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Evidence that both nitric oxide (NO) and a non-NO hyperpolarizing factor elicit NANC nerve-mediated relaxation in the rat isolated anococcygeus

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1 Responses to electrical field stimulation (EFS; 0.5–10 Hz, 0.2 ms duration, supramaximal voltage for 20 s) of non-adrenergic, non-cholinergic, (NANC) nerves were obtained in preparations of rat anococcygeus pre-contracted with titrated concentrations of phenylephrine (0.1–1 μM) to ~40% of their maximum contraction to phenylephrine (F_{max}) regardless of drug treatment.

2 With this set level of active force, NANC nerve stimulation resulted in relaxations that were maximal (peak relaxation) at 0.5–1 Hz, abolished by tetrodotoxin (1 μM) but only minimally blocked by the nitric oxide synthase (NOS) inhibitor, N^G-nitro-L-arginine, (L-NOARG; 100 μM). Furthermore, the nitric oxide (NO) scavenger, oxyhaemoglobin (HbO; 30 μM) gave no further block alone or in combination with L-NOARG (100 μM). By comparison, in preparations contracted with phenylephrine to ~70% F_{max}, relaxations to NANC nerve stimulation were markedly reduced or abolished by combined treatment with L-NOARG (100 μM) and HbO (30 μM).

3 Nifedipine (0.3 μM) significantly inhibited NANC nerve-mediated relaxations, which became frequency-dependent and abolished those resistant to L-NOARG (100 μM) and HbO (30 μM).

4 These data suggest that a non-NO, hyperpolarizing factor and NO both contribute to NANC nerve-mediated inhibitory responses in the rat anococcygeus. However, responses to the non-NO factor were observed only in preparations contracted sub-maximally by a nifedipine-sensitive mechanism.

Keywords: Anococcygeus (rat); NANC nerves; nitrergic nerves; nifedipine; NO; hyperpolarization

Introduction

Non-adrenergic, non-cholinergic, (NANC) inhibitory neurotransmission of the rat anococcygeus muscle is regarded to be solely nitrergic since potent inhibitors of nitric oxide synthase (NOS), such as N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine (L-NOARG), block relaxations to NANC nerve stimulation (Gillespie *et al*., 1989; Li & Rand, 1989; Hobbs & Gibson, 1990; Liu *et al*., 1991; Rand, 1992; Rand & Li, 1995). Furthermore, the NO scavenger, oxyhaemoglobin (HbO), has been shown to inhibit NANC transmission in the same tissue (Li & Rand, 1993; La *et al*., 1996). However, in most of these studies, NANC nerve-mediated relaxations have been examined in preparations contracted to near-maximum levels of active force, such that excessive functional antagonism may have exaggerated the potency of NOS inhibitors. This may be relevant for transmitters that evoke a hyperpolarization since excessive functional antagonism has been shown to block the effects of an endothelium-derived hyperpolarizing factor (EDHF; Garland *et al*., 1995) in blood vessels such as the pig (Kilpatrick & Cocks, 1994) and cow (Drummond & Cocks, 1996) coronary arteries. Therefore, the aim of the present study was to examine the effect of L-NOARG on NANC nerve-mediated relaxations in the rat anococcygeus, but at levels of active force maintained at ~40% of the maximum contraction of the tissue to phenylephrine. We have demonstrated that under these conditions, the relaxations to NANC nerve-stimulation are largely resistant to a combination of L-NOARG plus HbO, yet they are powerfully inhibited by nifedipine.

Methods

Sprague-Dawley rats (200–300 g; of either sex) were killed by CO_2 asphyxia and hemi-anococcygeus muscles removed and suspended between a force-displacement transducer (model FTO3, Grass, Quincy, MA, U.S.A.) and a micrometer driven support in 25 ml organ baths containing carbogenated (95% O_2, 5% CO_2) Krebs solution (composition in mM; Na^+ 143.1, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 127.8, HCO_3^- 25.0, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11.0) at 37°C. Changes in isometric force were amplified (model 108, BMRI, Australia) and recorded with Rikadenki (model R-01, Japan) chart recorders. After a 30 min equilibration period, preparations were stretched to obtain a resting tension of 0.5 g and allowed to equilibrate for a further 30 min, after which they were treated with guanethidine (30 μM), atropine (1 μM) and propranolol (1 μM) for 30 min. The indirect sympathomimetic effect of guanethidine resulted in a contraction of 7.1 ± 0.22 g ($n = 28$). When the contraction to guanethidine reached a plateau, preparations were then further contracted with phenylephrine (30 μM) to a maximum (F_{max}) of 10.7 ± 0.26 g ($n = 28$). Tissues were then repeatedly washed every 10 min with Krebs solution containing guanethidine, atrophine and propranolol until force was restored to pre-guanethidine plus phenylephrine resting levels. This took between 60 and 90 min to achieve. All tissues then remained exposed to guanethidine, atropine and propranolol for the duration of the experiment. Tissues were then contracted to ~40% F_{max} with titrated concentrations of phenylephrine (0.1–1 μM) and once a stable level of contraction was achieved, relaxations were obtained to electrical field stimulation (EFS; 0.5–10 Hz, 0.2 ms duration, supramaximal voltage for 20 s, every 3–6 min) via platinum ring electrodes.

Tissues were either left untreated (time control) or treated for 30 min with (a) L-NOARG (100 μM), (b) HbO (10 μM), (c) nifedipine (0.3 μM) or the following combination treatments of (d) L-NOARG (100 μM) and HbO (10 μM) and (e) L-NOARG (100 μM), HbO (10 μM) and nifedipine (0.3 μM). Preparations were then re-contracted with phenylephrine to ~40% F_{max} or in some cases to ~70% F_{max} regardless of drug treatment and again responses to EFS were obtained. In preparations that were exposed to HbO either alone or in combination with L-NOARG, a further 20 μM HbO was added to the bath after

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the contraction to phenylephrine to account for any protein denaturation. The final concentration of HbO was taken as 30 μM.

Statistics

Peak relaxation responses to EFS were expressed as percentages of the level of active force to phenylephrine. Differences in mean peak relaxations within groups were tested for significance by use of Student's paired t test. In all cases, significance was accepted at the $P<0.05$ level.

Drugs

Bovine haemoglobin, N^G-nitro-L-arginine, guanethidine sulphate, tetrodotoxin (Sigma, MO, U.S.A.); nifedipine, 3-bromo-7-nitroindazole (Sapphire Bioscience, N.S.W., Australia); atropine sulphate and phenylephrine hydrochloride (Research Biochemicals International, U.S.A.). Haemoglobin was dissolved in 0.9% NaCl to make a stock solution of 1 mM and then reduced with sodium dithionite ($Na_2S_2O_4$). Excess $Na_2S_2O_4$ was removed by running the solution through a Sephadex column (PD 10). Stock solutions of N^G-nitro-L-arginine (100 mM) and 3-bromo-7-nitroindazole (100 mM) were prepared in 1 M $NaHCO_3$ and 100% dimethyl-sulphoxide (DMSO) respectively. Stock solutions (10 mM) of guanethidine, atropine and phenylphrine were prepared in distilled water and those of tetrodotoxin and nifedipine were prepared in citrate buffer and 100% ethanol, respectively. All stock solutions were further diluted with distilled water.

Results

EFS (0.5–10 Hz) of NANC inhibitory nerves in the absence of any drug treatment resulted in tetrodotoxin (1 μM)-sensitive relaxations (data not shown) in tissues pre-contracted to $\sim$40% F_{max} with phenylephrine (0.1–0.5 μM). These peak relaxations were not frequency-dependent, however, they were reproducible with time since there were no significant differences between the first and second control relaxations (time control) for each frequency of stimulation (Figure 1).

Effect of L-NOARG and the NO scavenger, HbO

L-NOARG (100 μM) and HbO (30 μM) either alone or in combination caused a small, variable contraction and consequently in those preparations a lower concentration of phenylephrine was required to obtain an $\sim$40% F_{max} contraction (Figure 2). Whilst combined treatment of L-NOARG (100 μM) and HbO (30 μM) had no significant effect on the peak relaxations and time course to EFS at frequencies of 0.5–2 Hz (Figure 2), the peak relaxations to 5 Hz (81.9$\pm$2.4%) and 10 Hz (81.3$\pm$2.5%), were significantly reduced ($P<0.05$, $n=4$) to 61.5$\pm$7.8% and 52.7$\pm$7.1%, respectively (Figure 2). Similar results were obtained in tissues treated with either L-NOARG ($n=5$) or HbO ($n=4$) alone (data not shown). Also, no further reductions of peak relaxations to EFS (0.5–10 Hz) were obtained when 3-bromo-7-nitroindazole (100 μM), was added in combination with L-NOARG (100 μM) and HbO (30 μM) ($n=3$; data not shown). DMSO (1%) the amount of solvent for 3-bromo-7-nitroindazole (100 μM) had no effect on the responses to EFS at all frequencies of stimulation.

Effect of different levels of active force on NANC relaxations

Figure 3 shows a comparison of the relaxation to EFS in a single preparation contracted firstly to $\sim$40% F_{max} and then further contracted to $\sim$70% F_{max}. The relaxations to EFS with identical stimulation parameters were markedly reduced at $\sim$70% F_{max}. The relaxations that were resistant to the combination treatment of L-NOARG and HbO in preparations contracted to $\sim$40% F_{max} were completely blocked by this increase in active force (Figure 3). Similar results were obtained in a further 4 preparations.

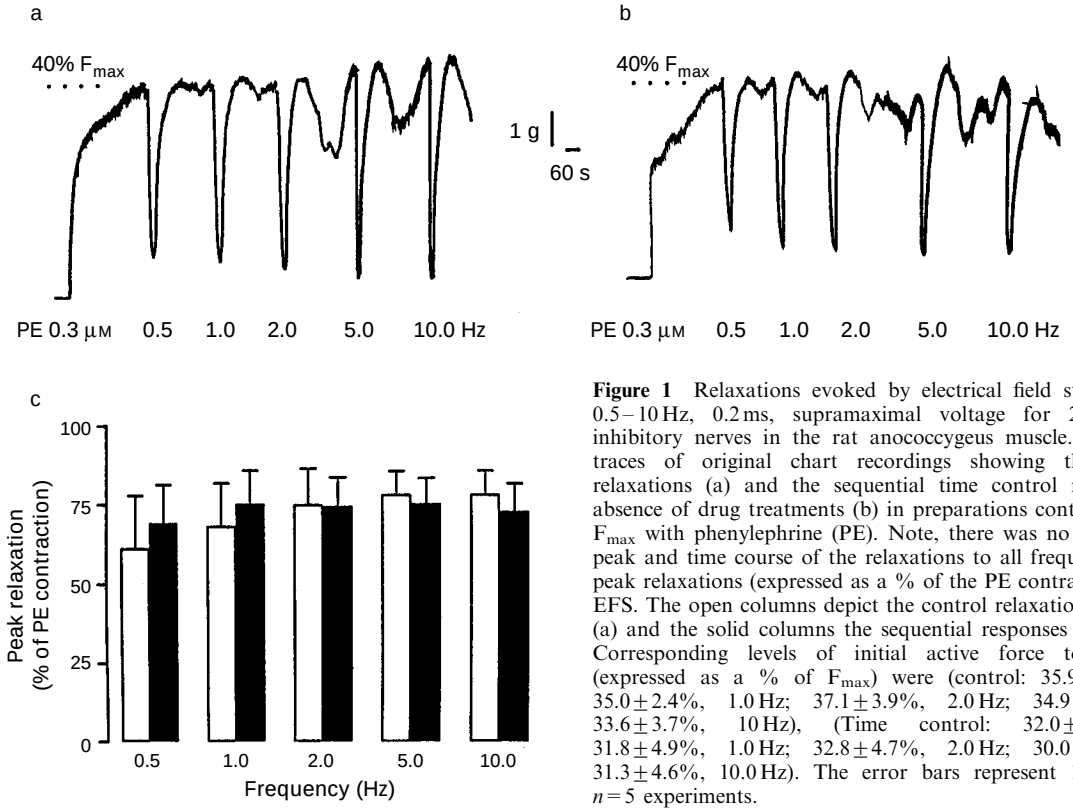

Figure 1 Relaxations evoked by electrical field stimulation (EFS; 0.5–10 Hz, 0.2 ms, supramaximal voltage for 20 s) of NANC inhibitory nerves in the rat anococcygeus muscle. (Top) Digitized traces of original chart recordings showing the first control relaxations (a) and the sequential time control responses in the absence of drug treatments (b) in preparations contracted to $\sim$40% F_{max} with phenylephrine (PE). Note, there was no difference in the peak and time course of the relaxations to all frequencies. (c) Mean peak relaxations (expressed as a % of the PE contraction) elicited by EFS. The open columns depict the control relaxations represented in (a) and the solid columns the sequential responses as shown in (b). Corresponding levels of initial active force to phenylephrine (expressed as a % of F_{max}) were (control: 35.9$\pm$2.5%, 0.5 Hz; 35.0$\pm$2.4%, 1.0 Hz; 37.1$\pm$3.9%, 2.0 Hz; 34.9$\pm$3.6%, 5.0 Hz; 33.6$\pm$3.7%, 10 Hz), (Time control: 32.0$\pm$3.1%, 0.5 Hz; 31.8$\pm$4.9%, 1.0 Hz; 32.8$\pm$4.7%, 2.0 Hz; 30.0$\pm$4.5%, 5.0 Hz; 31.3$\pm$4.6%, 10.0 Hz). The error bars represent 1 s.e.mean from $n=5$ experiments.

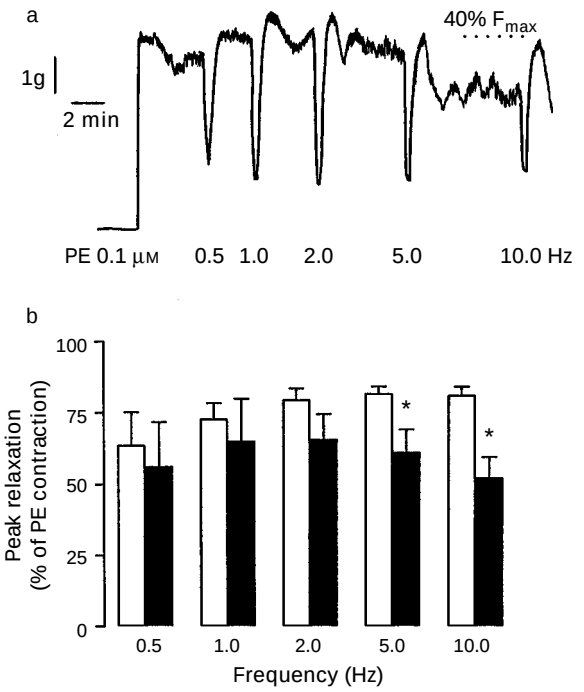

Figure 2 Relaxations elicited by electrical field stimulation (EFS) (0.5–10 Hz, 0.2 ms, supramaximal voltage for 20 s) of NANC inhibitory nerves in the rat isolated anococcygeus. (a) Digitized trace of original chart recording showing only the second responses to NANC nerve-stimulation in a preparation contracted to ~40% F_{max} with phenylephrine (PE), in the presence of a combination of L-NOARG (100 μM) and HbO, (30 μM). (b) Mean peak relaxations (expressed as a % of the PE contraction) elicited by EFS. The open columns represent the control relaxations and the solid columns the sequential responses obtained in the presence of a combination of L-NOARG (100 μM) and HbO (30 μM). Corresponding levels of initial active force to phenylephrine (expressed as a % of F_{max}) were (control: 39.3±1.6%, 0.5 Hz; 40.1±2.0%, 1.0 Hz; 37.2±2.5%, 2.0 Hz; 36.6±3.2%, 5.0 Hz; 33.5±2.8%, 10.0 Hz), (L-NOARG+HbO: 44.7±2.3%, 0.5 Hz; 46.6±0.9%, 1.0 Hz; 47.3±0.5%, 2.0 Hz; 47.3±0.9%, 5.0 Hz; 49.4±1.8%, 10.0 Hz). The error bars represent 1 s.e.mean from $n=4$. * Denotes significance at the $P<0.05$ level.

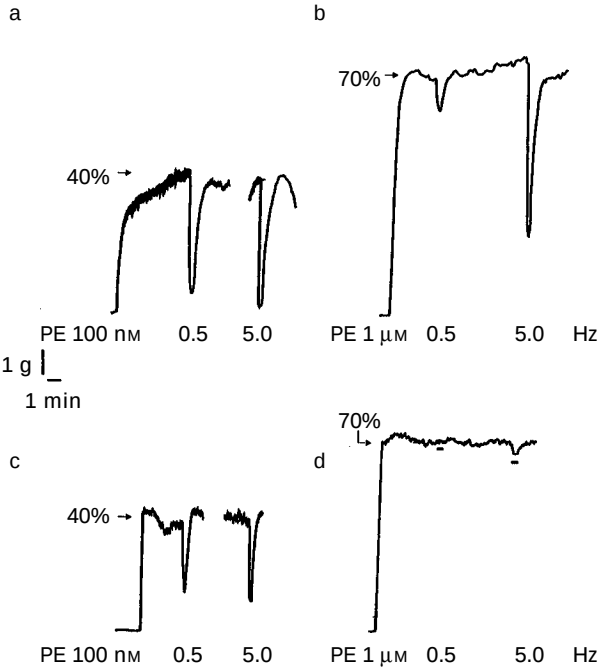

Figure 3 The effect of different levels of active force on NANC nerve-mediated relaxation in the rat anococcygeus. (a) and (b) Digitized traces of original chart recordings showing the responses to EFS (0.5, 5.0 Hz, 0.2 ms, supramaximal voltage for 20 s) of NANC inhibitory nerves in a single preparation contracted firstly to (a) ~40% F_{max} and then further contracted to (b) ~70% F_{max} with phenylephrine (PE). (c) and (d) Same experimental procedure as (a) and (b) except the responses to EFS were obtained in the presence of a combination of L-NOARG (100 μM) and HbO (30 μM) in a separate preparation. Traces are representative of $n=5$ experiments.

Effect of nifedipine

In the presence of nifedipine (0.3 μM), an ~3 fold higher concentration of phenylephrine was required to obtain a contraction of ~40% F_{max} (Figure 4). Under these conditions, EFS elicited frequency-dependent relaxations that were markedly suppressed compared to controls (Figure 4). Furthermore, relaxations to all frequencies of stimulation that were resistant to nifedipine were abolished by combined treatment with L-NOARG (100 μM) and HbO (30 μM) (Figure 5). Ethanol (0.03%), the amount of solvent for 0.3 μM nifedipine, had no effect on the relaxations to EFS in preparations contracted to ~40% F_{max} in the absence of any drug treatment.

Discussion

The present study has demonstrated that NO is unlikely to be the sole NANC inhibitory neurotransmitter in the rat anococcygeus. In fact, at low frequencies of stimulation (0.5–1 Hz) nearly all the response was due to a non-NO mechanism which was completely inactivated by nifedipine. Also, in preliminary studies we have shown that like nifedipine, a 'cocktail' of K^+ channel inhibitors, glibenclamide, charybdotoxin and apamin also inhibited these non-NO mediated relaxations (Selemidis & Cocks, unpublished observations). In the presence of either nifedipine or the K^+ channel inhibitors though, NANC nerve-mediated relaxations did not involve the closure of L-type voltage-operated Ca^{2+} channels (VOCCs) and were abolished by L-NOARG and HbO. This suggests that when preparations of rat anococcygeus are contracted by a non-depolarizing (nifedipine-insensitive) stimulus, any relaxation to EFS is due to NO only. However, as in most previous studies, we too were able to demonstrate readily near-complete block of NANC responses (in the absence of nifedipine) when tissues were contracted to ~70% F_{max}. Therefore, we suggest that a non-NO, hyperpolarization-dependent mechanism contributes to most of the NANC nerve-mediated relaxation, when active force is kept at low to medium levels and is dependent on Ca^{2+} influx through L-type VOCCs.

If NOS activity was not completely blocked by L-NOARG in our study, then the results could imply that residual, low levels of NO elicited the nifedipine-sensitive and presumably hyperpolarization-mediated relaxation. However, such an assumption seems unlikely since HbO, which has been shown to inhibit NANC nerve-mediated relaxations in rat anococcygeus preparations with high active force (Li & Rand, 1993), gave no additional block of these responses at ~40% F_{max} either alone or in combination with L-NOARG. Furthermore, the relaxation elicited by the lowest frequency of stimulation tested in the present study (ie. 0.5 Hz), where the release of NO would be expected to be small, was completely resistant to combined L-NOARG and HbO treatment. Moreover, the novel neuronal NOS inhibitor, 3-bromo-7-nitroindazole (Bland-Ward *et al*., 1994) gave no additional block in combination with L-NOARG and HbO, further support for our conclusion that the relaxation was independent of NO. However, it is possible that the slightly higher concentrations of phenylephrine required to raise active force to approximately 40% F_{max} in the presence of nifedipine somehow influenced the nature of NANC (NO)

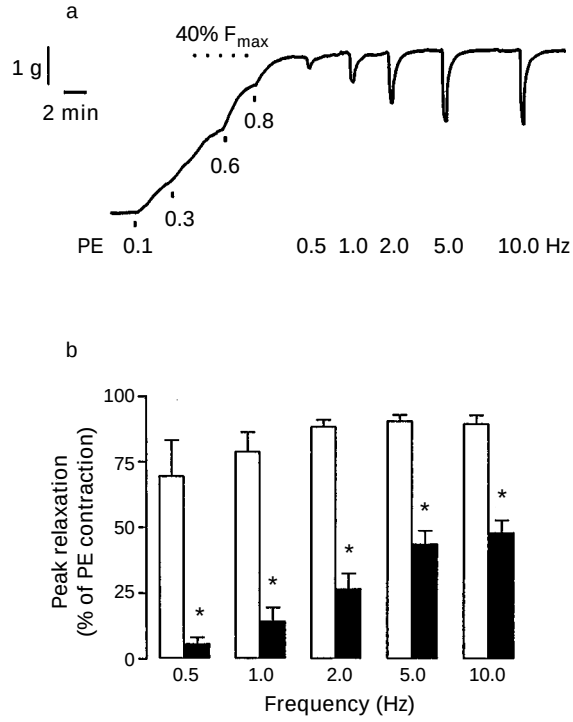

Figure 4 (a) Digitized trace of original chart recording showing NANC nerve-mediated relaxations to EFS (0.5–10.0 Hz, 0.2 ms, supramaximal voltage for 20 s) in a preparation of rat anococcygeus contracted to ~40% F_{max} with phenylephrine (PE), in the presence of nifedipine (0.3 μM). Note, the higher concentration of PE (in μM) required to obtain a ~40% F_{max} contraction. (b) Mean peak relaxations (expressed as a % of the PE contraction) elicited by EFS. The open columns represent control relaxations and the solid columns the sequential responses obtained in the presence of nifedipine (0.3 μM). Corresponding levels of initial active force to phenylephrine (expressed as a % of F_{max}) were (control: 36.8±1.4%, 0.5 Hz; 38.5±1.7%, 1.0 Hz; 40.1±1.9%, 2.0 Hz; 41.7±3.1%, 5.0 Hz; 40.0±2.3%, 10.0 Hz), (nifedipine: 38.7±0.9%, 0.5 Hz; 39.1±1.2%, 1.0 Hz; 39.5±1.2%, 2.0 Hz; 39.1±1.3%, 5.0 Hz; 39.1±1.6%, 10.0 Hz). The error bars represent 1 s.e.mean from $n=5$. * Denotes significance at the $P<0.05$ level.

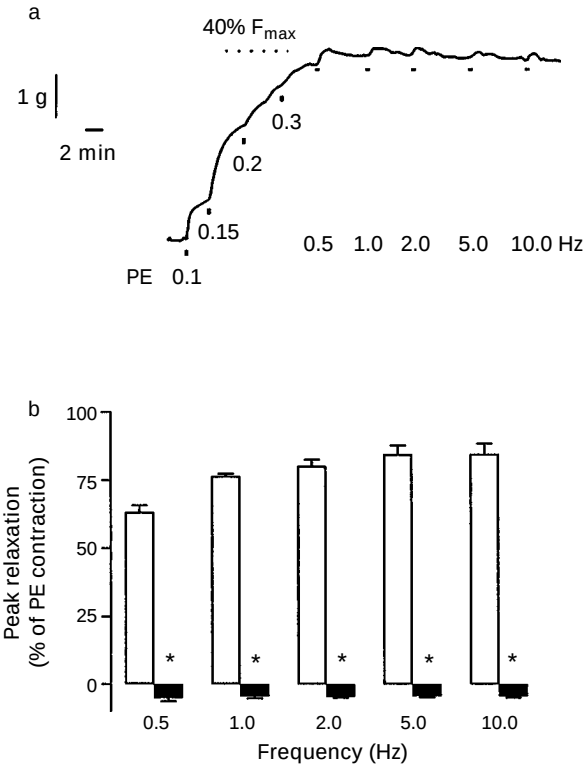

Figure 5 (a) Digitized trace of original trace chart recording showing NANC nerve-evoked responses to EFS (0.5–10.0 Hz, 0.2 ms, supramaximal voltage for 20 s) in a preparation of rat anococcygeus contracted to ~40% F_{max} with phenylephrine (PE, in μM), in the presence of a combination of nifedipine (0.3 μM), L-NOARG (100 μM) and HbO, (30 μM). (b) Mean peak responses (expressed as a % of the PE contraction) elicited by EFS. The open columns represent the control relaxations and the solid columns the sequential responses obtained in the presence of a combination of nifedipine (0.3 μM), L-NOARG (100 μM) and HbO (30 μM). Corresponding levels of initial active force to phenylephrine (expressed as a % of F_{max}) were (control: 39.6±4.1%, 0.5 Hz; 41.1±5.9%, 1.0 Hz; 39.6±7.1%, 2.0 Hz; 38.8±6.6%, 5.0 Hz; 38.9±5.8%, 10.0 Hz), (nifedipine + L-NOARG + HbO: 39.0±1.0%, 0.5 Hz; 40.2±1.3%, 1.0 Hz; 40.2±1.3%, 2.0 Hz; 39.8±1.3%, 5.0 Hz; 39.4±1.3%, 10.0 Hz). The error bars represent 1 s.e.mean from $n=3$. * Denotes significance at the $P<0.05$ level.

relaxations. This is unlikely given our findings that NO appears to have little or no role in mediating responses to NANC inhibitory nerve stimulation at frequencies $\leqslant$2 Hz in the absence of nifedipine.

Our results also demonstrate that the maximal relaxation to EFS in tissues contracted to ~40% F_{max} had already occurred at 0.5 to 1 Hz. This was unlikely to be due to too little functional antagonism by the contracting agent, phenylephrine, since all frequencies of stimulation gave peak responses no greater than ~75% of the contraction to phenylephrine. Although small, NO-dependent responses at frequencies of stimulation $\leqslant$2 Hz occurred in the presence of nifedipine they were not apparent in the presence of the putative hyperpolarizing factor, ie. in the absence of nifedipine. This implies that up to approximately 2 Hz these small amounts of NO released with the non-NO factor contribute little to the relaxation response. Therefore, our findings not only raise the important issue of physiological significance of neuronal NO at least in these NANC nerves, but also suggest that the mechanism and amplitude of contraction in the anococcygeus may markedly influence the responses to both NO and the non-NO transmitter. For example, in tissues contracted via α_1-adrenoceptor activation to about half their maximal capacity, low frequency NANC nerve stimulation elicits powerful relaxations via a hyperpolarizing mechanism which is independent of NO. If the tissue is contracted to the same level of active force by another stimulus, which does not involve the influx of Ca^{2+} via VOCCs, then higher frequencies of stimulation are required to release sufficient NO to produce equivalent levels of relaxation.

Many blood vessels similarly utilize a hyperpolarizing factor, EDHF together with NO to mediate endothelium-dependent relaxation of vascular smooth muscle (Garland *et al.*, 1995). However, the type and relative contribution of EDHF to this response varies considerably. For example in rat mesenteric arteries, EDHF acts in parallel with NO (Parsons *et al.*, 1994), whereas in the pig (Kilpatrick & Cocks, 1994) and cow coronary arteries (Drummond & Cocks, 1996) it acts as a backup system for NO. However, in all cases NO remains the dominant relaxing factor with EDHF release activated at higher stimulus strengths. Also, as found in this study, the effects of the vascular hyperpolarizing factor are readily masked by excessive levels of active force such that all the endothelium-dependent relaxations are due to NO (Kilpatrick & Cocks, 1994). However, unlike EDHF in blood vessels, the nerve-derived hyperpolarizing factor in the anococcygeus appears to be the dominant relaxing factor over a lower stimulus strength range.

In conclusion, we propose that a non-NO, nerve-derived hyperpolarizing factor (which we have termed NDHF) not NO, elicits nearly all the relaxation responses to low frequency

stimulation of NANC nerves in the rat isolated anococcygeus, particularly when the tissue is submaximally contracted by a depolarizing (nifedipine-sensitive) stimulus. In contrast, NO is the only relaxing factor evident in preparations with high active force and only mediates relaxations at higher frequencies of stimulation (>1 Hz). Finally, whilst our results suggest that endothelial cells and NANC inhibitory nerves share similar mechanisms for mediating smooth muscle relaxation, the type of contribution by each mechanism to the overall response may differ. Without knowledge of *in vivo* levels of active force or tone in both preparations, we can only speculate as to the physiological significance of our findings. However, one possibility is that NO in NANC inhibitory nerves act as a backup for NDHF, the reverse of which occurs for EDHF and NO in blood vessels.

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