Actions and interactions of N^G-substituted analogues of L-arginine on NANC neurotransmission in the bovine retractor penis and rat anococcygeus muscles

¹William Martin, John S. Gillespie & Ian F. Gibson

Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ

1 The effects and interactions of a series of N^G-substituted analogues of L-arginine known to inhibit nitric oxide synthase were examined on non-adrenergic, non-cholinergic (NANC) neurotransmission in the bovine retractor penis (BRP) and rat anococcygeus muscles.

2 Treatment of BRP muscle strips with either N^G-nitro L-arginine (L-NOARG; 0.1–10 μ M) or N^G-nitro L-arginine methyl ester (L-NAME; 0.1–100 μ M) produced a concentration-dependent blockade of NANC relaxation: blockade was complete at the highest concentration of each.

3 Pretreatment with L-arginine (1–10 mM) had no effect on NANC relaxation by itself, but inhibited, in a concentration-dependent manner, the subsequent ability of both L-NOARG (0.1–300 μ M) and L-NAME (0.1–1 mM) to produce blockade. L-Arginine (1–10 mM) reversed established submaximal blockade of NANC relaxation induced by L-NOARG (1 μ M) or L-NAME (1 μ M), but had little effect on maximal blockade induced by these agents.

4 In contrast to L-NOARG and L-NAME, N^G-monomethyl L-arginine (L-NMMA; 1 μ M–1 mM) had no effect by itself on NANC relaxation of the BRP. L-NMMA (0.1–1 mM) did, however, like L-arginine, inhibit, in a concentration-dependent manner, the subsequent ability of both L-NOARG (0.1–1 mM) and L-NAME (0.1–3 mM) to produce blockade, but was more potent. As with L-arginine, L-NMMA (0.1–1 mM) reversed established submaximal blockade of NANC relaxation induced by L-NOARG (1 μ M) or L-NAME (1 μ M), but had little effect on maximal blockade induced by these agents.

5 In contrast to the effects on BRP, treatment of rat anococcygeus muscle with either L-NOARG (0.1–10 μ M) or L-NMMA (1–100 μ M) produced concentration-dependent inhibition of NANC relaxation: the maximal inhibition induced by L-NOARG and L-NMMA was 100% and $40.1 \pm 5.9\%$ ($n = 8$), respectively. L-Arginine (1–10 mM) reversed established submaximal inhibition of NANC relaxation induced by L-NOARG (1 μ M), had little effect on maximal blockade by this agent, and reversed maximal blockade induced by L-NMMA (100 μ M).

6 In the presence of partial blockade of NANC relaxation on rat anococcygeus by a maximal concentration of L-NMMA (100 μ M), subsequent blockade by L-NOARG (0.1–100 μ M) was inhibited. L-NMMA (100 μ M) produced a partial reversal of established submaximal blockade of NANC relaxation induced by L-NOARG (1 μ M), but had little effect on maximal blockade induced by this agent.

7 These findings suggest a complex series of interactions between L-arginine and certain of its N^G-substituted analogues that are commonly used to inhibit nitric oxide synthase. The most striking new finding is that L-NMMA does not block NANC relaxation in the BRP, but acts with greater potency than the endogenous substrate, L-arginine, to inhibit the blockade induced by L-NOARG or L-NAME. Even on rat anococcygeus where L-NMMA acts as a partial blocker of NANC relaxation, further blockade by L-NOARG is inhibited.

Keywords: Anococcygeus muscle; retractor penis muscle; NANC nerves; nitric oxide; L-arginine; N^G-monomethyl L-arginine; N^G-nitro L-arginine; N^G-nitro L-arginine methyl ester.

Introduction

There is increasing evidence that the L-arginine-nitric oxide pathway mediates neurotransmission by certain non-adrenergic, non-cholinergic (NANC) nerves in the peripheral autonomic nervous system (Gillespie *et al.*, 1989; Li & Rand, 1989), as well as neurotransmission or neuromodulation in the central nervous system (Garthwaite *et al.*, 1989; Bredt *et al.*, 1990). Early evidence of a link between the L-arginine-nitric oxide system and NANC nerves came from experiments on the rat and mouse anococcygeus and bovine retractor penis (BRP) muscles in which haemoglobin, methylene blue, free radical generators and hypoxia, which inhibit the formation or actions of endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980; Martin *et al.*, 1985; Moncada *et al.*, 1986), were shown to inhibit neurogenic

relaxation (Bowman *et al.*, 1982; Bowman & McGrath, 1985; Bowman *et al.*, 1986; Gibson & Mirzazadeh, 1989; Gillespie & Sheng, 1990). Furthermore, NANC transmission, like relaxation mediated by EDRF (Rapoport & Murad, 1983; Griffith *et al.*, 1985; Martin *et al.*, 1986), was shown to proceed through elevation of cellular guanosine 3':5'-cyclic monophosphate (cyclic GMP) content and was potentiated by inhibitors of cyclic GMP phosphodiesterase (Bowman & Drummond, 1984). The most compelling evidence linking nitric oxide to NANC transmission was obtained, however, when N^G-monomethyl L-arginine (L-NMMA), an inhibitor of nitric oxide synthase (Rees *et al.*, 1989), was shown to produce a partial inhibition of neurogenic relaxation in the anococcygeus of the rat and mouse (Gillespie *et al.*, 1989; Li & Rand, 1989; Gibson *et al.*, 1990). The inhibition was stereospecific and competitive in nature, i.e., the D-isomer was inactive, and blockade was reversed by L-, but not D-arginine; it therefore exhibited a profile similar to that for

¹ Author for correspondence.

inhibition of EDRF synthesis (Rees *et al.*, 1989). N^G -nitro L-arginine (L-NOARG) and its methyl ester (L-NAME) were subsequently shown to be more potent inhibitors of NANC relaxation than L-NMMA in the anococcygeus of the rat and mouse, and could abolish relaxation (Gibson *et al.*, 1990; Hobbs & Gibson, 1990; Liu *et al.*, 1991). Following these earlier studies, inhibitors of nitric oxide synthase were shown to inhibit certain forms of NANC inhibitory transmission in the gut (Bult *et al.*, 1990; Dalziel *et al.*, 1991; Desai *et al.*, 1991), respiratory tract (Tucker *et al.*, 1990; Li & Rand, 1991), reproductive system (Ignarro *et al.*, 1990; Liu *et al.*, 1991) and cerebral (Toda & Okamura, 1990) and penile arteries (Liu *et al.*, 1991). Nitric oxide synthase has recently been identified in homogenates of BRP and rat anococcygeus muscle (Mitchell *et al.*, 1991; Sheng *et al.*, 1991; 1992) and consists of two fractions; a soluble form similar to that isolated from brain (Bredt & Snyder, 1990; Schmidt *et al.*, 1991) and a particulate form, both of which are activated by calcium, and like NANC relaxation, are blocked by L-NOARG. Furthermore, a nitric oxide-like substance has been detected in the superfusion fluid following stimulation of NANC nerves in the isolated ileocolonic sphincter of the dog (Bult *et al.*, 1990).

Although NANC relaxation of the BRP and bovine penile artery is abolished by L-NOARG (Liu *et al.*, 1991), suggesting involvement of the L-arginine-nitric oxide pathway, we have surprisingly found that relaxation is completely unaffected by another inhibitor of nitric oxide synthase, L-NMMA. In this paper we show that L-NMMA, like the endogenous precursor, L-arginine, inhibits the ability of L-NOARG and L-NAME to block NANC relaxation, but is considerably more potent.

Methods

Preparation of tissues

BRP muscles were obtained from a local abattoir and transported to the laboratory. Some tissues were used that day,

but others were stored at 4°C in Krebs solution for use the following day. BRP muscle strips 2–3 mm wide and 1 cm long were cut for tension recording. Rat anococcygeus muscles were dissected out and freed of connective tissue as previously described (Gillespie, 1972). BRP and anococcygeus muscle strips were mounted under 2 g and 1 g resting tension, respectively, within Ag-AgCl ring electrodes in 12 ml organ chambers and bathed at 37°C in Krebs solution of the following composition (mM): NaCl 118, KCl 4.8, CaCl_2 2.5, MgSO_4 1.2, KH_2PO_4 1.2, NaHCO_3 24, glucose 11 and gassed with 95% O_2 and 5% CO_2 . Tension was measured with Grass FTO3C isometric transducers and displayed on a Grass Polygraph. In all experiments, adrenergic motor responses were blocked and the tone raised on BRP and anococcygeus muscle strips by use of guanethidine (30 μM). Electrical field stimulation was delivered at a pulse width of 0.5 ms and at supramaximal voltage.

Experimental protocols

Two separate protocols were adopted to examine the effects of L-arginine and L-NMMA on the ability of L-NOARG and L-NAME to block NANC relaxation. In the first, tissues were pretreated with L-arginine or L-NMMA to determine if this inhibited the subsequent blockade of NANC relaxation induced by L-NOARG or L-NAME. In the second, blockade of NANC relaxation was first established with L-NOARG or L-NAME and the subsequent ability of L-arginine or L-NMMA to reverse blockade was determined.

Drugs

L-Arginine hydrochloride, N^G -nitro L-arginine (L-NOARG) and N^G -nitro L-arginine methyl ester hydrochloride (L-NAME) were purchased from Sigma. N^G -monomethyl L-arginine and N^G -monomethyl D-arginine acetate were generously provided by Wellcome Laboratories, Beckenham. Guanethidine sulphate was purchased from CIBA. All drugs were dissolved in saline (0.9%).

Figure 1 In the presence of guanethidine (30 μM)-induced tone, electrical field stimulation of strips of bovine retractor penis muscle at 10 Hz (100 pulses, 0.5 ms, supramaximal voltage) elicited a powerful non-adrenergic, non-cholinergic (NANC) relaxation. This relaxation was blocked in a concentration-dependent manner by N^G -nitro L-arginine (L-NOARG, 0.3–10 μM). Neither L-arginine (L-Arg, 1 mM) nor N^G -monomethyl L-arginine (L-NMMA, 100 μM) had any effect by itself on NANC relaxation, but both inhibited the ability of L-NOARG to block relaxation. All final molar concentrations are given in log units.

Results

Effects of L-NMMA, L-NOARG and L-NAME on the bovine retractor penis muscle

On strips of BRP muscle in the presence of guanethidine (30 μ M)-induced tone, electrical field stimulation induced frequency (0.1–20 Hz)-dependent NANC relaxation. Treatment with L-NOARG (0.1–10 μ M) or L-NAME (0.1–100 μ M) produced a concentration-dependent blockade of NANC relaxation at all frequencies and complete abolition was obtained with the highest concentration of each. In contrast, L-NMMA (1 μ M–1 mM) had no effect on NANC relaxation at any frequency. Figures 1–5 show the effects of these treatments on responses obtained following stimulation at 10 Hz (100 pulses).

Pretreatment with L-arginine (1–10 mM) had no effect by itself on NANC relaxation (Figure 1), but produced a concentration-dependent inhibition of the blockade induced by subsequent treatment with L-NOARG (0.1–300 μ M; Figures 1 and 3a) or L-NAME (0.1–1 mM; data not shown). L-NMMA, but not D-NMMA, was also able to inhibit the blockade induced by subsequent treatment with L-NOARG (0.1–300 μ M; Figures 1 and 3b) or L-NAME (0.1–3 mM; Figure 4), but was more potent than L-arginine: a concentration-dependent inhibition of the effects of L-NOARG was seen with L-NMMA over the range 100–300 μ M, but concentrations of 1 mM or greater had no additional effect, whereas, the effects of L-NAME were blocked concentration-dependently by L-NMMA over the range 0.1–1 mM. In a second series of experiments, attempts were made to reverse L-NOARG- and L-NAME-induced blockade of NANC relaxation by subsequent addition of L-arginine or L-NMMA. Both L-arginine (1–10 mM) and L-NMMA (0.1–1 mM) reversed established submaximal inhibition of NANC relaxation induced by L-NOARG (1 μ M) or L-NAME (1 μ M), but had little effect on maximal inhibition by these agents (data not shown).

Effects of L-NMMA and L-NOARG on the rat anococcygeus muscle

In keeping with our previous findings (Liu *et al.*, 1991) on the rat anococcygeus muscle in the presence of guanethidine

(30 μ M)-induced tone, L-NOARG (0.1–10 μ M) and L-NMMA (1–100 μ M) each produced a concentration-dependent blockade of the NANC relaxation induced by electrical field stimulation: at 10 Hz (100 pulses) L-NOARG produced complete blockade (Figure 5), whereas the maximum blockade induced by L-NMMA was $40.1 \pm 5.9\%$ ($n = 8$). L-Arginine (1–10 mM) reversed established submaximal blockade of NANC relaxation induced by L-NOARG (1 μ M), had little effect on maximal blockade by this agent, and reversed maximal blockade induced by L-NMMA (100 μ M) (data not shown). Under conditions where the NANC response was partially blocked by a maximal concentration of L-NMMA (100 μ M), protection was seen against the inhibitory actions of L-NOARG (0.1–100 μ M; Figure 5). L-NMMA (100 μ M) was able to produce a partial reversal of established submaximal blockade of NANC relaxation induced by L-NOARG (1 μ M), but had little effect on maximal blockade induced by L-NOARG (10–30 μ M) (data not shown).

Discussion

With the advent of compounds that inhibit the enzyme nitric oxide synthase (Rees *et al.*, 1989; 1990), the concept of the L-arginine-nitric oxide pathway mediating neurotransmission in the peripheral autonomic nervous system (Gillespie *et al.*, 1989; Li & Rand, 1989; Gibson *et al.*, 1990) as well as neurotransmission or neuromodulation in the central nervous system (Garthwaite *et al.*, 1988; 1989; Knowles *et al.*, 1989; Bredt *et al.*, 1990) has become widely accepted. In the autonomic nervous system L-NMMA, L-NOARG or L-NAME inhibit NANC relaxation of smooth muscle in a diverse array of tissues such as rat and mouse anococcygeus (Gillespie *et al.*, 1989; Li & Rand, 1989; Gibson *et al.*, 1990), dog and opossum lower oesophageal sphincter (De Man *et al.*, 1991; Tottrup *et al.*, 1991), guinea-pig and rat gastric fundus (Desai *et al.*, 1991; Boeckstaens *et al.*, 1991; Lefebvre *et al.*, 1992), human ileum (Maggi *et al.*, 1991), dog ileocolonic sphincter (Bult *et al.*, 1990) and proximal colon (Dalziel *et al.*, 1991), guinea-pig trachea (Tucker *et al.*, 1990; Li & Rand, 1991), rabbit and human corpus cavernosum (Ignarro *et al.*, 1990; Pickard *et al.*, 1991), and dog cerebral arteries (Toda & Okamura, 1990).

The NANC relaxant responses of the BRP and bovine

Figure 2 The non-adrenergic, non-cholinergic (NANC) relaxant response of strips of bovine retractor penis muscle obtained following electrical field stimulation at 10 Hz (100 pulses, 0.5 ms, supramaximal voltage) in the presence of guanethidine (30 μ M)-induced tone was blocked in a concentration-dependent manner by N^G-nitro L-arginine methyl ester (L-NAME, 0.3–30 μ M). Pretreatment with N^G-monomethyl L-arginine (L-NMMA, 100 μ M) had no effect by itself on NANC relaxation, but inhibited the ability of L-NAME to block relaxation. All final molar concentrations are given in log units.

Figure 3 N^G-nitro L-arginine (L-NOARG, 1–30 μM, O) produced a concentration-dependent blockade of the non-adrenergic, non-cholinergic (NANC) relaxant response of strips of bovine retractor penis muscle obtained upon electrical field stimulation at 10 Hz (100 pulses, 0.5 ms, supramaximal voltage). Pretreatment with L-arginine (a) at 1 mM (●) and 10 mM (▼) or with N^G-monomethyl L-arginine (b) at 100 μM (●), 300 μM (▼) and 1 mM (■) inhibited the ability of L-NOARG to block NANC relaxation. Points are the mean, and vertical bars indicate the s.e.mean of 6–12 observations.

penile artery (Liu *et al.*, 1991) are also certain to be mediated by the L-arginine-nitric oxide pathway since they are abolished by L-NOARG. Surprisingly, however, we have found that L-NMMA does not inhibit NANC relaxation in these tissues. This result is all the more surprising since nitric oxide synthase activity isolated from the BRP is reported to be sensitive to inhibition by L-NMMA (Sheng *et al.*, 1991; 1992). Furthermore, in the rat and mouse anococcygeus, where L-NOARG and L-NAME each abolish NANC relaxation, L-NMMA is only partially effective, blocking relaxation by no more than 50% (Gillespie *et al.*, 1989; Gibson *et al.*, 1990).

The major new observation made in this study is that, in the BRP, L-NMMA behaves similarly to the endogenous precursor of nitric oxide synthase, L-arginine, and protects NANC relaxation against the blocking actions of both L-NOARG and L-NAME, but is considerably more potent. The protection afforded by L-NMMA or L-arginine was concentration-dependent; this was seen with all concentrations of L-NMMA (0.1–1 mM) tested against L-NAME, but

Figure 4 N^G-nitro L-arginine methyl ester (L-NAME, 0.1–100 μM, O) produced a concentration-dependent blockade of the non-adrenergic, non-cholinergic (NANC) relaxant response of strips of bovine retractor penis muscle obtained upon electrical field stimulation at 10 Hz (100 pulses, 0.5 ms, supramaximal voltage). Furthermore, pretreatment with N^G-monomethyl L-arginine at 100 μM (●), 300 μM (▼) and 1 mM (■) inhibited the ability of L-NAME to block NANC relaxation. Points are the mean, and vertical bars indicate the s.e.mean, of 6–12 observations.

Figure 5 N^G-nitro L-arginine (L-NOARG, 0.1–30 μM, ●) produced a concentration-dependent blockade of the non-adrenergic, non-cholinergic (NANC) relaxant response of rat anococcygeus muscle obtained upon electrical field stimulation at 10 Hz (100 pulses, 0.5 ms, supramaximal voltage). Furthermore, pretreatment with N^G-monomethyl L-arginine at 100 μM (▼) inhibited the ability of L-NOARG to block NANC relaxation. Points are the mean, and vertical bars indicate the s.e.mean, of 6 observations.

against L-NOARG the concentration-dependence of blockade was seen only with L-NMMA over the range 100–300 μM, with higher concentrations producing no further effect. Why L-NMMA exhibits only a partial concentration-dependent inhibition of the action of L-NOARG is not clear, but could result from a limitation on access or metabolism of L-NMMA. Nevertheless, the data with both L-NMMA and L-arginine suggest the interactions with L-NOARG and L-NAME to be competitive in nature. Despite this, we found

that neither L-NMMA nor L-arginine was able to reverse convincingly blockade induced by L-NOARG or L-NAME, unless the degree of blockade was substantially sub-maximal. Others working on the vasculature and central nervous system have reported that the blocking actions of L-NOARG cannot be reversed by L-arginine (Unmans, 1990; Dwyer *et al.*, 1991) or are only partially reversible (Steinberg *et al.*, 1990). Randall & Griffith (1991) have shown that the ability of L-NAME to inhibit basal release of EDRF in the rabbit isolated perfused ear is reversed by L-arginine, but inhibition of agonist-induced release is not. When taken together, these findings from the different laboratories suggest that competition does occur, but the rate of dissociation of L-NOARG or L-NAME from nitric oxide synthase is so slow as to give the impression of irreversibility in the normal time course of experiments.

The mechanism by which L-NMMA protects against the blocking actions of L-NOARG and L-NAME on NANC relaxation of BRP is not clear, but certain potential explanations can be excluded. One of these is that the protective effect of L-NMMA results from conversion to L-arginine. It has been shown previously that a deiminase enzyme in bovine aortic endothelial cells can convert L-NMMA, but not L-NOARG, to L-citrulline, which can be metabolized further to L-arginine (Hecker *et al.*, 1990). Alternatively, L-NMMA could potentially be metabolized by a demethylase enzyme directly to L-arginine. Neither of these is possible, however, since L-NMMA is more potent than L-arginine in protecting against the blockade by L-NOARG. It is equally unlikely that L-NMMA acts as an alternative substrate to L-arginine in the formation of nitric oxide by the neuronal nitric oxide synthase, since it partially blocks NANC transmission in the rat anococcygeus. Another possible explanation is that L-NMMA blocks the entry of L-NOARG into the NANC nerves. Again, this is unlikely, since previous work on bovine aortic endothelial cells showing the ability of L-NMMA, but not L-NOARG, to block competitively uptake of L-arginine by the y^+ cationic amino acid transporter (Bogle *et al.*, 1992), suggests separate uptake mechanisms for the two N^G -

substituted analogues. Furthermore, L-NMMA also inhibits the blocking action of L-NAME, a lipophilic ester, which is likely to enter cells by simple diffusion rather than by a carrier-mediated process. Our favoured, but speculative explanation is that there is a complex interaction between L-NMMA, L-NOARG and the endogenous precursor, L-arginine, at the level of the neuronal nitric oxide synthase. In this scheme, L-NMMA behaves as a partial inhibitor of nitric oxide synthase, in a manner similar to the actions of a partial agonist at a drug receptor, and thereby produces a variable degree of blockade by itself (low in BRP but around 50% in rat anococcygeus), but inhibits subsequent blockade by full inhibitors such as L-NOARG and L-NAME. For this to occur, L-NMMA would be required to have a higher affinity as well as a faster rate of dissociation from nitric oxide synthase than L-NOARG or L-NAME and so its presence would provide greater potential for the substrate, L-arginine, to interact with the enzyme than would be possible in the presence of L-NOARG or L-NAME alone. A definitive test of this hypothesis will require a detailed study of the interactions between L-NMMA, L-arginine and L-NOARG or L-NAME on nitric oxide synthase isolated from the NANC nerves, and this is beyond the scope of the present investigation.

In summary, on the BRP where NANC inhibitory neurotransmission is mediated by the L-arginine-nitric oxide pathway, we have found that L-NMMA, a putative inhibitor of nitric oxide synthase, does not block relaxation. Surprisingly, L-NMMA behaves in a similar manner to the endogenous precursor, L-arginine, and blocks competitively the ability of two other inhibitors of nitric oxide synthase, namely L-NOARG and L-NAME, to block NANC relaxation. On the basis of these findings it would seem unwise to use L-NMMA as a sole tool for investigating the role of the L-arginine-nitric oxide pathway in physiological or pathological processes.

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