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The effects of pyrogallol and hydroquinone on the response to NANC nerve stimulation in the rat anococcygeus and the bovine retractor penis muscles

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1 The effects of pyrogallol and hydroquinone on the bovine retractor penis (BRP) and rat anococcygeus muscles to non-adrenergic, non-cholinergic (NANC) nerve stimulation have been examined. Both drugs at a concentration of 10^{-4} M significantly reduced the response in the rat anococcygeus muscle but had no effect in the BRP muscle.

2 The inhibition of the NANC response in the rat anococcygeus muscle by pyrogallol was completely reversed by superoxide dismutase suggesting it was due to the generation of superoxide anions.

3 Pyrogallol inhibited the response to nitric oxide (NO) in the rat anococcygeus muscle but not that to 3-isobutyl-1-methyl-xanthine (IBMX) which confirmed a selective action.

4 These results suggest that the NANC neurotransmitter in the rat anococcygeus muscle is susceptible to superoxide anions and may be NO or a substance that can liberate NO.

Introduction

The existence of a powerful non-adrenergic, non-cholinergic (NANC) inhibitory innervation in the rat anococcygeus and bovine retractor penis (BRP) muscles was first reported by Gillespie (1972) and Klinge & Sjöstrand (1974). The identity of the inhibitory neurotransmitter however remains unknown (Gillespie, 1982; 1987). The mechanism of action of these nerves is through stimulation of guanylate cyclase and an increase in guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Bowman & Drummond, 1984). The effects of nerve stimulation are blocked by haemoglobin (Bowman & Gillespie, 1981; 1982; Bowman *et al.*, 1982) and by hypoxia (Bowman & McGrath, 1985). Another powerful smooth muscle relaxant with a similar profile of drug interaction is the endothelium-derived relaxant factor (EDRF). The relaxant effect of EDRF is also associated with the stimulation of guanylate cyclase (Rapoport & Murad, 1983) and is blocked by haemoglobin (Martin *et al.*, 1985). EDRF is almost certainly nitric oxide (NO) and its precursor is L-arginine (Palmer *et al.*, 1987; 1988). We have recently found that L-N^Gmonomethyl-L-arginine (L-NMMA), a competitive inhibitor of L-arginine, will inhibit the response to NANC nerve stimulation in the rat anococcygeus muscle but not in the BRP muscle (Gillespie & Xiaorong, 1989; Gillespie *et al.*, 1989) suggesting the neurotransmitter, at least in the rat anococcygeus muscle, may also be NO. NO is rapidly destroyed particularly in the presence of superoxide radicals and this effect can be prevented by superoxide dismutase (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986). If the NANC neurotransmitter is NO then the response to nerve stimulation should be reduced or abolished by drugs which generate superoxide anions and this effect should be reversed by superoxide dismutase. This paper describes results with two such drugs, pyrogallol, a potent generator of superoxide anions (Murklund &Murklund, 1974) and hydroquinone, a hydroxy radical scavenger which prevents the activation of guanylate cyclase by superoxide dismutase (Mittal & Murad, 1977).

Methods

BRP muscle was obtained from the local abattoir and kept in cold Krebs saline at 4°C until used usually within 3 h of death but on occasions up to two days later. Muscles were cleaned of connective tissue and thin slips, 2–3 mm in diameter and 1.5 cm in length, were removed. The rat anococcygeus muscle was isolated as previously described (Gillespie, 1972). Both the BRP and the rat anococcygeus preparations were suspended between pairs of Ag–AgCl ring electrodes under 1 g resting tension in a 10 ml organ bath. The tension was measured by a Grass FTO3 transduce and displayed on a Grass polygraph. The organ bath contained Krebs saline at 36°C and was bubbled with 95% O_2 + 5% CO_2. The motor adrenergic nerves were blocked and tone raised by addition of guanethidine (3×10^{-5} M). Field stimulation through the ring electrodes was applied from a Grass S44 stimulator at supramaximal voltage, 1 ms pulse width and trains of stimuli lasting 10 s. The composition of the Krebs saline in mM was: Na^+ 145, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 127, HCO_3^- 25, HPO_4^{2-} 1.2, SO_4^{2-} 1.2 and dextrose 11. Drugs were made up from 10^{-2} M stock solution in distilled water; final solutions were in normal saline. The following drugs were used: guanethidine sulphate (CIBA); hydroquinone (Sigma); 3-isobutyl-1-methyl-xanthine (Sigma); pyrogallol (BDH); superoxide dismutase (Sigma). NO stock solution at 220 μM was prepared freshly each day from gaseous NO, 99% pure from BDH as described previously (Gillespie & Sheng, 1988). Briefly, a soft rubber tube was attached to the cylinder with its other end under water and enough gas allowed to escape to expel all air from the tubing. The tubing wall close to the cylinder was then pierced with a needle attached to a syringe and 330 μl of gas removed. This was then injected into a rubber-sealed 65 ml brown bottle completely filled with normal saline previously purged for 1 h with helium.

Results

The effects of pyrogallol and hydroquinone on the response of BRP to NANC nerve stimulation

The effects of pyrogallol and hydroquinone on the frequency-response curves to NANC nerve stimulation between 0.2 and 10 Hz in the BRP muscle are illustrated in Figure 1. Both drugs at 10^{-4} M had no effect on the frequency-response curves to NANC nerve stimulation. This concentration level

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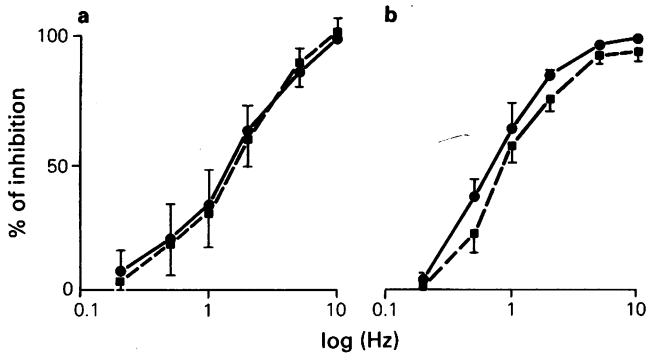

Figure 1 Log frequency-response curves to field stimulation of NANC nerves at frequencies between 0.2 and 10 Hz for 10 s in the bovine retractor penis muscle. Tone was induced by guanethidine (30 μM). Control responses (●) and reponses in the presence of pyrogallol (a) or hydroquinone (b) both at 0.1 mM (■) are indistinguishable. Bars represent ± s.e. $n = 4$ for with pyrogallol, $n = 5$ with hydroquinone.

was subsequently shown to produce significant inhibition in the rat anococcygeus muscle.

The effects of pyrogallol and hydroquinone on the response of the rat anococcygeus muscle to NANC nerve stimulation

The effects of pyrogallol and hydroquinone were also tested on the rat anococcygeus muscle response to NANC nerve stimulation. The experimental arrangements and the concentration (10^{-4} M) of both drugs were the same as used in the BRP muscle. As Figure 2 shows, the results were quite different and both pyrogallol and hydroquinone reduced the response, with pyrogallol proving more effective than hydroquinone. The effect of different concentrations of pyrogallol on the frequency-response curves was examined and the results are shown in Figure 3a. Pyrogallol over the dose range 10^{-5} to 10^{-4} M produced a concentration-related inhibition of the NANC response. At 10^{-5} M the reduction in response was not significant but at 3×10^{-5} M and at 10^{-4} M the effect was statistically significant. If the inhibitory effect of both drugs is due to the generation of superoxide anion, it should be reversed by superoxide dismutase, a superoxide anion scavenger. This was tested by adding superoxide dismutase (100 u ml^{-1}) to the bath solution 10 min after pyrogallol (10^{-4} M). The results are illustrated in Figure 3b. Pyrogallol inhibited the response to NANC nerve stimulation and this inhibition was completely reversed by superoxide dismutase.

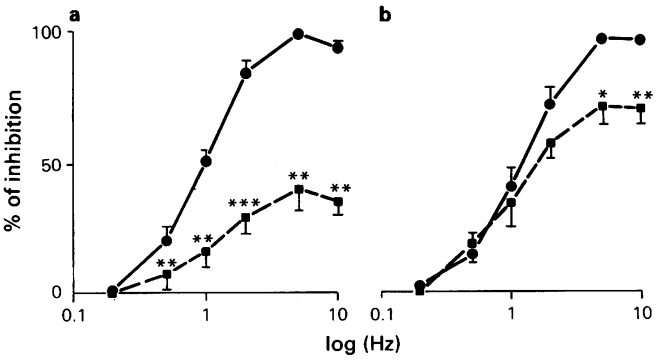

Figure 2 Log frequency-response curves to field stimulation of NANC nerves at frequencies between 0.2 and 10 Hz for 10 s in the rat anococcygeus muscle. Tone was induced by guanethidine (30 μM). Comparison of control responses (●) and responses in the presence of pyrogallol (a) or hydroquinone (b), both at 0.1 mM (■), show a significant reduction particularly with pyrogallol. Bars represent ± s.e., $n = 5$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

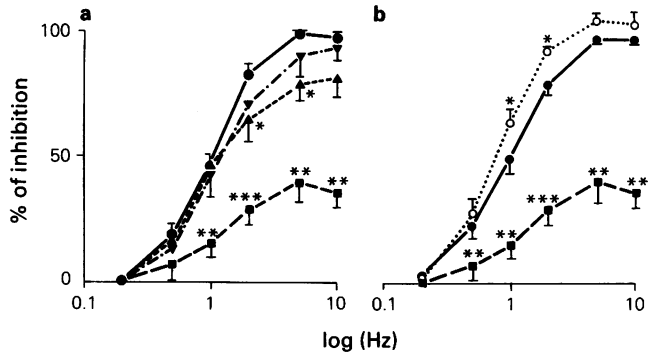

Figure 3 Log frequency-response curves to field stimulation of NANC nerves at frequencies between 0.2 and 10 Hz for 10 s in the rat anococcygeus muscle. Panel (a) shows the graded inhibitory effect of pyrogallol at 10 μM (▼), 30 μM (▲) and 100 μM (■) in comparison with the control (●). Panel (b) shows the control responses (●), their inhibition by pyrogallol (100 μM, ■) and the complete reversal of that inhibition by superoxide dismutase (100 u ml^{-1}, ○). Tone was induced by guanethidine (30 μM). Bars represent ± s.e., $n = 4$–6. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Indeed the amplitude of the responses in the presence of both pyrogallol and superoxide dismutase exceeded the control response suggesting that even in the absence of pyrogallol, the generation of superoxide radicals in a vigorously oxygenated solution is sufficient to produce some reduction in the response. These results suggest that the effect of pyrogallol on the response to NANC nerve stimulation in this tissue is indeed due to the generation of superoxide anions.

Response to other relaxants

The concentrations of pyrogallol used in these experiments were fairly high and it was possible that the inhibitory effect was non-specific. The absence of any inhibitory effect of this same concentration on the NANC response of the BRP was reassuring but other controls were desirable. If the effect of pyrogallol is entirely due to the generation of superoxide radicals then it should inhibit the response to NO which is highly sensitive to such radicals. On the other hand, IBMX which acts by inhibiting phosphodiesterase should be unaffected. The effect of pyrogallol (10^{-4} M) on the concentration-response curves to these two drugs is shown in Figure 4. Pyrogallol shifted the concentration-response curve to NO to the right but had no effect on the inhibitory response to IBMX. These results are further confirmation that pyrogallol acts by superoxide anion generation and has no direct effect on guanylate cyclase or cyclic nucleotide formation.

Discussion

The selective inhibition of the NANC response in the rat anococcygeus but not in the BRP muscle by pyrogallol and its reversal by superoxide dismutase is suggestive not simply that the effect is mediated by the generation of superoxide anions but that there is some difference in the neurotransmitters in the two muscles. Other evidence supporting such a difference has been found in this laboratory. For example, the response to NANC nerve stimulation in the rat anococcygeus but not the BRP muscle is blocked by L-NMMA a competitive inhibitor of L-arginine (Gillespie & Xiaorong, 1989; Gillespie *et al.*, 1989), as a source of EDRF in endothelial cells (Palmer *et al.*, 1988). The simplest explanation would be to assume that the neurotransmitters in the two tissues are different and NO may be involved only in the rat anococcygeus muscle. We have no direct evidence against such a proposition but much indirect evidence makes us reluctant to accept it. The ability of haemoglobin to block the response in both tissues, the implication of guanylate cyclase in the response and the homology of the

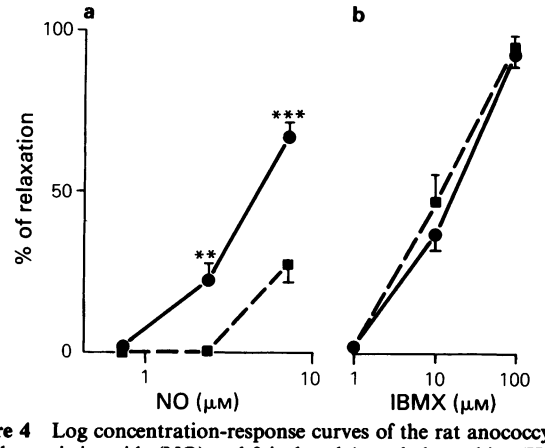

Figure 4 Log concentration-response curves of the rat anococcygeus muscle to nitric oxide (NO) and 3-isobutyl-1-methyl-xanthine (IBMX) and the effect of pyrogallol (10^{-4} M) on these inhibitory responses. (a) The control curve (●), for nitric oxide is shifted to the right by pyrogallol (■), consistent with the accelerated destruction by superoxide anions whereas the curve for IBMX (b) is unaffected by pyrogallol. Tone was induced by guanethidine (30 μM). Bars represent ± s.e., $n = 6$.

two muscles make it unlikely. In those animals which possess a well developed retractor penis muscle this is a direct continuation of the anococcygeus muscle and the pharmacology and histochemistry of the two muscles, at least in the ox, are identical. An alternative proposition would be to assume the neurotransmitter is the same but the interaction with the two muscles differs in some significant way. One possibility is to assume a neurotransmitter which readily liberates NO within the tissues i.e. a substance functionally related to the nitrovasodilator. If this neurotransmitter liberates NO intracellularly then the ability of superoxide anions to inhibit its effect will depend on the ease of penetration of the superoxide anions into the smooth muscle cells and the concentration of endogeneous superoxide dismutase within the cell. If the concentration of the latter within the muscle cells of the BRP is high then pyrogallol might have little effect. It would also explain the high sensitivity of the BRP and the low sensitivity of the rat anococcygeus muscle to NO and to EDRF (Gillespie & Sheng, 1988). If the released neurotransmitter was not itself NO but a less readily diffusible precursor of higher molecular weight, it would also explain the inability of erythrocytes to reduce the response to NANC nerve stimulation, even though they do reduce the response to EDRF released *in situ* (Gillespie & Sheng, 1989). A major objection to such an explanation remains the ability of L-NMMA to inhibit the response to NANC nerve stimulation in the rat anococcygeus muscle but not the BRP muscle, suggesting the synthetic pathway to neurotransmitter formation is different.

Recently Gibson & Mirzazadeh (1989) failed to find any effect of hydroquinone at a concentration of 5×10^{-5} M on the response to NANC nerve stimulation in the mouse anococcygeus muscle. In view of the weaker effect of hydroquinone than pyrogallol in the present results and the threshold for a statistically significant study of the latter at 3×10^{-5} M, it may be the ineffectiveness in the mouse is a concentration effect and it, like the muscle in the rat, would prove sensitive to pyrogallol at an appropriate concentration.

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