



Effects of hydroxocobalamin and carboxy-PTIO on nitrenergic transmission in porcine anococcygeus and retractor penis muscles

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1 The effects of carboxy-PTIO and hydroxocobalamin were studied on nitrenergic transmission in anococcygeus and retractor penis muscles taken during *post mortem* examination from young male pigs.

2 In both muscles under resting conditions, electrical field stimulation (EFS) caused contractions that were sensitive to tetrodotoxin (1 μ M) and were greatly inhibited by prazosin (1 μ M) and guanethidine (10–30 μ M), but were not significantly affected by atropine (1 μ M). In the anococcygeus muscle, but not in the retractor penis muscle, guanethidine produced a prolonged contraction.

3 After tone was raised by guanethidine in the anococcygeus or by phenylephrine (1 μ M) in the presence of guanethidine in the retractor penis, EFS caused tetrodotoxin-sensitive relaxations. The EFS-induced relaxations were abolished by the NO synthase inhibitor N^G-L-nitro-arginine methyl ester (L-NAME; 100 μ M) and its effect was partly overcome by L-arginine (1 mM), indicating it was mediated by nitrenergic nerves.

4 Carboxy-PTIO (0.1–1 mM) had no significant effect in reducing stimulation-induced nitrenergic relaxations in either muscle. However, hydroxocobalamin (0.1–1 mM) caused concentration-dependent reductions of nitrenergic relaxations in both muscles. Relaxations to exogenous nitric oxide (1 μ M) in both muscles were abolished by carboxy-PTIO (0.3 mM) and hydroxocobalamin (0.1 mM).

5 There were no differences in reactivity to carboxy-PTIO or hydroxocobalamin between anococcygeus and retractor penis muscles from the same species (pig). The finding also confirms earlier observations that the nitrenergic transmitter is generally resistant to the NO-scavenger carboxy-PTIO.

Keywords: Anococcygeus (porcine); carboxy-PTIO; hydroxocobalamin; nitric oxide; nitrenergic transmission; retractor penis muscle (porcine)

Abbreviations: ANOVA, one-way analysis of variance; EFS, electrical field stimulation; L-NAME, N^G-L-nitro-arginine methyl ester; NANC, non-adrenergic non-cholinergic; L-NMMA, N^G-monomethyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; PSS, physiological salt solution; TTX, tetrodotoxin

Introduction

Most of the research which led to the initial recognition of nitrenergic transmission at autonomic neuroeffector junctions was carried out with the rat anococcygeus and bovine retractor penis muscles, and these were the first tissues in which peripheral nitrenergic transmission was demonstrated (for reviews, see: Gillespie *et al.*, 1990; Martin & Gillespie, 1991; Rand & Li, 1995a,b).

Although there is indisputable evidence for nitrenergic transmission in both muscles, a number of differences have been reported in their responsiveness to various agents. The first difference that was noted was that the nitric oxide synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA; methylarginine) readily inhibited nitrenergic transmission in rat anococcygeus muscle (Li & Rand, 1989; Gillespie *et al.*, 1989), but did not in the bovine retractor penis muscle (Gillespie *et al.*, 1989). In fact L-NMMA, like L-arginine, restored nitrenergic relaxations of the bovine retractor penis that had been blocked by the more potent NOS inhibitor N^G-nitro-L-arginine (Martin *et al.*, 1993). More recently, it has been reported that the NO-sequestering substance hydroxocobalamin produces concentration-dependent reductions in nitrenergic nerve stimulation-induced relaxations in the bovine retractor

penis muscle (Paisley & Martin, 1996), but did not reduce nitrenergic transmission in rat anococcygeus muscles, at least in concentrations that blocked responses to exogenous nitric oxide (Rajanayagam *et al.*, 1993; Li & Rand, 1993). Furthermore, the NO scavenger carboxy-PTIO did not inhibit nitrenergic relaxations in rat and mouse anococcygeus muscles (Rand & Li, 1995c; Lilley & Gibson, 1996) but blocked them in the bovine retractor penis muscle (Paisley & Martin, 1996).

The fact of these discrepancies raises the question as to whether they are due to species or tissue differences. It seems unlikely that there is a tissue difference since the retractor penis is contiguous with the anococcygeus muscles (Klinge & Sjöstrand, 1974; Martin & Gillespie, 1991). The two bands of the anococcygeus muscles unite in a bar on the ventral aspect of the terminal portion of the colon and then project forward as the single retractor penis muscle (or pair of muscles in some species) to their attachment to the cartilaginous shaft of the penis. The muscle is absent in primates and rabbits and is vestigial in rats. Both the anococcygeus and the retractor penis muscles have a noradrenergic sympathetic motor innervation and a NANC parasympathetic inhibitory innervation (Sjöstrand & Klinge, 1995). However, anatomical facts about the two muscles do not provide adequate grounds for surmise about the nature of their neurotransmitter mechanisms.

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We had the opportunity to obtain anococcygeus and retractor penis muscles from freshly killed pigs that were undergoing *post mortem* examination in the course of another series of experiments (see Methods). Therefore, we set out to test in them the agents that had different effects between the rat anococcygeus muscle and the bovine retractor penis. The preliminary results of the findings reported here have been communicated to the Australian Society of Clinical and Experimental Pharmacologists and Toxicologists (Li *et al.*, 1996).

Methods

Young male pigs (40–50 kg) which acted as controls in a study on vaccine production were stunned and bled. During *post mortem* examination, the anococcygeus and retractor penis muscles were removed, transported to the laboratory in cold oxygenated physiological salt solution (PSS), and longitudinal portions of muscle strips 2–3 mm wide and 1 cm long of each were set up in organ baths at a resting tension of 1 g as described previously for the rat anococcygeus muscle (Gillespie 1972; Li & Rand, 1989). The Royal Melbourne Institute of Technology animal ethics committee approved these additional experiments.

Responses were recorded as changes in isometric tension in response to electrical field stimulation of intrinsic nerves and exogenously applied NO in aqueous solution. The effects of

NO-related agents were studied after the tone of the muscle was raised (see Results). Nitroergic relaxations were evoked by electrical field stimulation (EFS) with 1 ms pulses of supramaximal voltage at 2 Hz for 10 s at 2 min intervals applied through a pair of platinum wire electrodes at either side of the muscle. Parallel experiments without addition of drugs acting on nitroergic mechanisms were carried out with tissues from the same donor animal as time controls.

The composition of the PSS was as follows (mM): NaCl, 118; KCl, 4.7; NaHCO₃, 25; MgSO₄, 0.45; KH₂PO₄, 1.03; CaCl₂, 2.5; d-(+)-glucose, 11.1; disodium edetate, 0.067.

The following drugs were used: L-arginine, atropine sulphate, guanethidine sulphate, hydroxocobalamin hydrochloride, N^G-nitro-L-arginine methyl ester (L-NAME), L-phenylephrine hydrochloride, tetrodotoxin (TTX) (Sigma Chemical Co., U.S.A.); carboxy-PTIO (Sapphire Bioscience, Australia); prazosin hydrochloride (Pfizer, U.S.A.). The NO solution (2 mM) was prepared from NO compressed gas (Commonwealth Industrial Gases, Melbourne, Australia) as previously described (Rajanayagam *et al.*, 1993).

Data are expressed as means and standard errors. The significance of differences between means was determined by Student's *t*-test or one-way analysis of variance (ANOVA). Values of *P* < 0.05 were considered as significant.

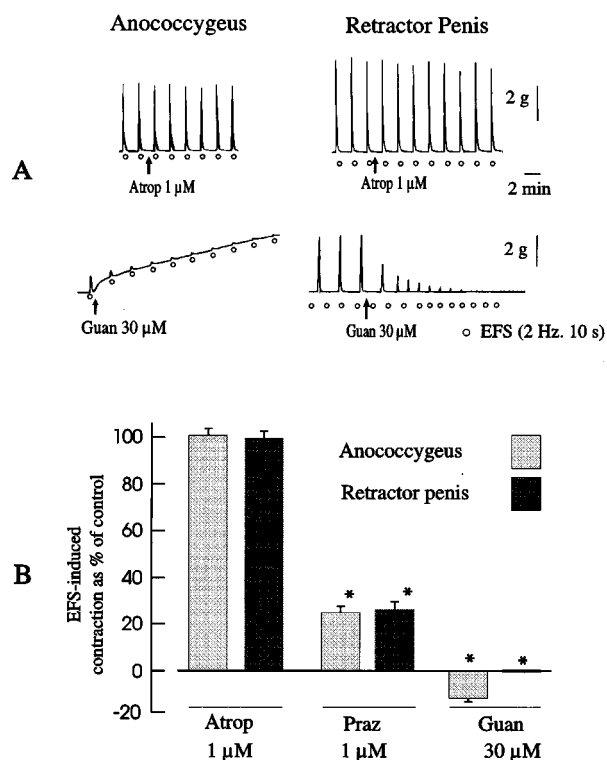


Figure 1 (A) Tracings illustrating the effects of atropine and guanethidine on field stimulation-induced contractile responses of the pig anococcygeus and retractor penis muscle. (B) Mean data for the effects of atropine, guanethidine and prazosin on field stimulation-induced contractions of the pig anococcygeus and retractor penis muscles. Columns represent means and I-bars indicate the s.e.mean of 4–5 experiments. *Indicates a significant difference (*P* < 0.05, Student's *t*-test) from the corresponding control group.

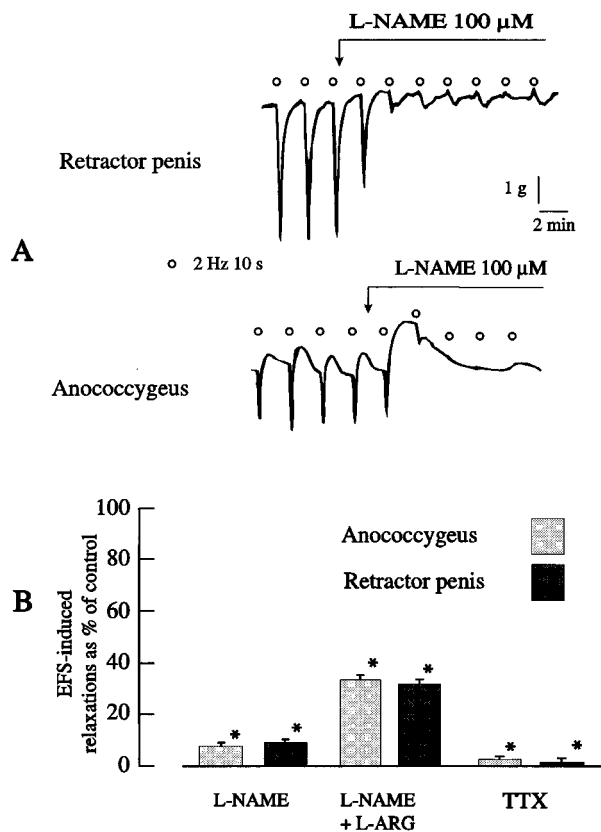


Figure 2 (A) Tracings illustrating the effects of L-NAME on field stimulation-induced relaxant responses of the pig anococcygeus and retractor penis muscles. (B) Mean data for the effects of L-NAME (100 μM), L-NAME plus L-arginine (1 mM) and tetrodotoxin (1 μM) on field stimulation-induced relaxations of the pig anococcygeus and retractor penis muscles. Columns represent means and I-bars indicate the s.e.mean of 4–5 experiments. *Indicates a significant difference (*P* < 0.05, Student's *t*-test) from the corresponding control group.

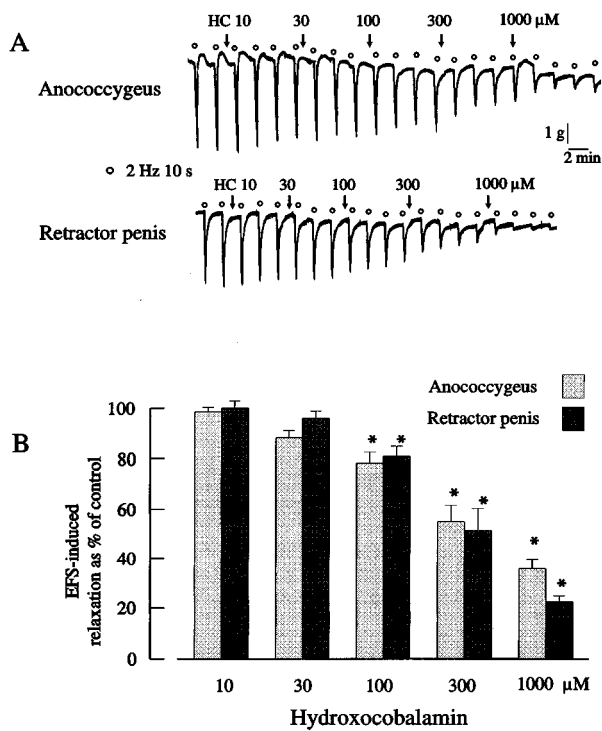


Figure 3 (A) Tracings illustrating the effects of hydroxocobalamin on field stimulation-induced relaxations of the porcine anococcygeus and retractor penis muscles. (B) Mean data for effects of hydroxocobalamin on field stimulation-induced relaxations of the pig anococcygeus and retractor penis muscles. Columns represent means and I-bars indicate the s.e.mean of 4–5 experiments. *Indicates a significant difference ($P < 0.05$, ANOVA followed by the Bonferroni t -test) from the corresponding control group.

Results

In both anococcygeus and retractor penis muscles under resting conditions, EFS (1–5 Hz, 10 s) produced frequency-dependent contractions which were abolished by tetrodotoxin (1 µM) (data not shown). Contractions induced by EFS at 2 Hz were 5.2 ± 0.6 g ($n = 7$) and 5.6 ± 0.4 g ($n = 8$), respectively, in the anococcygeus and the retractor penis muscles. EFS-induced contractions were not significantly affected by atropine (1 µM, Figure 1A), but were significantly reduced by prazosin (1 µM, Figure 1B). The noradrenergic nerve blocking agent guanethidine (10–30 µM) blocked EFS-induced contractile responses in both muscles and increased the tone in the anococcygeus but not in the retractor penis muscle (Figure 1).

In anococcygeus muscles, guanethidine alone raised the tone and relaxant responses to EFS were revealed. In retractor penis muscles, the tone was raised by phenylephrine (1 µM) so that relaxations could be manifested. In both muscles, the EFS-induced relaxations were abolished by tetrodotoxin (1 µM, Figure 2B) and by the NO synthase inhibitor L-NAME (100 µM; Figure 2). The effects of L-NAME were partly overcome by L-arginine (1 mM; Figure 2B).

Hydroxocobalamin (0.1–1 mM) caused concentration-dependent reductions of nitric relaxations in both muscles (Figure 3). Carboxy-PTIO (0.01–1 mM) had no significant effect on stimulation-induced nitric relaxations in either muscle (Figure 4). In both muscles, relaxations in response to exogenous NO (1 µM) were very similar in shape and amplitude as those to electrical stimulation. They were reduced or abolished by carboxy-PTIO (0.1 and 0.3 mM) or hydrox-

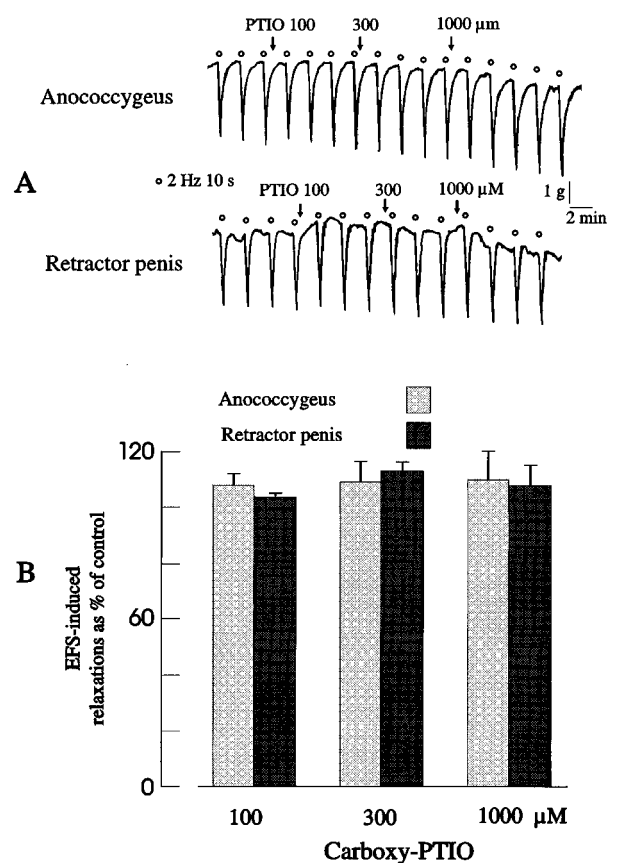


Figure 4 (A) Tracings illustrating the effects of carboxy-PTIO on field stimulation-induced relaxations of the porcine anococcygeus and retractor penis muscles. (B) Mean data for the effects of carboxy-PTIO on field stimulation-induced relaxations of the pig anococcygeus and retractor penis muscles. Columns represent means and I-bars indicate the s.e.mean of 4–5 experiments.

ocobalamin (30 and 100 µM). The responses to exogenous NO in the presence of 0.1 and 0.3 mM carboxy-PTIO were $19.3 \pm 4.4\%$ ($n = 4$) and $0.2 \pm 0.3\%$ ($n = 4$), respectively, of the control response ($P < 0.01$, Student's t -test), and $59.7 \pm 4.3\%$ ($n = 4$) and $4.5 \pm 0.6\%$ ($n = 4$) of the control response ($P < 0.01$, Student's t -test), respectively, in the presence of 30 and 100 µM hydroxocobalamin.

Discussion

The innervations of the anococcygeus muscle and retractor penis muscle have been described by a number of workers in several species. In general, there is a dense network of typical noradrenergic fibres and a less prominent network of nitric fibres (nNOS immunoreactive and NADPH-diaphorase positive) (Klinge *et al.*, 1970; Gillespie & Lüllmann-Rauch, 1974; Gibbins & Haller, 1979; Byrne & Muir, 1984; Sheng *et al.*, 1992; Brave *et al.*, 1993; Dail *et al.*, 1990, 1993). The finding that the contractile responses to EFS in both anococcygeus and retractor penis muscles of young pigs were greatly inhibited or blocked by the α_1 -adrenoceptor antagonist prazosin and by tetrodotoxin indicate that these responses are largely noradrenergic in origin. Similarly, the findings that EFS-induced relaxations in both tissues are blocked by the NOS inhibitor L-NAME, and the effect of L-NAME was partly reversed by L-arginine, indicates that these relaxations

are mediated by nitrenergic nerves, as already established in these tissues from other species (see Introduction). We are not aware of any previous studies on EFS-induced responses in pig anococcygeus muscle; however, an earlier study by Ambache & Killick (1978) reported a species difference in EFS-induced contractile responses of retractor penis muscles from pig and sheep compared with horse and dog. In their study, a portion of the EFS-induced contraction in adult pig retractor penis muscle was resistant to phentolamine. It seems that a non-adrenergic component may also be involved in EFS-induced contractions in the immature pigs used in the present study, as a small portion of the EFS-induced contractions was prozosin resistant. However, the nature of the non-adrenergic component is not clear. Nevertheless, the main difference in contractile responses we observed is that guanethidine increased the tone in the anococcygeus muscle but not in the retractor penis muscle; this effect may be related to the density of the excitatory nerve fibres in these tissues.

Although nitrenergic transmission is absolutely dependent on the functional integrity of nitric oxide synthase, it has been found that responses to exogenous NO and nitrenergic nerve stimulation are differentially affected by certain agents such as superoxide generators and NO scavengers including carboxy-PTIO (Rand & Li, 1995a,b). These agents generally block the responses to exogenous NO but have little effect on responses to nitrenergic nerve stimulation (Hobbs *et al.*, 1991; Rand & Li, 1995c; Lefebvre, 1996).

Carboxy-PTIO is a NO scavenger that inactivates free radical NO (Akaike *et al.*, 1993). It has been shown that carboxy-PTIO inhibits responses to exogenous NO in the rat and mouse anococcygeus muscles but not those induced by nitrenergic nerve stimulation (Rand & Li, 1995c; Lilley & Gibson, 1996). However, in the bovine retractor penis, nitrenergic responses were inhibited by carboxy-PTIO (Paisley & Martin, 1996). In the present study, carboxy-PTIO in concentrations that blocked responses to exogenous NO failed to inhibit EFS-induced nitrenergic relaxations in either the anococcygeus muscle or the retractor penis muscle of the pig, indicating that the differences between rat anococcygeus and bovine retractor penis muscles are likely to be due to a species difference.

Recent debate on the mechanism of nitrenergic transmission has offered several explanations for the contrasting actions of certain superoxide generators and NO-scavengers on authentic NO and nitrenergic transmission. These include the protection of NO by endogenous superoxide dismutase from inactivation by superoxide (Martin *et al.*, 1994) and the protection of NO by antioxidant agents from inactivation by some NO-scavengers (Lilley & Gibson, 1997), as well as the hypothesis that the nitrenergic transmitter may not be free radical NO (Rand & Li, 1995a). Since the effect of carboxy-PTIO is not affected by

SOD inhibitors (Rand & Li, 1995c), the involvement of superoxide is unlikely to be involved. On the other hand, the release of antioxidant ascorbate from the nitrenergically innervated tissues upon electrical field stimulation (Lilley & Gibson, 1997) may explain the inability of carboxy-PTIO to block the responses to exogenous NO. However it is not clear whether a sufficient amount of ascorbate is released from nitrenergic nerves to interact with carboxy-PTIO. Further experiments are necessary to explore the possible mechanism of nitrenergic transmission that is resistant to carboxy-PTIO in pig anococcygeus and retractor penis muscles.

Hydroxocobalamin interacts with NO to form an unidentified NO-adduct (Rochelle *et al.*, 1995). Hydroxocobalamin blocked responses to exogenous NO in concentrations that only slightly inhibited or had no effect on responses to nitrenergic nerve stimulation in the rat anococcygeus muscle (Li & Rand, 1993; La *et al.*, 1996) and other nitrenergically-innervated tissues such as rat gastric fundus (Jenkinson *et al.*, 1995; Lefebvre, 1996) and dog ileocolonic junction (De Man *et al.*, 1995), but it inhibited responses to nitrenergic nerve stimulation in the mouse anococcygeus muscle (Lilley & Gibson, 1996) and the bovine retractor penis (Paisley & Martin, 1996) as well as in the guinea-pig basilar artery (Jiang *et al.*, 1997). In the present study, hydroxocobalamin produced concentration-dependent inhibitions of responses to nitrenergic nerve stimulation in both the anococcygeus muscle and retractor penis muscle. Explanations for the difference between tissues in regard to actions of hydroxocobalamin may include differences in experimental conditions (La *et al.*, 1997) and may involve tissue factors, although inhibition of superoxide dismutase did not affect the actions of hydroxocobalamin (Lefebvre, 1996; Paisley & Martin, 1996).

In conclusion, the results reveal that there are no differences in reactivity to carboxy-PTIO or hydroxocobalamin between anococcygeus and retractor penis muscles from the same species (pig), and indicate that the difference between rat anococcygeus and bovine retractor penis muscles is due to a species difference. Furthermore, the observations are in accord with the view that the nitrenergic transmitter is generally resistant to the NO scavenger carboxy-PTIO, indicating either that the transmitter is protected by one or more endogenous substances or that it may not be free radical NO.

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