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The effect of hypoxia on neuroeffector transmission in the bovine retractor penis and rat anococcygeus muscles

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1 The effects of reducing the P_{O_2} of the bathing fluid were studied on non-adrenergic non-cholinergic (NANC) transmission in isolated preparations of the bovine retractor penis muscle, the rat anococcygeus muscle, the guinea-pig taenia caeci and the guinea-pig urinary bladder.

2 Hypoxia rapidly and reversibly impaired NANC transmission in the bovine retractor penis and rat anococcygeus muscles but did not affect transmission in the guinea-pig taenia caeci or bladder, suggesting that different NANC mechanisms are involved.

3 Although neurally-evoked relaxation of the bovine retractor penis was impaired by hypoxia, relaxations produced by vasoactive intestinal peptide, prostaglandin E_1, sodium nitroprusside or an inhibitory factor isolated from the bovine retractor penis were unaffected. Since the inhibitory factor is similar to, and may actually be the NANC transmitter, the results suggest that the site of action of hypoxia in impairing transmission is prejunctional at the inhibitory nerve endings.

Introduction

Reduction of the P_{O_2} in the fluid bathing smooth muscle preparations has various effects on their responsiveness to drugs or to nerve stimulation in different organs (Garry, 1928; Day & Vane, 1963; Smith & Vane, 1966; De Mey & Vanhoutte, 1981; Chang & Detar, 1980). We now describe two examples, the bovine retractor penis and the rat anococcygeus muscles, in which two types of nerve-mediated response within the same tissue are differently affected by P_{O_2}. These muscles are innervated by motor fibres that are noradrenergic and inhibitory fibres whose transmitter is unknown (Gillespie, 1972; Klinge & Sjöstrand, 1974). In some experiments we noted that inhibitory transmission in the bovine retractor penis muscle was drastically impaired after even a brief period of hypoxia, and at a time when adrenergically-mediated responses persisted. We have now studied the phenomenon in greater detail, both in the bovine retractor penis muscle and in the related anococcygeus muscle of the rat, and have compared the responsiveness of these organs with that of other organs known to possess a non-adrenergic, non-cholinergic (NANC) innervation. Some of the results have been published in abstract form (Bowman & McGrath, 1982; Bowman *et al.*, 1983).

Methods

Bovine penises, with attached retractor penis muscles, were collected from the abattoir during the morning. Thin strips of retractor penis muscle, about 2 cm long, were cut off, and used that afternoon, or the next day after storage overnight in Krebs solution in the refrigerator.

Male Wistar rats (250–300 g) were killed by a blow on the head and exsanguinated. Anococcygeus muscles were isolated; the portion taken was that dorsal to the colon, ending where some fibres attach to the colon wall.

Taeniae caeci and bladders were taken from male guinea-pigs (300–600 g) which had been killed by a blow on the head; strips of bladder were cut as described by Ambache & Zar (1970). In all experiments on preparations of taenia caeci and bladder, atropine (0.5 μM) and guanethidine (5 μM) were present in the reservoir of Krebs solution.

All tissues were attached to silver: silver chloride ring and hook electrodes and placed in Krebs solution at 35–37°C. Tissues were connected by thread to Grass FTO3 transducers and tension was recorded on a Grass polygraph. In experiments with taeniae caeci,

Grass copper springs were inserted between the muscle and the transducer so that contractions were recorded semi-isometrically. Field stimulation was applied from Grass S88 or Square One Instruments stimulators (supramaximal voltage, pulse width 0.1–0.5 ms, frequencies as stated in the text).

The Krebs solution which was initially used had the following composition (mM): NaCl 119, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 11.1; and was gassed with 5% CO_2 in O_2 but the gassing mixture was changed from time to time during the experiments. The gassing mixtures contained 5% CO_2 and varying proportions of O_2 and N_2. They were either bubbled directly from pressurized cylinders, or were made up in Douglas bags with the required volumes of O_2, CO_2 and N_2 and were pumped through the saline. Composition was checked by monitoring O_2 and CO_2, and it was assumed that the balance was N_2.

Drugs

The following drugs were obtained from Sigma, unless otherwise stated: aspirin, atropine sulphate (B.D.H.), 5,8,11,14-eicosatetraynoic acid (ETYA, Wellcome), guanethidine sulphate (Ciba), hydroquinone, indomethacin, mepacrine, phenidone, prostaglandin E_1 (Upjohn), sodium nitroprusside (B.D.H.), vasoactive intestinal peptide and xylazine hydrochloride (Bayer). Methods for the preparation of the inhibitory factor from the bovine retractor penis muscle (Gillespie *et al.*, 1981) and haemolysate (Bowman & Gillespie, 1982; Bowman *et al.*, 1982) have been described before.

Results

Bovine retractor penis muscle

Motor responses to field stimulation Initially when strips of bovine retractor penis muscle are set up in the organ bath they lack tone and respond to field stimulation with contraction due to excitation of the noradrenergic nerves. Oxygen lack increased the amplitude of these contractions. Figure 1 illustrates an experiment in which noradrenergic motor responses were evoked at 3.5 min intervals by field stimulation at 10 Hz for 10 s. When the normal gassing mixture (5% CO_2 in O_2) was replaced by 5% CO_2 in N_2, there was a marked but waning augmentation of the contractions. On restoration of the normal gassing mixture, the motor responses were temporarily depressed below the control level.

In previous experiments, we have shown (Bowman & Gillespie, 1982; Bowman *et al.*, 1982) that the oxyhaemoglobin in haemolysed erythrocytes blocks the non-adrenergic, non-cholinergic inhibitory responses of the bovine retractor penis muscle. However, haemolysate increased the amplitude of the noradrenergic motor responses of this muscle. Unlike the effect of hypoxia, the effect of haemolysate did not wane during its presence in the bath (Figure 1).

Inhibitory responses to field stimulation With time, tone develops in the bovine isolated retractor penis muscle, and gradually this completely masks the noradrenergic motor response to field stimulation. Instead, a powerful non-adrenergic, non-cholinergic neurally-mediated inhibitory response is revealed.

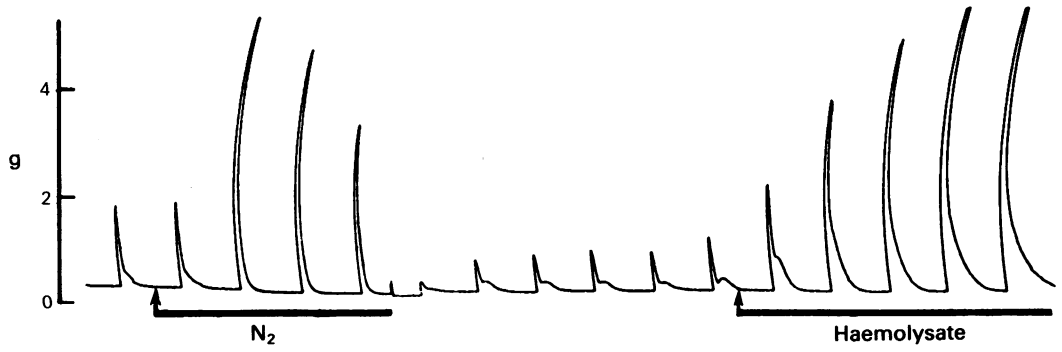

Figure 1 The effects of hypoxia and oxyhaemoglobin (haemolysate) on motor responses to field stimulation of bovine isolated retractor penis muscle. Field stimulation (10 Hz, 0.5 ms) was applied for 10 s every 3.5 min. Gassing mixture was changed from 5% CO_2 in O_2 to 5% CO_2 in N_2 as indicated at the first horizontal bar; this caused a marked but waning augmentation of the contractions. After oxygenation was resumed, the contractions were reduced in size, but were augmented again in the presence of haemolysate (oxyhaemoglobin content, 10 μM).

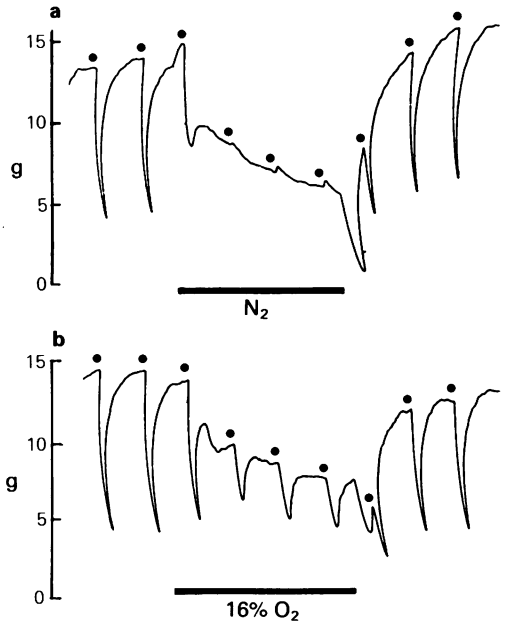

Figure 2 The effects of hypoxia on inhibitory responses to field stimulation of the bovine isolated retractor penis muscle. Field stimulation (5 Hz, 100 μs) was applied (at ●) for 10 s at 3.5 min intervals. The bath was bubbled with 5% CO_2 in O_2 except where shown by the horizontal bars where gassing was with 5% CO_2 in N_2 (a) or 5% CO_2 + 16% O_2 in N_2 (b).

Although it was not necessary for the production of tone in this tissue, guanethidine (10 μM) was added in these experiments to block noradrenergic transmission.

Figure 2 illustrates inhibitory responses to field stimulation under these conditions and the effects of hypoxia upon them. In the experiment illustrated in Figure 2a, the normal gassing was replaced by 5% CO_2 in N_2, and this had two effects: it caused a gradually developing fall in tone of the smooth muscle and a rapid abolition of the inhibitory responses to field stimulation. On returning to 5% CO_2 in O_2, there was a sharp and transient drop in tone followed by a return to the control high tone level. During the restoration of tone, the inhibitory responses to field stimulation reappeared.

Figure 2b illustrates a continuation of the experiment of Figure 2a, but here the normal gassing mixture was replaced by 5% CO_2 plus 16% O_2 in N_2. The spontaneous tone fell to about the same extent. The inhibitory responses to field stimulation were reduced but not abolished.

The degree of fall in spontaneous tone was variable between tissues and was not related to the reduction in inhibitory response to field stimulation. Figure 3 (a and b) illustrates the extremes of the effects of hypoxia on spontaneous tone. In Figure 3a, there is virtually no change in tone, whereas in Figure 3b, tone fell to a point lower than the maximum depression in the control inhibitory responses, yet in both instances, the inhibitory responses were abolished. Furthermore, on restoration of oxygenation, the inhibitory responses reappeared before tone was fully re-established. These observations make it clear that the abolition of the inhibitory responses was not simply a consequence of the fall in background tone preventing their manifestation. The two effects appear to be unrelated.

In the experiments described above, the changes in gas tensions were achieved simply by changing the gas mixture with which the organ bath was bubbled; the time taken for equilibration with the new gas mixture was 1–2 min. In 4 experiments, the bath fluid was changed rapidly for fluid that had been previously equilibrated with the desired gas mixture at the same time as the gas bubbling line was changed. The results showed that when oxygen was removed from the gas mixture, abolition of the inhibitory response occurred in less than 2 min.

Inhibitory responses to agonists The bovine retractor penis muscle relaxes in response to the inhibitory factor extracted from that muscle (Ambache *et al.*, 1975; Gillespie & Martin, 1978; 1980), and to sodium nitroprusside, to prostaglandin E_1 and to vasoactive

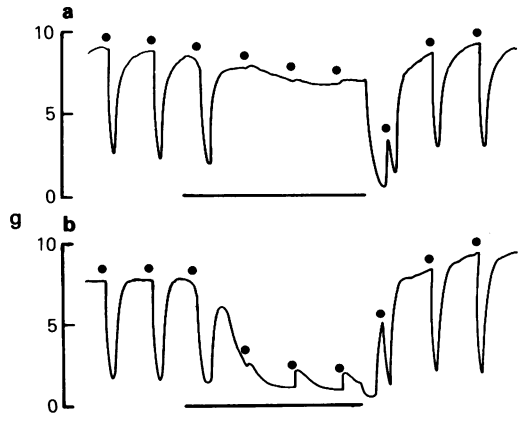

Figure 3 The effects of hypoxia on basal tone and on inhibitory responses of the bovine retractor penis muscle to field stimulation (at ●, 5 Hz, 100 μs, train 10 s, at 3.5 min intervals). The bath was bubbled with 5% CO_2 in O_2 except where shown by the horizontal bars where gassing was with 5% CO_2 in N_2. Muscle strips used for panels (a) and (b) were taken from the same part of the same retractor penis muscle, and were treated similarly. Why tone was maintained in one and lost in the other is not known.

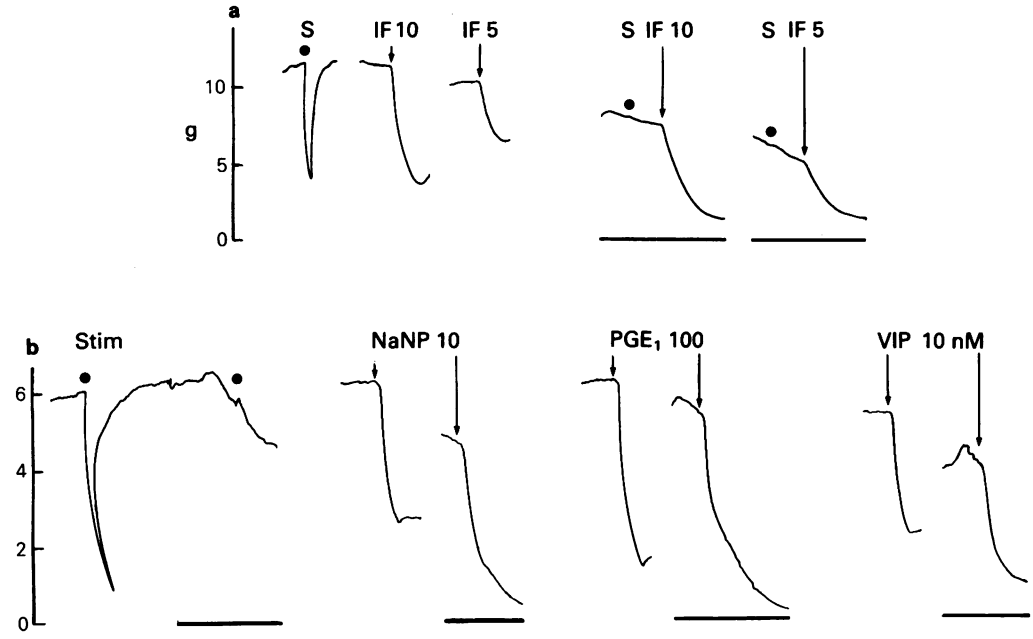

Figure 4 The effect of hypoxia on relaxations of the bovine isolated retractor penis muscle produced by field stimulation (5 Hz, 100 μs for 10 s) or by agonists. (a) Control relaxations elicited by field stimulation (at ●) and inhibitory factor (IF) from the bovine retractor penis (10 and 5 μl ml^{-1}) are followed by responses elicited under hypoxic conditions. The bath was bubbled with 5% CO_2 in N_2 (at horizontal bars), which abolished the responses to field stimulation but not to the inhibitory factor. (b) After each control relaxation, the bath was bubbled with 5% CO_2 in N_2 for 5 min, and then the stimulus or application of agonist was repeated. Hypoxia (at horizontal bars) abolished the response to field stimulation (at ●) but not to sodium nitroprusside (NaNP, 10 nM), prostaglandin E_1 (100 nM) or vasoactive intestinal peptide (VIP, 10 nM).

intestinal polypeptide (VIP). When hypoxia had abolished the inhibitory responses to nerve stimulation, relaxations induced by the above named agonists persisted (Figure 4). The experiments were done in two ways. In some, control responses to nerve stimulation and to an agonist were obtained first with normal gas mixture. Subsequently oxygen was excluded from the gas mixture, and all the responses were retested (Figure 4a). This protocol necessitated a prolonged period of hypoxia by the time the later responses were obtained. In other experiments, each individual agonist or nerve stimulation in turn was tested in the presence and absence of oxygen from the gas mixture (Figure 4b). Similar results were obtained by both methods.

Role of arachidonic acid metabolites Impairment of the inhibitory responses to field stimulation by oxygen lack suggested the possibility that a product of lipoxygenase or cyclo-oxygenase action on arachidonic acid might be involved in the inhibitory transmission mechanism. Several agents that directly or indirectly interfere with these enzymes (see Flower, 1974; Blackwell & Flower, 1978) were tested on the inhibitory responses to nerve stimulation. The agents used were aspirin (50 μM) and indomethacin (40 μM) which inhibit cyclo-oxygenase, and 5,8,11,14-eicosatetraynoic acid (ETYA, 100 μM) and phenidone (100 μM) which inhibit both cyclo-oxygenase and lipoxygenase. The effects of mepacrine (100 μM), which inhibits phospholipase A_2, and of hydroquinone (100 μM), a free-radical quencher, were also tested. None of these substances impaired the relaxations of the bovine retractor penis muscle evoked by field stimulation.

Rat anococcygeus muscle

The mean amplitudes of noradrenergically-evoked responses to field stimulation (2–20 Hz, 20 s) were usually not altered across the range of oxygen tensions produced by bubbling with gas containing 6–95% O_2. However, in a few preparations there was an increase in response on changing from 95% to a lower level, e.g., 16%. Since this could result from loss of the inhibitory nerve response rather than from enhancement of the motor response, the experiment was

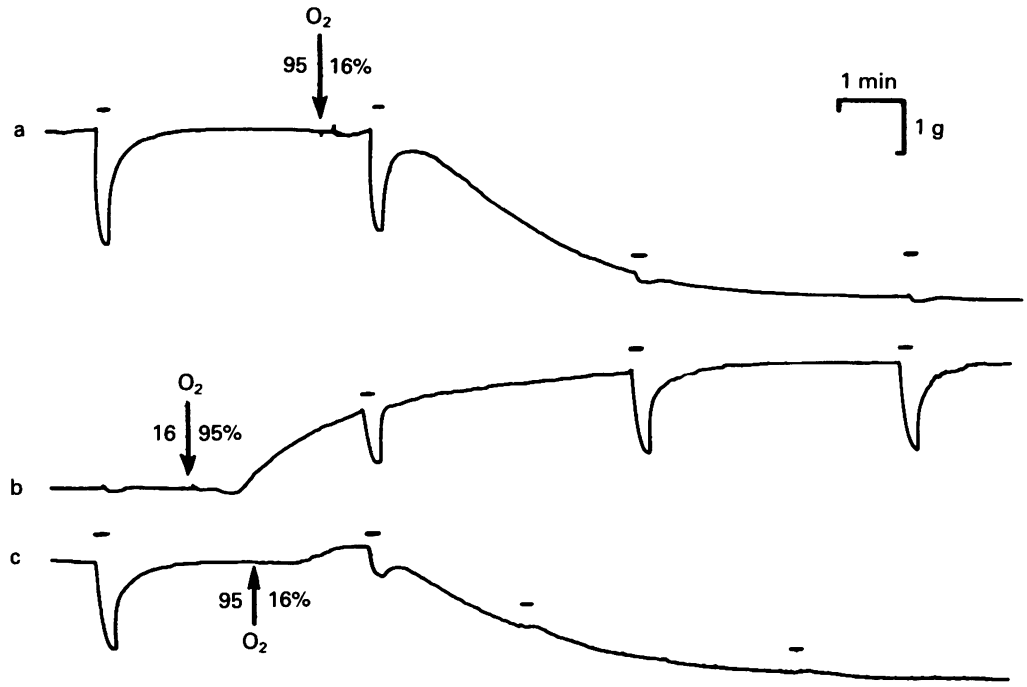

Figure 5 The effects of hypoxia on the inhibitory responses to field stimulation in rat anococcygeus muscle. Field stimulation, 0.5 ms pulses, 5 Hz, 10 s trains, is indicated by the horizontal bars. Xylazine (100 μM) was present throughout to block motor adrenergic transmission and raise the muscle tone. The three records are sequential. (a) On changing from 95% to 16% O_2, stimulation within 30 s produced a small reduction in response. After a further 3 min, the nerve-induced response was lost but interpretation is difficult because tone fell. (b) Tone and nerve-induced responses were rapidly restored by returning to 95% O_2. (c) O_2 was changed from 95% to 16% as in (a) but the response to stimulation, after 90 s, before tone fell, was attenuated. This illustrates that, although the tone of the anococcygeus is not maintained when O_2 tension is lowered, the inhibitory nerve response can be shown to be blocked before this happens.

repeated in the presence of guanethidine (30 μM) or of xylazine (100 μM). The latter produces pharmacological blockade of the noradrenergic nerves (pre-junctional α_2-adrenoceptor agonism) and elevates the tension (post-junctional α_1-agonism) (McGrath, 1984). Under these conditions, relaxations in response to field stimulation were attenuated when the Krebs was bubbled with a reduced percentage of O_2 compared with the 95% O_2 at which they had been studied previously. It was not necessary to exclude oxygen completely in order to show an effect. The response to inhibitory nerve stimulation was restored on return of the O_2 content of the gassing mixture to 95% (Figure 5).

Guinea-pig taenia caeci and bladder

These two organs each possess a non-adrenergic non-cholinergic innervation, that of the taenia caeci being inhibitory and that of the bladder excitatory. Changing the gas mixture from 95% O_2 plus 5% CO_2 to 95% N_2 plus 5% CO_2 produced a slowly developing loss of spontaneous tone of the taenia caeci, just as it did in the bovine retractor penis muscle. However, the relaxations evoked by inhibitory nerve stimulation were not impaired except for the reduction in amplitude consequent upon the fall in background tone (Figure 6a, b). In the bladder, where background tone is always low, changing from O_2 to N_2 was without effect on resting tone and had little or no effect on the amplitude of contractions evoked by non-adrenergic non-cholinergic nerve stimulation (Figure 6c).

Discussion

The results show that hypoxia produces a rapid and selective impairment of non-adrenergic, non-cholinergic (NANC) transmission in the bovine retractor penis

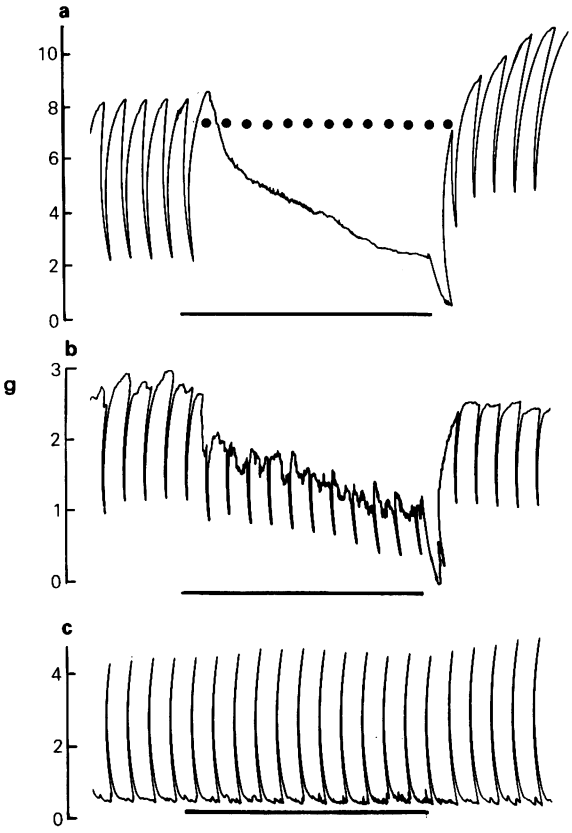

Figure 6 A comparison of the effects of hypoxia on responses to field stimulation of non-adrenergic non-cholinergic nerves in (a) bovine retractor penis muscle (b) guinea-pig taenia caeci and (c) guinea-pig bladder. Field stimulation (2 Hz for 10 s, 100 μs pulses) was applied at intervals of 100 s in the presence of atropine (0.5 μM) and guanethidine (5 μM). For the periods indicated by the horizontal bars, the usual gas mixture (5% CO_2 in O_2) was replaced by 5% CO_2 in N_2.

and rat anococcygeus muscles but not in the guinea-pig taenia caeci and bladder. It should be emphasized that it is the rapidity of the hypoxic transmission block that is the unique feature at the NANC transmission sites in the bovine retractor penis and rat anococcygeus. Obviously, eventually, all types of transmission will be impaired by metabolic failure consequent upon oxygen lack.

The observation that noradrenergic transmission in the bovine retractor penis and rat anococcygeus muscles was apparently enhanced by hypoxia should not be taken as evidence that oxygen lack exerts the opposite action on this type of transmission, for abolition of concomitant inhibitory transmission is probably sufficient to account for the apparent increase in the motor responses.

Whether or not the NANC transmission mechanism is of the type that is impaired by hypoxia, background tone in the smooth muscle, when present, was reduced. The two effects were clearly independent of each other. Reduction in background tone may simply reflect the metabolic dependence of the membrane potential or the filamentary resting tension in the smooth muscle; it is probably unrelated to neurotransmission.

The experiments do not allow an absolute conclusion as to whether the hypoxia-induced transmission failure in the bovine retractor penis and rat anococcygeus is a pre- or a post-junctional phenomenon. However, evidence from the bovine retractor penis suggestive of a pre-junctional site was obtained. Thus, the cause of the block was clearly not an inability of the muscle to relax, since agonists (sodium nitroprusside, prostaglandin E_1, VIP, the inhibitory factor) continued to produce relaxation under hypoxic conditions. The inhibitory factor, if not identical, at least closely resembles the transmitter and the fact that it remained effective during severe hypoxia, when neurotransmission was blocked, indicates a pre-junctional site of impairment. The site of the impairment seems unlikely to be axonal conduction or action potential-release coupling since these processes are essentially similar at all autonomic junctions, yet other such junctions were not affected by hypoxia to such an extent. Since the release mechanism is probably not involved, it might be speculated that it is the transmitter synthesizing process that is affected. Possibly there exists a novel transmission mechanism at the affected types of junction in which, on demand, an oxygen-requiring enzyme converts an inactive precursor of the transmitter into its functional form. If this is so, the oxygen-requiring enzyme involved does not appear to be one of those associated with arachidonic acid metabolism.

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