Br. J. Pharmacol. (1990), **100**, 749–752

L-N^G-nitro-arginine and its methyl ester are potent inhibitors of non-adrenergic, non-cholinergic transmission in the rat anococcygeus

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1 The effects of L-N^G-nitro-arginine (L-NOARG) and some other arginine analogues on non-adrenergic, non-cholinergic (NANC) relaxations of the rat anococcygeus muscle were investigated.

2 L-NOARG (5–200 μM) produced concentration-related inhibition of the NANC response; 100 μM L-NOARG produced 90% inhibition.

3 L-Arginine (5–200 μM) produced a concentration-related reversal of the inhibitory effect of 20 μM L-NOARG; a five fold excess of L-arginine (100 μM) was required to obtain the maximum reversal of 90%. D-Arginine (100 μM) produced no such reversal, but significant reversal was produced by L-citrulline, L-arginine-L-aspartate, L-homoarginine and L-arginine-methyl-ester (all at 100 μM).

4 L-N^G-nitro-arginine-methyl-ester (L-NAME; 5–200 μM) also reduced NANC relaxations, with a potency similar to that of L-NOARG; both L-NOARG and L-NAME were some ten times more potent than L-N^G-monomethyl-arginine (L-NMMA). Like L-NOARG, the effects of L-NAME (20 μM) were reversed by 100 μM L- but not D-arginine.

5 Neither L-NOARG nor L-NAME (both 20 μM) affected submaximal relaxations induced by 10 μM sodium nitroprusside or 20 μM hydroxylamine.

6 D-NOARG, L-N^G-tosyl-arginine and L-N^{α}-(*t*-butyl-oxycarbonyl)-N^G-nitro-arginine (all at 100 μM) had no effect on NANC relaxations.

7 Thus, in the rat anococcygeus, L-NOARG and L-NAME are more potent than L-NMMA as prejunctional inhibitors of NANC transmission. The reversibility of the effect of L-NOARG by arginine analogues suggests that the NANC system of the anococcygeus shows similarities to the endogenous nitrate system recently described in the brain.

Introduction

Since the original description of the phenomenon by Gillespie (1972) the identity of the transmitter responsible for non-adrenergic, non-cholinergic (NANC) relaxations of the anococcygeus muscle has been the subject of much research (Gillespie, 1980; 1987). An inhibitory substance, which mimics the actions of the NANC transmitter and has the characteristics of a nitrovasodilator-like material, has been extracted from the anococcygeus and the related retractor penis muscle (Gillespie & Martin, 1980; Bowman & Gillespie, 1982; Bowman & Drummond, 1984; Gillespie & Sheng, 1988). Further, the pharmacological actions of the inhibitory substance have been attributed to nitric oxide (NO; Martin *et al.*, 1988). Such observations have suggested that NANC transmission in the anococcygeus may represent another physiological system involving the release of an endogenous nitrate, similar to the endothelium-derived relaxing factor (EDRF; Moncada *et al.*, 1989). This suggestion has been strengthened by the recent observation that in both the rat (Gillespie *et al.*, 1989) and mouse (Gibson *et al.*, 1990) anococcygeus the NANC responses are reduced by L-N^G-monomethyl-arginine (L-NMMA) which blocks the synthesis of NO from L-arginine in several cell types (Moncada *et al.*, 1989). However, in both species, L-NMMA is relatively weak; we have recently shown that another guanidino-substituted arginine derivative, L-N^G-nitro-arginine (L-NOARG), is some 30–50 times more potent than L-NMMA in the mouse anococcygeus (Gibson *et al.*, 1990). In this paper, we show that L-NOARG is also more potent than L-NMMA in the rat anococcygeus and that the potency of L-NOARG is matched by its methyl-ester; we also demonstrate the modulatory actions of some other arginine analogues on NANC transmission in this tissue.

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Methods

Male rats (Wistar strain; 200–400 g) were killed by stunning and cervical dislocation. The anococcygeus muscles were dissected (Gillespie, 1972) and set up in 2 ml glass organ baths containing Krebs-bicarbonate buffer (mM: NaCl 118.1, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 25.0 and glucose 11.1) which was maintained at 37°C and aerated continuously with 95% O_2:5% CO_2. A resting tension of 1 g was placed on the tissue and changes in tension recorded with a Grass FTO3 force-displacement transducer attached to a Graphtec pen-recorder (WR3101). Muscles were allowed to equilibrate for 30 min before beginning the experiment.

Field stimulation was applied by two parallel platinum electrodes running down either side of the tissue; these were attached to a Grass S48 stimulator (1 ms width; 70 V). To observe NANC relaxations to field stimulation in the anococcygeus it is necessary to raise muscle tone and to attenuate sympathetically induced contractions. Tone was raised with 50 μM carbachol in all cases; sympathetic responses were inhibited by the addition of 1 μM phentolamine to the Krebs solution and the pre-incubation of each muscle with 30 μM guanethidine for 10 min during the 30 min equilibration period. In the case of relaxant drugs, responses were calculated as the % reduction of carbachol-induced tone occurring within 3 min of the addition of the relaxant to the organ bath. Both carbachol and the relaxant were then washed out of the bath and the muscle allowed to rest for 15 min before continuing the experiment. Results are expressed as mean ± standard error of the mean (s.e.mean). Statistical analysis was by Student's *t* test (unpaired); a probability (*P*) value of <0.05 was taken to indicate significance.

All drugs used in this study were dissolved in distilled water and were obtained as follows: L-arginine-L-aspartate (Sigma), L-arginine-methyl-ester (Sigma), carbachol (BDH), L-citrulline

(Sigma), guanethidine sulphate (Ciba), L-homoarginine hydrochloride (Sigma), hydroxylamine hydrochloride (Sigma), L-N^G-monomethyl-arginine (kindly supplied by Dr S. Moncada, Wellcome Research Laboratories, Beckenham, Kent), D-N^G-nitro-arginine (Bachem), L-N^G-nitro-arginine (Bachem, Aldrich or Sigma), L-N^G-nitro-arginine-methyl-ester (Sigma), L-N^α-(*t*-butyl-oxycarbonyl)-N^G-nitro-arginine (Calbiochem), phentolamine mesylate (Ciba), sodium nitroprusside (Sigma) and L-N^G-tosyl-arginine (Peninsula).

Results

Effect of L-N^G-nitro-arginine on NANC relaxations

Field stimulation (15 Hz; 10 s train every 100 s) of the rat anococcygeus muscle elicited reproducible NANC relaxations of carbachol-induced tone (Figure 1). These relaxations were reduced, in a concentration-related manner, by L-NOARG (5–200 μM); 100 μM L-NOARG reduced the NANC response by 90% (Figure 2). To determine the arginine-reversibility of this effect, standard inhibitions of the NANC response were induced by 20 μM L-NOARG (as in Figure 1); the ability of various concentrations of L-arginine to reverse this inhibition was then measured (as in Figure 1a). L-Arginine (5–200 μM) produced a concentration-related reversal of the effect of L-NOARG (Figure 3); a five fold excess of L-arginine (100 μM) was required to produce the maximum reversal of 90% (Figures 1a and 3). D-Arginine (100 μM) failed to produce significant reversal (Figure 1b), but the inhibitory effects of 20 μM L-NOARG (58.4 ± 1.6% inhibition; $n = 60$) were significantly reversed by the following (all at 100 μM; $n > 6$): L-citrulline (by 60.3 ± 6.1%), L-arginine-L-aspartate (by 100.5 ± 7.4%), L-homoarginine (by 55.5 ± 5.6%) and L-arginine-methyl-ester (by 67.6 ± 10.2%).

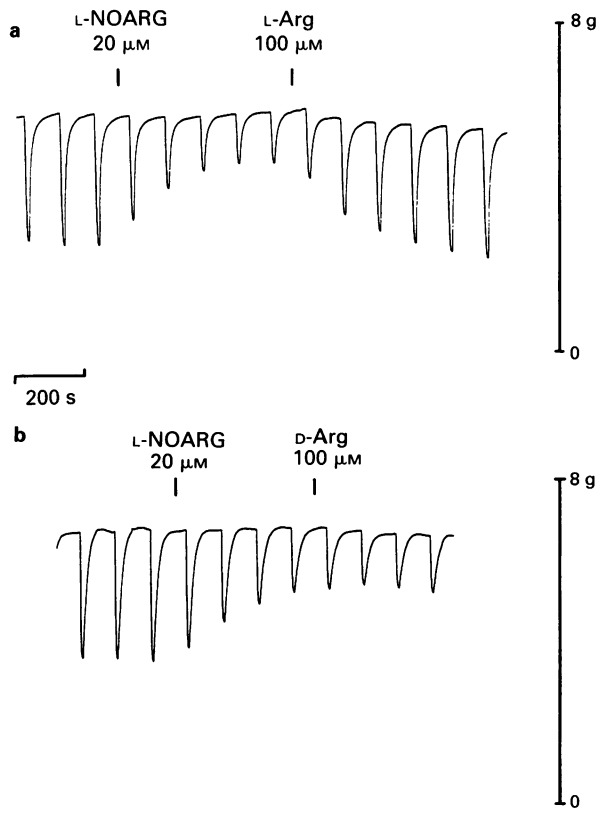

Figure 1 The effect of L-N^G-nitro-arginine (L-NOARG) on non-adrenergic, non-cholinergic (NANC; 15 Hz, 10 s train every 100 s)-induced relaxations of the rat anococcygeus muscle. Muscle tone was raised with 50 μM carbachol. L-NOARG reduced NANC relaxations, this effect being reversed by L-arginine (L-Arg; a) but not D-arginine (D-Arg; b).

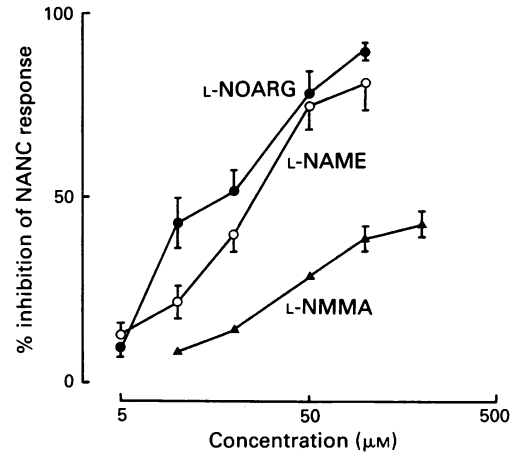

Figure 2 Concentration-response curves for L-N^G-nitro-arginine (L-NOARG; ●), L-N^G-nitro-arginine-methyl-ester (L-NAME; ○) and L-N^G-monomethyl-arginine (L-NMMA; ▲) causing inhibition of non-adrenergic, non-cholinergic (NANC) relaxations of the rat anococcygeus muscle. Each point represents the mean from at least 6 individual muscle preparations; s.e.mean shown by vertical bars.

Effect of other arginine analogues on NANC relaxations

L-N^G-nitro-arginine-methyl-ester (L-NAME; 5–200 μM) reduced NANC relaxation of the rat anococcygeus, with a potency similar to that of L-NOARG (Figure 2). NANC relaxations were also reduced by L-NMMA (10–200 μM), as shown by Gillespie *et al.* (1989), although it was much less potent than either L-NOARG or L-NAME (Figure 2). The inhibitory effect of L-NAME was reversed by L- but not D-arginine, in a ratio similar to that found with L-NOARG (data not shown). D-NOARG, L-N^G-tosyl-arginine and L-N^α-(*t*-butyl-oxycarbonyl)-N^G-nitro-arginine (all at 100 μM) had no effect on NANC relaxations, or on carbachol-induced tone.

Effect of L-N^G-nitro-arginine and L-N^G-nitro-arginine-methyl-ester on nitrovasodilator-induced relaxations

The nitrovasodilator drugs sodium nitroprusside (SNP; 1–200 μM) and hydroxylamine (1–200 μM) caused concentration-related relaxations of carbachol-induced tone; submaximal relaxations induced by 10 μM SNP and 20 μM hydroxylamine

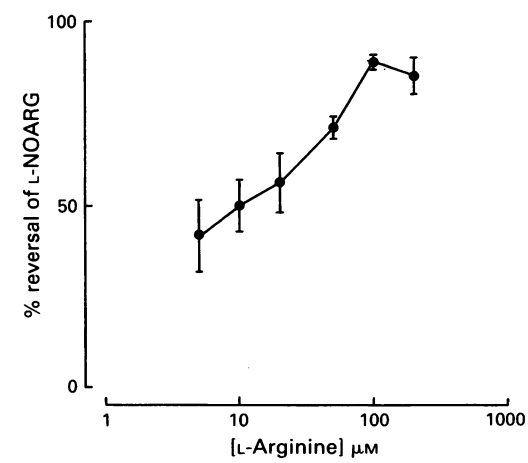

Figure 3 Concentration-response curve for the reversal of L-N^G-nitro-arginine (L-NOARG; 20 μM) inhibition of non-adrenergic, non-cholinergic (NANC) relaxations of the rat anococcygeus muscle (protocol similar to that shown in Figure 1a) by L-arginine. Each point represents the mean from at least 6 individual muscle preparations; s.e.mean shown by vertical bars.

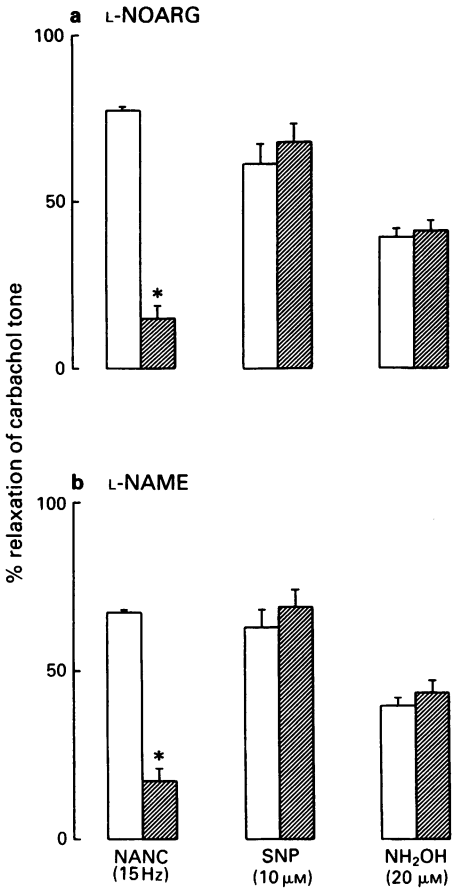

Figure 4 Histogram showing the effect of L-N^G-nitro-arginine (L-NOARG; a) and L-N^G-nitro-arginine-methyl-ester (L-NAME; b) on relaxations of the rat anococcygeus in response to non-adrenergic, non-cholinergic field stimulation (NANC), sodium nitroprusside (SNP; 10 μM) and hydroxylamine (NH_2OH; 20 μM). Muscle tone was raised with 50 μM carbachol. Open columns, controls; hatched columns, in presence of either 20 μM L-NOARG (a) or 20 μM L-NAME (b). Each point represents the mean from at least 6 individual muscle preparations; s.e.mean shown by vertical bars. $^*P < 0.05$, significant reduction of response. Both L-NOARG and L-NAME reduced NANC relaxations but not those due to nitrovasodilators.

were unaffected by L-NOARG or L-NAME (both at 20 μM; Figure 4).

Discussion

L-NMMA is believed to be a specific, competitive inhibitor of the enzyme(s) which convert L-arginine to NO (Moncada *et al.*, 1989) and has been widely used to investigate possible physiological roles for the endogenous nitrate systems in various tissues, including the anococcygeus (Gillespie *et al.*, 1989; Gibson *et al.*, 1990), vascular endothelial cells, macrophages and brain cells (Moncada *et al.*, 1989). L-NOARG also inhibits NO formation by vascular endothelial cells (Moore *et al.*, 1990); the results of the present study show that L-NOARG is more potent than L-NMMA as an inhibitor of NANC relaxations in the rat anococcygeus, confirming and extending previous observations in the mouse (Gibson *et al.*, 1990). A further new finding is that L-NAME is equipotent with L-NOARG in the rat anococcygeus. The inhibitory effects of all three compounds (L-NMMA, L-NOARG, L-NAME) were reversed by L- but not D-arginine and neither L-NOARG nor L-NAME inhibited relaxations induced by nitrovasodilator drugs; Gillespie *et al.* (1989) have already demonstrated that L-NMMA does not inhibit NO-induced relaxations of the rat anococcygeus. These results are consistent with the proposal that L-NMMA, L-NOARG and L-NAME inhibit NANC transmission in the anococcygeus by blocking the production of an endogenous nitrate material derived from L-arginine; the stereospecificity of this inhibition was confirmed by the lack of effect of D-NOARG. It seems that the crucial modification of the arginine molecule which produces effective inhibitors of endogenous nitrate synthesis is N^G-substitution. However, the substituting group must be small (as in L-NMMA and L-NOARG) since L-N^G-tosyl-arginine was ineffective; similarly, bulky substitution of the N^α atom removes the inhibitory effect of L-NOARG (as in L-N^α-(*t*-butyl-oxycarbonyl)-N^G-nitro-arginine), although addition of smaller groups to the N^α region (as in L-NAME) appears not to affect potency. Further modification of the N^G atom in L-arginine may provide even more powerful inhibitors of NO synthesis.

The concentration-dependent reversibility of L-NOARG by L-arginine provides further evidence that L-NOARG is a competitive inhibitor of the NO-generating system. An equimolar concentration of L-arginine will only reverse L-NOARG induced inhibitions by approximately 56% (Figure 3), a 5 fold excess being required for full reversal, in agreement with results already found in rat aorta (Moore *et al.*, 1990). L-Arginine produced a maximum reversal of about 90%, and this is consistent with the observations of Gillespie *et al.* (1989) that L-arginine, by itself, did not potentiate NANC relaxations of the rat anococcygeus. In the mouse anococcygeus, however, 100 μM L-arginine did potentiate the NANC response and produced a reversal of L-NOARG-induced inhibition of about 130% (Gibson *et al.*, 1990). This difference may reflect variations in the rate of transmitter turnover between the two species or in the rate of diffusion of L-arginine; alternatively, it may be that the levels of free L-arginine are lower in the NANC nerves of the mouse anococcygeus. In addition to L-arginine, the inhibitory effects of L-NOARG were reversed by L-citrulline, L-arginine-L-aspartate, L-homoarginine and L-arginine-methyl-ester. Apart from L-citrulline, these substances have been shown to be substrates for the NO-synthesizing enzyme extracted from the brain (Moncada *et al.*, 1989; Collier & Vallance, 1989). Palmer *et al.* (1988) found that L-citrulline enhanced NO release from vascular endothelial cells but suggested that this action was due to its prior conversion to L-arginine; it may be that this also occurs in the anococcygeus thereby explaining the reversal of L-NOARG.

The present observations in the anococcygeus, together with recent findings in brain tissue (Garthwaite *et al.*, 1988; Knowles *et al.*, 1989) strongly suggest that the L-arginine/NO pathway represents a novel, and unexpected, chemical neurotransmission process. The mechanisms regulating production and release of the neurotransmitter have yet to be determined, but it has been shown that the brain enzyme which converts L-arginine to NO is calcium-dependent (Garthwaite *et al.*, 1988; Knowles *et al.*, 1989) and is activated by calcium concentrations similar to those found intracellularly during nerve activation. In the anococcygeus, L-arginine does not relax the unstimulated muscle but it does reverse the inhibitory effect of L-NOARG during NANC stimulation; this observation could be explained if the NO-synthesising enzyme was inactive in the quiescent nerve but was activated by calcium moving into the cell during depolarization. Clearly, the anococcygeus will provide a useful model with which to investigate this new neurotransmission system and to identify drugs modifying its function.

In conclusion, the results of this study have confirmed that NANC transmission in the anococcygeus results from the release of an endogenous nitrate material (Gillespie, 1987; Gillespie *et al.*, 1989; Gibson *et al.*, 1990). In the rat anococcygeus, as in the mouse anococcygeus (Gibson *et al.*, 1990) and rat vascular tissue (Moore *et al.*, 1990), L-NOARG is considerably more potent than L-NMMA; further, in the rat anococcygeus, the potency of L-NOARG is matched by its methyl-ester.

A.J.H. is an SERC research student.

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(Received January 18, 1990
Revised March 14, 1990
Accepted March 30, 1990)