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No evidence for a significant non-nitrergic, hyperpolarising factor contribution to field stimulation-induced relaxation of the mouse anococcygeus

Mario Fonseca, Nazim Uddin & [1]Alan Gibson

Pharmacology Group, Biomedical Sciences Division, King's College London, Manresa Road, London SW3 6LX

1 The aim of the study was to determine whether a nerve-derived hyperpolarizing factor (NDHF) might contribute to non-adrenergic, non-cholinergic (NANC) relaxations of the mouse anococcygeus when low concentrations of contractile agent are used to raise tone and low frequencies of field stimulation applied; such a non-nitrergic NDHF has been proposed to contribute to NANC relaxations of the rat anococcygeus and guinea-pig taenia coli.

2 Phenylephrine (0.1–100 μM) produced concentration-related contractions of the mouse isolated anococcygeus muscle; 0.2 μM phenylephrine (EC_{26}) was used to raise tone in subsequent experiments.

3 Field stimulation (0.5, 1.0 and 5.0 Hz) produced frequency-dependent relaxations of phenylephrine-induced tone. In the presence of the nitric oxide synthase inhibitor L-N^G-nitro-arginine (L-NOARG; 100 μM), the soluble guanylate cyclase inhibitor 1H-[1,2,4]oxodiazolo[4,3-a]quinoxalin-1-one (ODQ; 5 μM), or a combination of these two drugs, relaxations to field stimulation were abolished at all frequencies studied. Relaxations to sodium nitroprusside (0.01–5 μM) were unaffected by L-NOARG but strongly inhibited by ODQ; neither enzyme inhibitor affected relaxations to 8-Br-cyclic GMP (10 μM).

4 Nifedipine (1 μM) reduced the contractile response to 0.2 μM phenylephrine by 38%; however, it had no effect on NANC relaxations.

5 It is concluded that NANC relaxations of the mouse anococcygeus are purely nitrergic and that there is no significant contribution from a putative NDHF.

Keywords: Anococcygeus (mouse); 8-Br-cyclic GMP; nerve derived hyperpolarizing factor; nifedipine; nitrergic; L-N^G-nitro-arginine; IH-[1,2,4]oxodiazolo[4,3-a]quinoxalin-1-one; phenylephrine; sodium nitroprusside

Introduction

Anococcygeus muscles from rats and mice were among the first tissues in which the involvement of the L-arginine/nitric oxide (NO) system in peripheral non-adrenergic, non-cholinergic (NANC) neurotransmission was clearly demonstrated (Gillespie *et al*., 1989; Li & Rand, 1989; Ramagopal & Leighton, 1989; Gibson *et al*., 1990). The anococcygeus has since become a widely used model for investigation of the mechanisms underlying nitrergic neurotransmission (see reviews by Rand & Li, 1995a, b). Early observations of a discrepancy between the effects of some NO blocking agents on relaxations to exogenous NO and those to nitrergic field stimulation (Gillespie & Sheng, 1990; Hobbs *et al*., 1991) led to a considerable debate on the nature of the transmitter actually released from the nitrergic nerves (Gibson *et al*., 1995; Rand & Li, 1995b). However, current evidence suggests that these discrepancies may arise from the protection afforded to endogenously-released NO by superoxide dismutase, and other antioxidants, within the tissue (Martin *et al*., 1994; De Man *et al*., 1996; Lefebvre, 1996; Lilley & Gibson, 1996) and therefore that free radical NO is likely to be the transmitter which traverses the neuroeffector junction. Recently, however, a further complication has arisen since Selemidis & Cocks (1997) have proposed, in the rat anococcygeus muscle, that an additional, non-nitrergic, transmitter acts to relax the tissue when it is contracted via a depolarizing stimulus. They have termed this transmitter 'nerve-derived hyperpolarizing factor' (NDHF) and argued that NDHF becomes the dominant NANC transmitter in the rat anococcygeus when low to medium (EC_{40} or less) concentrations of contractile agent are used to raise the tone of the tissue, and when low frequencies (5 Hz or less) of field stimulation are applied. Relaxations to NDHF are resistant to nitric oxide synthase inhibitors but can be reduced by calcium channel blocking agents such as nifedipine. The same authors (Selemidis *et al*., 1997) have proposed that NDHF is also the dominant transmitter in the guinea-pig taenia coli, where it is resistant to both nitric oxide synthase inhibitors and the guanylate cyclase inhibitor 1H-[1,2,4]oxodiazolo[4,3-a]quinoxalin-1-one (ODQ; Garthwaite *et al*., 1995; Cellek *et al*., 1996); in the taenia, NO only contributes to nerve-induced relaxations once the influence of NDHF has been negated (Selemidis *et al*., 1997). Since the presence of a functionally significant NDHF may have important implications for the interpretation of results obtained from investigations into nitrergic mechanisms, we have examined whether NDHF may contribute to field stimulation-induced relaxations in another model of nitrergic neurotransmission, the mouse anococcygeus (Gibson *et al*., 1995), using experimental conditions which should favour NDHF activity.

Methods

Male mice (LACA, 25–35 g) were killed by stunning and exsanguination. The two anococcygeus muscles were dissected out separately and set up in 1 ml glass organ baths containing Krebs-bicarbonate buffer (composition, mM: NaCl 118.1, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 11.1) which was maintained at 37°C and gassed with

[1] Author for correspondence.

95% O_2 : 5% CO_2. A resting tension of 200–400 mg was placed on the tissue and changes in tension recorded with a Grass FT03 force-displacement transducer attached to a pen-recorder (Graphtec or Lectromed). Muscles were allowed to equilibrate for 30 min before beginning experimental procedures.

Field stimulation was applied by two parallel platinum electrodes running down either side of the tissue. These were attached to square wave pulse generators (1 ms pulse width; 90 V; 10 s trains every 2 min). To record relaxations to field stimulation in the mouse anococcygeus it is necessary to negate the effects of the sympathetic nerves and to raise the tone of the tissue. Sympathetic function was inhibited by preincubation of each tissue with 30 μM guanethidine for 10 min during the equilibration period. In some tissues, this guanethidine caused a contraction which was reversed on washout of the drug, so that by the time experimental procedures were initiated tone had returned to baseline. Subsequently, muscle tone was raised with phenylephrine (normally 0.2 μM). Field stimulation (0.5, 1.0 and 5.0 Hz) was applied once phenylephrine had produced a stable increase in tone, and responses were calculated as the % relaxation of tone compared with the level just before each train of stimulation. Each time tone was raised with phenylephrine, a set of 3 responses (0.5, 1, 5 Hz) was obtained; the phenylephrine was then washed from the bath and the tissue allowed to rest for 20 min before tone was raised again. Once field stimulation was causing reproducible relaxations at each frequency, muscles were exposed to L-N^G-nitro-arginine (L-NOARG) and/or ODQ for 20 min before determining their effects on nerve-induced relaxations.

To record relaxations to drugs, tone was first raised with phenylephrine. Relaxant drugs were then applied when a stable elevation of tone had been achieved. Both sodium nitroprusside (SNP) and 8-Br-cyclic GMP were added cumulatively; in the case of SNP the peak relaxation of the preceding concentration was the signal to add the subsequent dose, while the 8-Br-cyclic GMP, which produced a much slower relaxation, each concentration was left in contact with the tissue for 2 min before addition of the next dose. Responses were calculated as the % relaxation of tone compared with the level just before addition of the first dose of relaxant drug. The contact time for L-NOARG/ODQ was again 20 min.

Results are expressed as mean ± s.e. mean and statistical analysis was by Student's unpaired t test, with a P value of 0.05 or less taken to indicate a significant difference.

Drugs were dissolved in distilled water, except ODQ (10 mM stock in DMSO) and nifedipine (1 mM stock in ethanol); the final bath concentrations of these solvents showed no significant biological effects. Drugs used during the study were: 8-Br-cyclic GMP (Sigma); guanethidine monosulphate (Sigma); nifedipine (Sigma); L-NOARG (Sigma); ODQ (Tocris); phenylephrine HCl (Sigma); sodium nitroprusside (Sigma).

Results

Since Selemidis & Cocks (1997) showed that NDHF became the dominant transmitter in the rat anococcygeus when low to medium concentrations (EC_{40} or less) of phenylephrine were used to raise the tone of the tissue, initial experiments were carried out to determine an appropriate concentration of phenylephrine with which to raise tone in the mouse anococcygeus to this level. Cumulative addition of phenylephrine (0.1–100 μM) produced concentration-related contractions of the mouse anococcygeus; the pD_2 value was 6.19 ± 0.09 and the maximum response was 816 ± 58 mg ($n = 12$). Phenylephrine 0.2 μM produced a contraction of 26 ± 5% ($n = 12$) of the maximum response, and was chosen as the standard concentration to raise tone in subsequent experiments.

When tone had been raised with 0.2 μM phenylephrine, field stimulation (0.5, 1.0 and 5.0 Hz) produced frequency-related relaxations (Figures 1 and 2). The effects on these relaxations of L-NOARG (100 μM; nitric oxide synthase inhibitor), ODQ (5 μM; guanylate cyclase inhibitor), and a combination of both L-NOARG and ODQ were examined. An example of the experimental traces obtained with the combination of inhibitors is given in Figure 1, and the overall results shown in Figure 2. Either drug alone, or the combination, significantly reduced field stimulation-induced relaxations at all the frequencies tested. In some muscles, small residual relaxations were still obtained, especially at 5.0 Hz; however, in each experimental group, the relaxations obtained in the presence of the inhibitors were not significantly different from zero ($P < 0.05$). In a small number of experiments ($n = 4$), tone was raised to near-maximal level with 15 μM phenylephrine (EC_{98}); under such conditions, field stimulation at 5 Hz produced relaxations of 20 ± 3% and these were completely abolished by 100 μM L-NOARG, confirming results of a

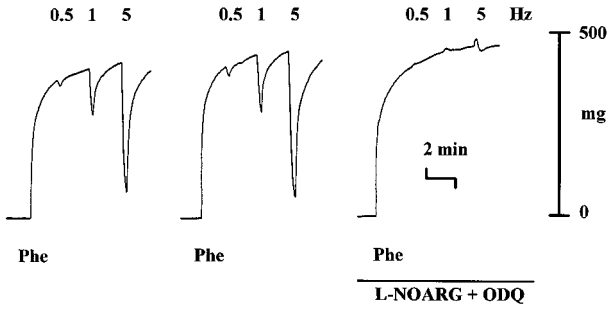

Figure 1 Traces showing the responses of a mouse anococcygeus muscle to NANC field stimulation (0.5, 1 and 5 Hz; 10 s trains) in the absence (two left hand traces) and presence (right hand trace) of a combination of L-N^G-nitro-arginine (L-NOARG; 100 μM) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ; 5 μM). Tone was raised with 0.2 μM phenylephrine (Phe).

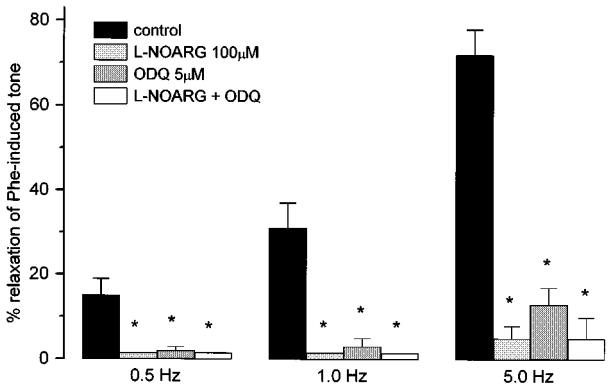

Figure 2 Histogram showing the effects of L-N^G-nitro arginine (L-NOARG; 100 μM) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ; 5 μM), and a combination of these two drugs, on NANC (10 s trains) relaxations of the mouse anococcygeus muscle; tone was raised with 0.2 μM phenylephrine (Phe). Each column represents the mean ± s.e.mean from 6 individual muscle preparations. *Significantly different from the control at that frequency.

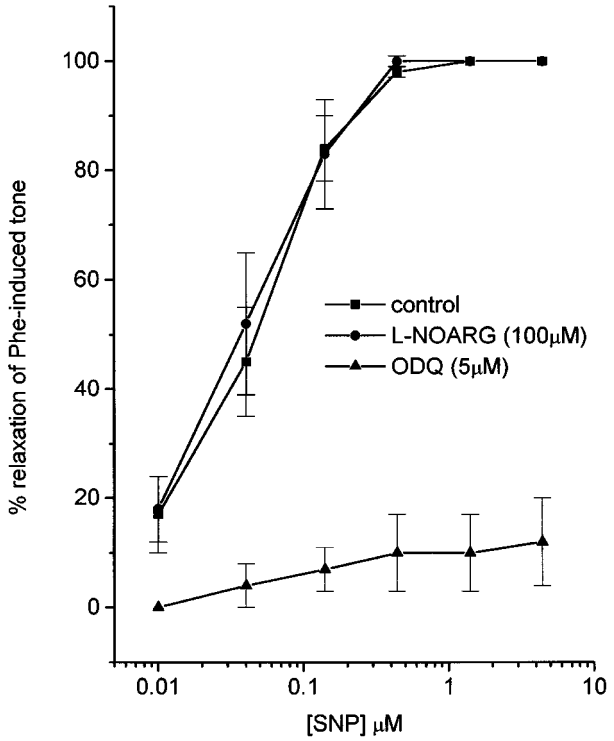

Figure 3 Concentration-response curves for sodium nitroprusside (SNP)-induced relaxations of the mouse anococcygeus, under control conditions and in the presence of L-N^G-nitro-arginine (L-NOARG; 100 μM) or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ; 5 μM). Each point represents the mean from 5 individual muscle preparations; vertical lines show s.e.mean. Tone was raised with 0.2 μM phenylephrine (Phe).

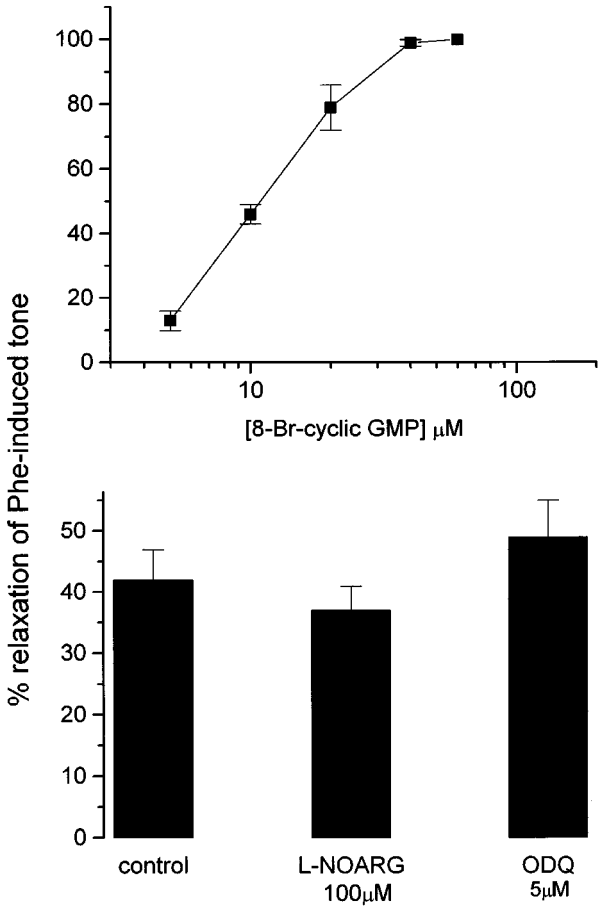

Figure 4 (a) Concentration-response curve for 8-Br-cyclic GMP-induced relaxations of the mouse anococcygeus. Each point represents the mean from at least 4 individual muscle preparations; vertical lines show s.e.mean. (b) Histogram showing relaxations of the mouse anococcygeus to 10 μM 8-Br-cyclic GMP under control conditions and in the presence of L-N^G-nitro-arginine (L-NOARG; 100 μM) or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ; 5 μM). Each column represents the mean$\pm$s.e.mean from at least 4 individual muscle preparations. In both (a) and (b) tone was raised with 0.2 μM phenylephrine (Phe).

previous study in which high-level tone was induced by carbachol (Gibson *et al*., 1990).

Experiments were carried out to determine the selectivity of the inhibitory actions of L-NOARG and ODQ on field stimulation. The contractile response to 0.2 μM phenylephrine was not altered by either 100 μM L-NOARG (tone in the presence of L-NOARG 101$\pm$3% of that in its absence, $n=16$) or 5 μM ODQ (tone in the presence of ODQ 103$\pm$3% of that in its absence, $n=19$). In addition, neither L-NOARG nor ODQ affected the resting tension. SNP (0.01–5 μM) produced concentration-related relaxations of phenylephrine (0.2 μM)-induced tone (Figure 3). In the presence of 100 μM L-NOARG, responses to SNP were unaffected, while almost complete suppression of the SNP concentration-response curve was observed with 5 μM ODQ (Figure 3). 8-Br-cyclic GMP (5–60 μM) also produced concentration-related relaxations of phenylephrine (0.2 μM)-induced tone (Figure 4); 10 μM 8-Br-cyclic GMP produced a relaxation of 42$\pm$5% ($n=6$) and this was unchanged in the presence of either 100 μM L-NOARG (37$\pm$4%; $n=6$) or 5 μM ODQ (49$\pm$6%; $n=6$).

Selemidis & Cocks (1997) found that nifedipine could block relaxations of the rat anococcygeus to the putative NDHF and therefore, in a final series of experiments, the effects of nifedipine were investigated in the mouse anococcygeus, using a concentration (1 μM) which has been shown to abolish K^+-induced contractions of this tissue (Gibson *et al*., 1994). In the presence of 1 μM nifedipine, the contractile response to 0.2 μM phenylephrine was reduced by 38$\pm$7% ($n=4$), but there was no significant effect on the relaxations to field stimulation (control relaxations at 0.5, 1, and 5 Hz, 27$\pm$8%, 53$\pm$7%, and 90$\pm$4%; in the presence of 1 μM nifedipine, 46$\pm$13%, 62$\pm$11%, and 95$\pm$6%; $n=4$ in each case; $P>0.05$ control vs nifedipine at each frequency).

Discussion

The object of this study was to investigate whether a NDHF might make a significant contribution to NANC relaxations of the mouse anococcygeus, as has been proposed in the rat anococcygeus by Selemidis & Cocks (1997). In this latter tissue, it has been suggested that NDHF is the dominant NANC relaxant transmitter when low to medium concentrations of contractile agent are used to raise the tone of the muscle, and when low frequencies of field stimulation are applied; under these conditions, NANC relaxations of the rat anococcygeus were reported to be largely resistant to nitric oxide synthase inhibitors but highly sensitive to block by nifedipine. In previous studies with the mouse anococcygeus, NANC relaxations were effectively abolished by nitric oxide synthase inhibitors (Gibson *et al*., 1990), but high concentrations ($>EC_{90}$) of contractile agent, and a stimulation frequency of 10 Hz, were used. In the present study, we

employed experimental conditions which should favour NDHF; a low concentration of phenylephrine, the agent used by Selemidis & Cocks (1997) in the rat anococcygeus, was used to raise tone and low frequencies of field stimulation applied.

However, even under these conditions, field stimulation-induced relaxations of the mouse anococcygeus were abolished by L-NOARG, but were unaffected by nifedipine. These results, by themselves, would suggest that there is no significant NDHF contribution to nerve-induced relaxations of the mouse anococcygens. However, in addition, we investigated the effects of the soluble guanylate cyclase inhibitor ODQ (Garthwaite *et al*., 1995), which has been shown to inhibit NANC relaxations of the rabbit anococcygeus (Cellek *et al*., 1996), but which was ineffective against NDHF in the guinea-pig taenia coli (Selemidis *et al*., 1997). In the mouse anococcygeus ODQ, either alone or in combination with L-NOARG, abolished NANC relaxations. These results strongly suggest that NO is the sole transmitter involved in NANC relaxations of the mouse anococcygeus. The selectivity of L-NOARG and ODQ was assessed using SNP and 8-Br-cyclic GMP. While both L-NOARG and ODQ inhibited relaxations to field stimulation, only ODQ reduced responses to SNP and neither agent had any effect on relaxations to 8-Br-cyclic GMP; such results would be expected for selective inhibitors of nitric oxide synthase (L-NOARG) and soluble guanylate cyclase (ODQ) respectively. ODQ has proved to be a useful and relatively selective inhibitor of soluble guanylate cyclase and has been shown to inhibit NANC relaxations in such tissues as the rabbit anococcygeus (Cellek *et al*., 1997) and the canine proximal colon (Franck *et al*., 1997). In the mouse anococcygeus, phenylephrine-induced contractions and basal tone were unaffected by either L-NOARG or ODQ suggesting that, in this tissue, background release of NO does not modulate responses to contractile agents and ODQ does not exert a non-specific relaxant effect, as reported in canine proximal colon (Franck *et al*., 1997).

In the presence of nifedipine, contractions to phenylephrine were reduced by some 38%. This partial inhibition would be consistent with the current model of excitation-contraction coupling in the mouse anococcygeus (Wayman *et al*., 1998). Agonists acting on receptors coupled to phospholipase C generate inositol 1,4,5-trisphosphate which initiates release of calcium from the sarcoplasmic reticulum (SR). The calcium so released stimulates calcium-activated chloride channels in the plasma membrane leading to depolarization and opening of L-type voltage-operated calcium channels (VOCCs). In addition, depletion of the SR results in the opening of store-operated channels (SOCCs) in the plasma membrane, through which calcium also enters the cell. Thus, sustained contractions of the mouse anococcygeus require extracellular calcium, some of which enters through VOCCs and the rest through SOCCs. Nifedipine would partially reduce the contraction to phenylephrine by inhibiting the VOCC component of calcium entry. Since nifedipine had no effect on NANC relaxations, it is unlikely that the NO/cyclic GMP pathway produces relaxations via a hyperpolarization-dependent closure of VOCCs. Indeed, recent evidence suggests that inhibition of the store-operated calcium entry process may be an important mechanism underlying the relaxations induced by nitric oxide and cyclic GMP in the mouse anococcygeus (Wayman *et al*., 1996).

In conclusion, the results of the present study, using the mouse anococcygeus, contrast sharply with those recently reported by Selemidis & Cocks (1997) for the rat anococcygeus; low frequency NANC relaxations were abolished by L-NOARG and by ODQ even when tone was raised by a concentration of phenylephrine which produced only 26% of the maximum contraction. In addition, the relaxations were resistant to nifedipine. NO is therefore the dominant transmitter in the mouse anococcygeus and there is no evidence for any significant contribution from a putative NDHF.

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