). Later when its effectiveness in inhibiting the response in the rat anococcygeus was demonstrated the BRP was reexamined with higher concentration of 10^{-3}M. L-NMMA and some inhibition of the response and a rise in the tone were observed (Fig. 43 & Fig. 44). L-Canavanine gave similar results i.e. no inhibitory effect up to 3×10^{-4}M. (Fig. 55 & Fig. 56) but some reduction in the response at 10^{-3}M. which was partially reversed by L-arginine (Fig. 57 & Fig. 58). These results are difficult to interpret. In the absence of a positive result in the rat anococcygeus by L-canavanine, the high concentrations of both L-NMMA and L-canavanine needed and the small inhibition observed in the BRP muscle might suggest the effects could be ignored as non-specific. Reinforcing this is the

observation that the evidence for L-arginine involvement is most convincing in the anococcygeus where L-NMMA is a most effective blocking drug yet in that tissue L-canavanine is without effect. The similar efficiency of both drugs in the BRP at such high concentrations suggests a non-specific effect. Against that is the ability of L-arginine to produce some reversal of inhibition. It may be that in the BRP the enzymes involved in synthesis differ from those in the anococcygeus and are more resistant to inhibition and alternatively there is more than one precursor and L-arginine plays only a part in the synthesis.

The effect of L-NMMA on the rat anococcygeus was dramatic. In a dose related fashion L-NMMA both raised the guanethidine-induced tone (Fig. 47; Fig. 48 & Fig. 50) and at the same level inhibited the NANC inhibitory relaxation to field stimulation (Fig. 45 & Fig. 46). These effects were easily reversed by washing out the L-NMMA (Fig. 47) or by L-arginine dose-dependently (Fig. 50 & Fig. 51) and prevented by L-arginine added with L-NMMA simultaneously (Fig. 52 & Fig. 53). The D-isomer of L-NMMA was without effects on either tone or the response to NANC stimulation. Since the postsynaptic sensitivity to nitric oxide was unaffected by L-NMMA, these results suggest the NANC inhibitory neurotransmitter is synthesized from L-arginine by L-arginine-utilizing enzymes and L-arginine is a major physiological precursor of the NANC inhibitory neurotransmitter in this muscle.

Given the effectiveness of L-NMMA, it was surprising to find that L-Canavanine was without effect on either tone or the NANC response in the rat anococcygeus in doses even higher than those inhibiting the BRP. This difference may be due to either a tissue difference or a species difference if one assures that the inhibitory effect of high dose (10^{-3}M.) of L-canavanine on the BRP is selective. It

may however be that L-canavanine is effective on the enzymes responsible for nitric oxide synthesis in macrophages but is not effective on the enzymes of either the endothelial cells or the NANC nerves. This is in spite of the reports by Schmidt et al. (1988a; 1988b) that L-canavanine does significantly inhibit endothelium-dependent relaxation in rat aortic rings, in my own experiments L-canavanine in concentrations up to 2×10^{-3}M. for one hour had no effect on the response to EDRF released by acetylcholine in the rabbit aortic ring. If this is so then the only results which have a significance bearing on NANC neurotransmission are those with L-NMMA inhibiting the response in the rat anococcygeus. The inhibition at high concentrations of L-NMMA in the BRP may not be specific. The explanation for the difference between the two muscles is not known.

It is unlikely that the failure of L-canavanine to inhibit the NANC response in the rat anococcygeus is due to a species difference since the report that L-canavanine inhibited endothelium-dependent relaxation in aortic rings was in the same species, the rat. But in our experiments L-canavanine had no effect on the endothelium-dependent relaxation induced by acetylcholine at concentrations from 3×10^{-5}M. up to 2×10^{-3}M. for one hour's pretreatment on the rabbit aortic strips (unreported). The very clear differences between the BRP and the rat anococcygeus in their sensitivity to L-NMMA could have several explanations. First the inhibitory neurotransmitter in both is the same, this explains the similar sensitivity to block by haemoglobin, but the precausor is not the same, only in the anococcygeus is L-arginine as essential precursor. Secondly the NANC inhibitory neurotransmitter and its precursor may be the same in both muscles but the enzymes which synthesize the neurotransmitter are not identical and have different sensitivities to L-NMMA and L-canavanine. Thirdly the NANC

inhibitory neurotransmitter might be different in the two muscles. This third hypothesis is unattractive since so many properties of NANC inhibitory neurotransmission are identical in the two tissues, their anatomical origins and function so similar and the effects of drugs such as haemoglobin and methylene blue on the NANC response identical. Resolution of the problem will require further work for example isolation of the enzymes responsible in the two tissues or perhaps study of nitrite formation from ^{15}N labelled precursors during nerve stimulation.

CONCLUSIONS

1. It has proved impossible to detect NANC inhibitory neurotransmitter in the BRP by bioassay.

2. L-Arginine does not potentiate the NANC nerve response in the fresh BRP tissues. Attempts to deplete endogenous arginine by either prolonged nerve stimulation or cold storage in an arginine free saline and uncover a potentiating effect of L-arginine were unsuccessful.

3. L-monomethyl-N-arginine (L-NMMA) inhibits the NANC response in the rat anococcygeus in a dose related fashion between 10^{-5}M. and 3×10^{-4}M. At these doses L-NMMA has no effect on the BRP. The effect on the anococcygeus is stereospecific and competitively reversed by L-arginine. These results suggest L-arginine is the main precursor of the NANC inhibitory neurotransmitter in the rat anococcygeus but not in the BRP.

4. L-Canavanine has no effect on either muscle in concentrations up to 3×10^{-4}M. At higher concentration of 10^{-3}M. it produces some inhibition of the NANC response in the BRP but not in the rat anococcygeus. The effect of L-canavanine may be non-specific but if not then the enzymes involved in synthesis of nitric oxide in the two tissues may be different.

5. The results though indirect favour nitric oxide on a substrate capable of liberating nitric oxide as neurotransmitter of the NANC nerves in the rat anococcygeus.

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THE EFFECT OF ARGININE AND L-N MONOMETHYL ARGININE ON THE RESPONSE OF THE BOVINE RETRACTOR PENIS TO STIMULATION OF ITS NANC NERVES

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Evidence suggests the EDRF is nitric oxide (NO) (Palmer et al., 1987). More recently the same group have suggested its' precursor is the guanidino group of arginine (Palmer et al., 1988). This last suggestion is based on the ability of arginine to increase the release of NO from cultured endothelial cells by bradykinin as measured both chemically and by bioassay. An even more recent reprint (Garthwaite et al., 1988) suggests cerebellar nerve cells may release EDRF through activation of N-methyl-D-aspartate receptors.

In the bovine retractor penis (BRP) the response to EDRF, to NO and to a smooth muscle inhibitory factor extracted from the tissue are similar (Gillespie and Sheng, 1988) and the response to NANC nerve stimulation and to EDRF have much in common (Gillespie, 1987) suggesting NO might be the neurotransmitter in these nerves. We have therefore tested the ability of arginine to increase and L-N monomethyl arginine (LNMMA) to block the response to field stimulation of the NANC nerves.

BRP's from the abattoir were cleaned and small strips 2 cm by approximately 2 mm in breadth removed and mounted in ring electrodes in 10 ml baths containing Krebs saline at 37 degrees centigrade and gassed with a mixture of 95% O_2 + 5% CO_2. Motor adrenergic nerves were blocked and tone raised by the addition of guanethidine 10^{-5}M. Frequency-response curves in response to field stimulation of NANC nerves were then constructed before the addition and in the presence of L arginine. Arginine in concentrations up to 3 x 10^{-4}M did not potentiate the response. Attempts were made to exhaust transmitter stores by long trains of stimulation before adding arginine. There was still no potentiation. Finally, in an attempt to deplete endogenous arginine preparations were kept in sterile Tyrodes' solution at 4 degrees centigrade for periods of up to ten days. The solution was changed daily to remove any arginine which might have leaked from the tissue. The responses of groups of tissue were studied at daily intervals from 1-10 days. Arginine did not potentiate responses at any time.

The effect of the blocking drug LNMMA was examined. It failed to inhibit the response to NANC nerve stimulation. As a control the effect of LMNA was examined on the response of fresh rabbit aortic strips to EDRF released by acetylcholine. LNMMA efficiently inhibited this response.

These results suggest arginine is not the precursor of the NANC neurotransmitter in the BRP. They do not however apply to the rat anococcygeus where LNMMA selectively inhibits NANC relaxation.

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