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Hydroxocobalamin and haemoglobin differentiate between exogenous and neuronal nitric oxide in the rat gastric fundus

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Abstract

In longitudinal strips of rat gastric fundus, hydroxocobalamin (30 μM) significantly reduced relaxations to sodium nitroprusside (100 nM), nitric oxide (NO; 5 μM) and *S*-nitrosocysteine (3 μM), whereas responses to non-adrenergic, non-cholinergic (NANC) nerve stimulation were only slightly reduced. The stimulation-induced relaxations were markedly reduced by the NO synthase inhibitor N^G-nitro-L-arginine (100 μM). Hydroxocobalamin (30 μM) enhanced relaxations to *S*-nitrosoglutathione (1 and 3 μM), and had no effect on responses to vasoactive intestinal polypeptide (1 nM). Haemoglobin (10 μM) significantly reduced relaxations to sodium nitroprusside, NO, *S*-nitrosocysteine and *S*-nitrosoglutathione, but did not affect responses to NANC nerve stimulation or vasoactive intestinal polypeptide. The results suggest that hydroxocobalamin and haemoglobin can differentiate between exogenous and neuronally released NO, and that the transmitter released from nitrergic nerves in the rat gastric fundus is not free NO or the nitrosothiols, *S*-nitrosocysteine and *S*-nitrosoglutathione.

Keywords: Gastric fundus, rat; Hemoglobin; Hydroxocobalamin; Nitric oxide (NO); N^G-Nitro-L-arginine; NANC (non-adrenergic, non-cholinergic) nerve; Nitrosothiol

1. Introduction

Nitric oxide (NO)-mediated neurotransmission, termed nitrergic transmission, has been identified at inhibitory non-adrenergic, non-cholinergic (NANC) neuroeffector junctions in a variety of tissues including sites throughout the gastrointestinal tract (Rand, 1992). Although there is no doubt that the mediator of nitrergic transmission is closely related to NO, its precise chemical nature remains uncertain. It has been suggested that the transmitter substance may not be free NO but a NO-yielding compound, such as a nitrosothiol (Knudsen et al., 1992; Gibson et al., 1992; Rand and Li, 1993).

The biological actions of NO can be inhibited by haemoglobin which binds NO to the iron atom in its haem group (Martin et al., 1985). Hydroxocobalamin (vitamin B_{12a}) has a cobalt-containing corrin core that is structurally analogous to the iron-containing porphyrin (haem) core in haemoglobin. Hydroxocobalamin is readily converted to nitrosocobalamin (vitamin B_{12c}; Kaczka et al., 1951) and may therefore sequester NO in a similar manner to haemoglobin. Rajanayagam et al. (1993) demonstrated that hydroxocobalamin (30 μM) significantly inhibited acetylcholine-induced endothelium-dependent relaxations in rat aortic rings. However, this concentration of hydroxocobalamin had no effect on responses to NANC nerve stimulation in the rat anococcygeus muscle, despite compelling evidence that the primary NANC transmitter in the anococcygeus is NO or a closely related compound (Li and Rand, 1989). Furthermore, hydroxocobalamin had no effect on relaxations to the nitrosothiols *S*-nitrosocysteine or *S*-nitroso-*N*-acetylpenicillamine, but reduced relaxations to NO in the anococcygeus muscle, suggesting that the nitrergic transmitter more closely resembled a nitrosothiol than free NO (Rand and Li, 1993).

In the present study we have used hydroxocobal-

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amin to investigate the nature of the nitrergic transmitter in the rat gastric fundus, in which inhibitory NANC transmission has been shown to be mediated by NO and vasoactive intestinal polypeptide (Li and Rand, 1990; D'Amato et al., 1992). The effects of hydroxocobalamin on relaxations to NANC nerve stimulation, NO and some NO-donating compounds, including the nitrosothiols *S*-nitrosocysteine and *S*-nitrosoglutathione, have been compared with those of haemoglobin.

2. Materials and methods

2.1. Tissue preparation

Male Sprague-Dawley rats (240–420 g) were stunned and killed by decapitation. The stomach was removed and two longitudinal strips (length 20 mm, width 3 mm) were prepared from the ventral part of the fundus as described by Li and Rand (1990). Each fundus strip was mounted under a resting tension of 1 g in physiological salt solution (PSS) of the following composition (in mM): NaCl 118; KCl 4.7; $CaCl_2$ 2.5; KH_2PO_4 1.03; $MgSO_4$ 0.45; $NaHCO_3$ 25.0; D-(+)-glucose 11.1; disodium edetate 0.067; ascorbic acid 0.14. The PSS was maintained at 37°C, gassed with 5% CO_2 in O_2, and contained atropine (3 μM) and guanethidine (5 μM) throughout the experiment to block cholinergic and noradrenergic responses to electrical field stimulation. Intramural nerves were electrically stimulated using two platinum wire electrodes, one placed on either side of the strip, with square wave pulses of 1-ms duration and supramaximal voltage (18 V/cm). Changes in tissue length were measured using a Ugo Basile isotonic transducer and recorded on a Rikadenki potentiometric recorder. Smooth muscle relaxations were measured as an increase in tissue length.

2.2. Experimental protocols

The fundus strip was allowed to equilibrate for at least 30 min before serotonin (10 μM) was added to produce a sustained, submaximal increase in tone. After a further 20-min equilibration period, relaxant responses were obtained to drugs and electrical field stimulation at 5-min intervals as specified below. All relaxant drugs were used at concentrations that gave responses between 35% and 80% of their maximum response.

Two sets of control relaxant responses were obtained according to one of three experimental protocols: (1) nerve stimulation (2 Hz, 30-s train) and sodium nitroprusside (100 nM); (2) nerve stimulation (1 Hz, 30-s train), vasoactive intestinal polypeptide (1 nM) and NO (5 μM); and (3) nerve stimulation (1 Hz, 30-s train), *S*-nitrosocysteine (3 μM) and *S*-nitrosoglutathione (1 and 3 μM). A third set of responses was then obtained either in the absence (time-control experiments) or presence of N^G-nitro-L-arginine (100 μM), hydroxocobalamin (30 or 100 μM), haemoglobin (10 μM) or oxyhaemoglobin (10 μM; protocol 2 only). The third set of responses obtained in each experiment has been expressed as a percentage of the averaged first and second sets of control responses. In some experiments N^G-nitro-L-arginine (100 μM) was introduced 15 min before the addition of hydroxocobalamin (100 μM), and remained present throughout the experiment. Experiments were also performed to examine the influence of haemoglobin (10 μM) on relaxant responses to nitrosocobalamin (100 μM).

All experiments involving haemoglobin or oxyhaemoglobin were conducted in the presence of antifoam A (1:10 000) and results were compared to those in the presence of antifoam A (1:10 000) alone. Antifoam A alone slightly but significantly reduced responses to field stimulation at 1 Hz (see Fig. 2; $P < 0.05$, MANOVA followed by Student's *t*-test), increased responses to vasoactive intestinal polypeptide (see Fig. 7, $P < 0.05$, MANOVA followed by Student's *t*-test), but had no effect on relaxant responses to sodium nitroprusside, nitric oxide, *S*-nitrosoglutathione and *S*-nitrosocysteine. The enhancement of responses caused by antifoam A is presumably due to a stabilising effect of the anti-foaming agent on vasoactive intestinal polypeptide.

2.3. Analysis of results

Data are expressed as means ± S.E.M. and *n* indicates the number of animals tested. Differences between means were assessed by paired two-tailed Student's *t*-test, or one-way multiple analysis of variance (MANOVA) followed by Student's *t*-test. Probability values less than 0.05 ($P < 0.05$) were taken to indicate statistical significance.

2.4. Drugs and drug solutions

The following drugs were used in the study: atropine sulphate (Sigma, USA), L-cysteine (Sigma, USA), glutathione (Sigma, USA), guanethidine sulphate (Ciba-Geigy, Australia), haemoglobin (bovine; Sigma, USA), hydroxocobalamin acetate (Sigma, USA), 5-hydroxytryptamine creatinine sulphate (serotonin; Sigma, USA), nitric oxide gas (NO; CIG, Australia), N^G-nitro-L-arginine (Sigma, USA), sodium nitroprusside (Sigma, USA), tetrodotoxin (Sigma, USA), vasoactive intestinal polypeptide (human; Auspep, Australia).

Saturated solutions of NO (2 mM) were prepared on the day of the experiment using a modification of the method described by Feelisch (1991). Briefly, vials of deionised water, deoxygenated by bubbling with argon

gas for 1 h, were bubbled with NO gas for 20 min to give saturated solutions of NO. Solutions of *S*-nitrosoglutathione and *S*-nitrosocysteine were prepared on the day of the experiment by acid-catalysed *S*-nitrosation of the respective thiols as previously described (Ignarro et al., 1981; Jansen et al., 1992); sodium nitrite dissolved in 0.1 M hydrochloric acid was added to an equimolar amount of either L-glutathione or L-cysteine to give a solution of the corresponding nitrosothiol. Nitrosocobalamin was prepared according to the method of Kaczka et al. (1951). Briefly, hydroxocobalamin (46 mg) and sodium nitrite (33 mg) were dissolved in water (5 ml) with 2 drops of acetic acid. Nitrosocobalamin was crystallised from this solution, and then twice recrystallised from water, by the addition of acetone (approximately 20 ml). Oxyhaemoglobin was generously supplied by Dr. T. Cocks (Department of Pharmacology, University of Melbourne). The effects of haemoglobin and oxyhaemoglobin were examined in the presence of the emulsifier antifoam A (Sigma, USA) at the minimum concentration necessary to stop foaming (1:10000 dilution). All drug solutions were kept on ice throughout the experiment.

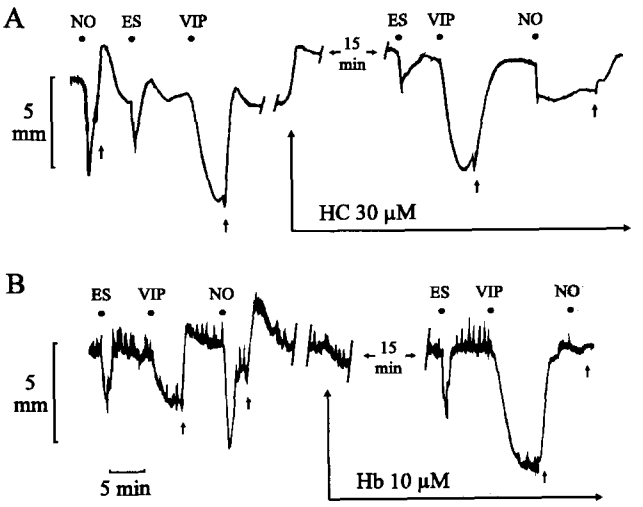

Fig. 1. Original trace showing the effect of (A) hydroxocobalamin (HC) and (B) haemoglobin (Hb; in the presence of 1:10000 antifoam A) on relaxant responses to electrical field stimulation (ES; 1 Hz, 30-s train), vasoactive intestinal polypeptide (VIP; 1 nM) and nitric oxide (NO; 5 μM) in precontracted strips of rat gastric fundus (↑ indicates wash).

3. Results

3.1. Direct effects on serotonin-induced tone

N^G-Nitro-L-arginine (10 μM) produced a small, sustained contraction of 0.54 ± 0.06 mm in 8 preparations, but had no effect on serotonin-induced tone in 15 preparations. Hydroxocobalamin (30 and 100 μM) caused significant, concentration-dependent, sustained contractions of 1.12 ± 0.12 mm ($n = 20$) and 2.34 ± 0.41 mm ($n = 7$), respectively (Fig. 1A; $P < 0.05$, paired *t*-test). The contraction produced by 100 μM hydroxocobalamin was significantly smaller when tissues had been exposed to N^G-nitro-L-arginine (100 μM) for 15 min (1.49 ± 0.16 mm, $n = 5$). Haemoglobin (10 μM) had no sustained effect on serotonin-induced tone (Fig. 1B).

3.2. Effects on NANC nerve-mediated relaxations

Field stimulation (1 and 2 Hz, 30-s train) produced frequency-dependent relaxations of 3.12 ± 0.20 mm ($n = 33$) and 4.73 ± 0.23 mm ($n = 32$), respectively, that remained consistent over a period of 150 min in time-control experiments (Fig. 2). Field stimulation-induced relaxations were abolished by a 10-min exposure to tetrodotoxin (1 μM, data not shown), demonstrating their neural origin. In addition, responses to nerve stimulation were markedly reduced following a 15-min exposure to 100 μM N^G-nitro-L-arginine (Fig. 2; $P <$ 0.05, MANOVA followed by Student's *t*-test), confirming that, at the frequencies tested, the responses were primarily mediated by NO as previously reported (Li and Rand, 1990; Boeckxstaens et al., 1991; D'Amato et al., 1992).

A 15-min exposure to hydroxocobalamin (30 and 100 μM) slightly but significantly reduced relaxations induced by field stimulation at a frequency of 1 Hz for 30 s (Figs. 1 and 2; $P < 0.05$, MANOVA followed by Student's *t*-test), whereas the response to field stimulation at 2 Hz (30-s train) was significantly reduced by hydroxocobalamin only at 100 μM (Fig. 2; $P < 0.05$, MANOVA followed by Student's *t*-test). Responses to field stimulation (1 and/or 2 Hz, 30-s train) were not significantly affected by 10 μM haemoglobin or 10 μM oxyhaemoglobin when compared to the effects of antifoam A (Figs. 1 and 2; $P > 0.05$, MANOVA followed by Student's *t*-test).

3.3. Effects on relaxations to sodium nitroprusside and NO

Addition of sodium nitroprusside (100 nM) and NO (5 μM) to precontracted fundus strips produced submaximal relaxations of 4.73 ± 0.35 mm ($n = 31$) and 4.32 ± 0.35 mm ($n = 31$), respectively, that did not alter significantly over a period of 150 min (Fig. 3). N^G-Nitro-L-arginine (100 μM) had no effect on responses to sodium nitroprusside or NO (Fig. 3). Exposure to hydroxocobalamin (30 and 100 μM) or haemoglobin (10 μM) for 15 min significantly reduced relaxant responses to both sodium nitroprusside (100 nM) and NO (5 μM; Figs. 1 and 3; $P < 0.05$, MANOVA fol-

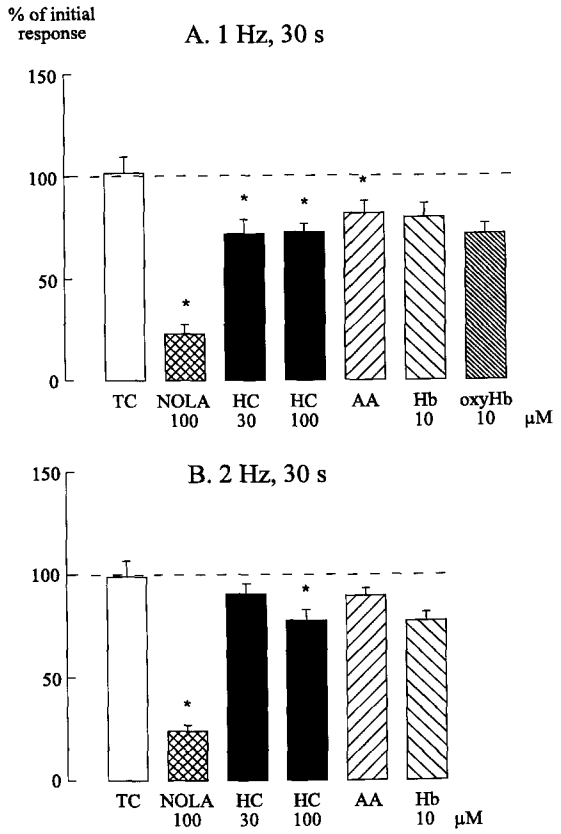

Fig. 2. Effects of N^G-nitro-L-arginine (NOLA, 100 μM), hydroxocobalamin (HC, 30 and 100 μM), haemoglobin (Hb, 10 μM with 1:10000 antifoam A), oxyhaemoglobin (oxyHb, 10 μM with 1:10000 antifoam A) and antifoam A alone (AA, 1:10000) on relaxant responses to field stimulation at (A) 1 Hz for 30 s and (B) 2 Hz for 30 s in precontracted strips of rat gastric fundus. Values are means ± S.E.M. for five or six experiments, expressed as percentages of the responses obtained before the addition of NOLA, HC, Hb, oxyHb or AA. Time controls (TC) indicate responses obtained after 150 min in the absence of NOLA, HC, Hb, oxyHb or AA. * Significant difference between TC and NOLA, HC or AA ($P < 0.05$, one-way MANOVA followed by Student's t-test). There was no significant difference between AA and Hb or oxyHb ($P > 0.05$, one-way MANOVA).

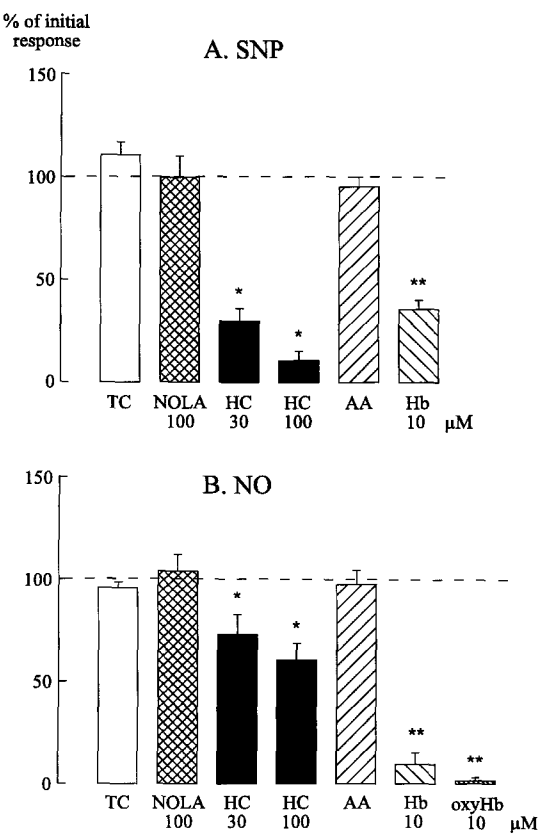

Fig. 3. Effects of N^G-nitro-L-arginine (NOLA, 100 μM), hydroxocobalamin (HC, 30 and 100 μM), haemoglobin (Hb, 10 μM with 1:10000 antifoam A), oxyhaemoglobin (oxyHb, 10 μM with 1:10000 antifoam A) and antifoam A alone (AA, 1:10000) on relaxant responses to (A) sodium nitroprusside (SNP; 100 nM), and (B) nitric oxide (NO; 5 μM) in precontracted strips of rat gastric fundus. Values are means ± S.E.M. for five or six experiments, expressed as percentages of the initial responses obtained before the addition of NOLA, HC, Hb, oxyHb or AA. Time controls (TC) indicate responses obtained after 150 min in the absence of NOLA, HC, Hb, oxyHb or AA. * Significant difference between TC and HC; ** significant difference between AA and Hb or oxyHb ($P < 0.05$, one-way MANOVA followed by Student's t-test).

lowed by Student's t-test). Oxyhaemoglobin (10 μM) significantly reduced responses to NO (Fig. 3; $P < 0.05$, MANOVA followed by Student's t-test).

While the magnitude of responses to NO was reduced in the presence of hydroxocobalamin, the duration of responses was markedly increased (Fig. 1A). It was hypothesised that if hydroxocobalamin sequestered NO to form nitrosocobalamin, the NO may be bound reversibly and gradually liberated, thus producing this change in response profile to exogenous NO. Therefore, we examined responses to nitrosocobalamin. Addition of nitrosocobalamin (100 μM) produced a slowly developing relaxation of approximately 4 mm that could be inhibited by haemoglobin ($n = 2$; Fig. 4).

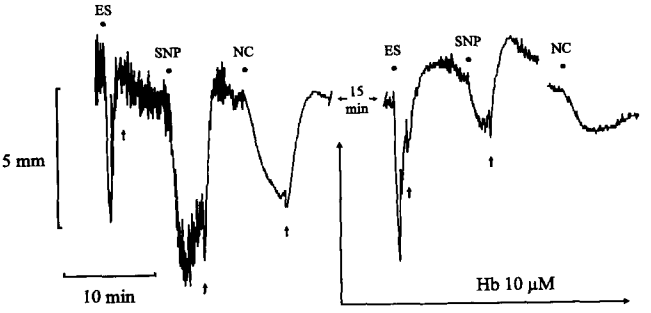

Fig. 4. Original trace showing the effect of haemoglobin (Hb; in the presence of 1:10000 antifoam A) on relaxant responses to electrical field stimulation (ES; 2 Hz, 30-s train), sodium nitroprusside (SNP; 100 nM) and nitrosocobalamin (NC; 100 μM) in precontracted strips of rat gastric fundus (↑ indicates wash).

3.4. Effects on relaxations to S-nitrosocysteine and S-nitrosoglutathione

Addition of the NO donor *S*-nitrosocysteine (3 μM) produced relaxations of 4.73 ± 0.41 mm ($n = 24$) that were slightly but significantly reduced over a period of 150 min in time-control experiments (Fig. 5; $P < 0.05$, MANOVA followed by Student's *t*-test). Addition of the NO donor *S*-nitrosoglutathione (1 and 3 μM) to precontracted fundus strips produced submaximal relaxations of 3.06 ± 0.20 mm ($n = 24$) and 5.51 ± 0.32 mm ($n = 24$), respectively, that did not alter significantly over a period of 150 min (Fig. 6). Hydroxocobalamin (30 μM) significantly reduced relaxant responses to *S*-nitrosocysteine (3 μM; Fig. 5; $P < 0.05$, MANOVA followed by Student's *t*-test), whereas responses to *S*-nitrosoglutathione (1 and 3 μM) were significantly enhanced (Fig. 6; $P < 0.05$, MANOVA followed by Student's *t*-test). Haemoglobin (10 μM) significantly reduced responses to *S*-nitrosocysteine (3 μM) and *S*-nitrosoglutathione (1 and 3 μM; Figs. 5 and 6; $P <$ 0.05, MANOVA followed by Student's *t*-test).

3.5. Effects on relaxations to vasoactive intestinal polypeptide

Addition of vasoactive intestinal polypeptide (1 nM) to precontracted fundus strips produced relaxations of 4.26 ± 0.31 mm ($n = 33$) that remained consistent for at least 150 min (Fig. 7). Relaxant responses to 1 nM vasoactive intestinal polypeptide were not affected by incubation with N^G-nitro-L-arginine (100 μM) or hydroxocobalamin (30 and 100 μM; Figs. 1 and 7). Haemoglobin (10 μM) and oxyhaemoglobin (10 μM) had no effect on responses to vasoactive intestinal polypeptide when compared to the effect of antifoam A alone (Figs. 1 and 7; $P > 0.05$, MANOVA followed by Student's *t*-test).

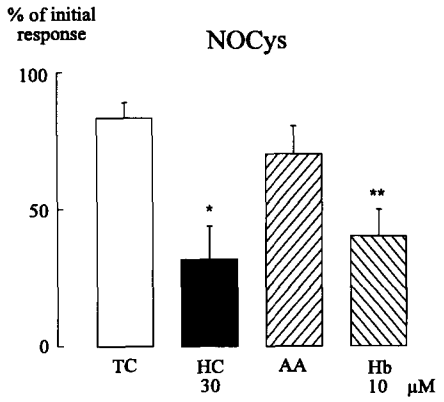

Fig. 5. Effects of hydroxocobalamin (HC, 30 μM), haemoglobin (Hb, 10 μM with 1:10000 antifoam A) and antifoam A alone (AA, 1:10000) on relaxant responses to *S*-nitrosocysteine (NOCys; 1 μM) in precontracted strips of rat gastric fundus. Values are means ± S.E.M. for five or six experiments, expressed as percentages of the initial responses obtained before the addition of HC, Hb or AA. Time controls (TC) indicate responses obtained after 150 min in the absence of HC, Hb or AA. * Significant difference between TC and HC; * * significant difference between AA and Hb ($P < 0.05$, one-way MANOVA followed by Student's *t*-test).

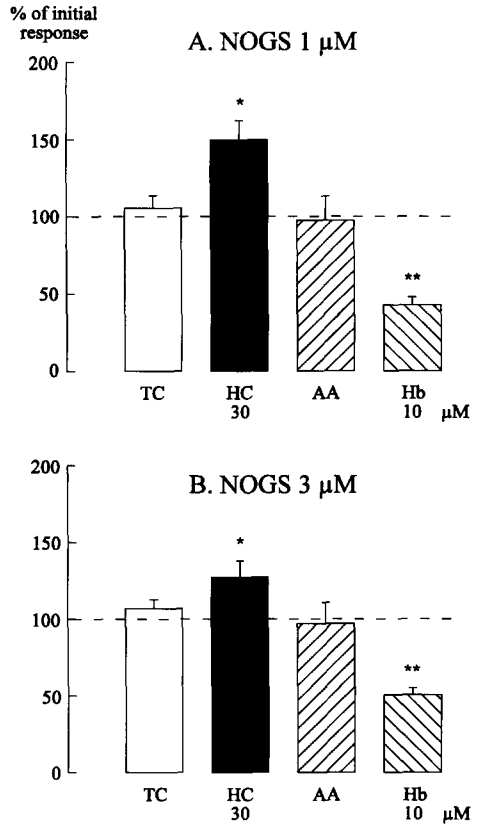

Fig. 6. Effects of hydroxocobalamin (HC, 30 μM), haemoglobin (Hb, 10 μM with 1:10000 antifoam A) and antifoam A alone (AA, 1:10000) on relaxant responses to *S*-nitrosoglutathione (NOGS) at (A) 1 μM and (B) 3 μM in precontracted strips of rat gastric fundus. Values are means ± S.E.M. for five or six experiments, expressed as percentages of the initial responses obtained before the addition of HC, Hb or AA. Time controls (TC) indicate responses obtained after 150 min in the absence of HC, Hb or AA. * Significant difference between TC and HC; * * significant difference between AA and Hb ($P < 0.05$, one-way MANOVA followed by Student's *t*-test).

4. Discussion

The present study has demonstrated that, in the rat gastric fundus, hydroxocobalamin reduces responses to exogenously applied NO and the NO donors, sodium nitroprusside and *S*-nitrosocysteine. Hydroxocobalamin had no effect on the NO-independent relaxations to vasoactive intestinal polypeptide, demonstrating that its inhibitory effects are specific to NO-mediated relaxations. In contrast to its effect on responses to exogenous NO, responses to NANC nerve stimulation were

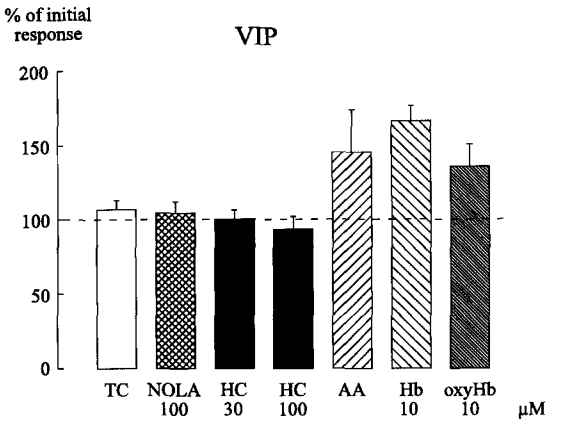

Fig. 7. Effects of N^G-nitro-L-arginine (NOLA, 100 μM), hydroxocobalamin (HC, 30 and 100 μM), haemoglobin (Hb, 10 μM with 1:10000 antifoam A), oxyhaemoglobin (oxyHb, 10 μM with 1:10000 antifoam A) and antifoam A (AA, 1:10000) on the relaxant response to vasoactive intestinal polypeptide (VIP; 1 nM) in precontracted strips of rat gastric fundus. Values are means ± S.E.M. for five or six experiments, expressed as percentages of the initial response obtained before the addition of NOLA, HC, Hb, oxyHb or AA. Time controls (TC) indicate responses obtained after 150 min in the absence of NOLA, HC, Hb, oxyHb or AA. There was no significant difference between TC and NOLA or HC, or between AA and Hb or oxyHb ($P > 0.05$, one-way MANOVA).

relatively refractory to inhibition by hydroxocobalamin, even though stimulation-induced responses were markedly inhibited by N^G-nitro-L-arginine. Therefore, the results of this study demonstrate that hydroxocobalamin can differentiate between exogenously added and neuronally released NO in the rat gastric fundus. These findings are compatible with the view that the nitrergic transmitter in the rat gastric fundus may not be free NO, but a closely related compound in which NO is bound to a carrier molecule. If the carrier molecule had a greater affinity for NO than hydroxocobalamin, the NO would remain preferentially bound to this carrier.

Rand and Li (1993) reported that the nitrergic transmitter released from NANC nerves in the rat anococcygeus muscle more closely resembled *S*-nitrosocysteine than NO. In the rat gastric fundus, however, hydroxocobalamin inhibited responses to *S*-nitrosocysteine to an even greater degree than responses to NO, and so *S*-nitrosocysteine is unlikely to be the nitrergic transmitter in this tissue. Similarly, it has been suggested that the nitrergic transmitter in the mouse anococcygeus muscle (Gibson et al., 1992) and the opossum lower oesophageal sphincter (Knudsen et al., 1992) is neither NO nor *S*-nitrosocysteine. It is possible that upon synthesis in nitrergic nerves, NO binds to available carrier molecules, such as thiol-containing amino acids. If this were the case, then the exact nature of the nitrergic transmitter may vary between tissues depending on the availability of suitable carrier molecules.

The possibility that NO as such is the nitrergic transmitter in the rat gastric fundus cannot be ruled out, since there are other considerations that may explain the lack of effect of hydroxocobalamin on responses to NANC nerve stimulation. One possibility is that the local concentration of transmitter released from nerves is high enough to overcome the inhibitory effect. However, this explanation is unlikely as the responses to NANC nerve stimulation at 1 and 2 Hz for 30 s are submaximal and so the full inhibitory effect of hydroxocobalamin should be evident. Another possible explanation is that either cellular transport mechanisms or enzymatic activity may inactivate or metabolise hydroxocobalamin, thus reducing its effective concentration specifically at the neuroeffector junction.

The findings of this study together with previous reports (Rajanayagam et al., 1993; Li and Rand, 1993; Rand and Li, 1993), suggest that hydroxocobalamin may sequester free NO to form nitrosocobalamin, thereby inhibiting NO-mediated responses. Given the similarity between the cobalt-containing corrin core in hydroxocobalamin and the iron-containing porphyrin core (which binds NO) in haemoglobin, it was postulated that the mechanism of action of the two compounds may be similar. Therefore the effects of hydroxocobalamin were compared to those of haemoglobin in the present study. Haemoglobin potently inhibited responses to exogenously applied sodium nitroprusside, NO, *S*-nitrosocysteine and *S*-nitrosoglutathione in the rat gastric fundus, but did not affect responses to NANC nerve stimulation or vasoactive intestinal polypeptide. The effects of oxyhaemoglobin on responses to NANC nerve stimulation, NO and vasoactive intestinal polypeptide were not different to those of haemoglobin, indicating that the lack of effect of haemoglobin on stimulation-induced responses was not due to a predominance of the oxidised form of haemoglobin (methaemoglobin). The profile of inhibition of relaxant responses by hydroxocobalamin at 30 μM was very similar to that by haemoglobin at 10 μM, supporting the proposal that both compounds sequester NO in a similar manner. There were, however, some differences between the effects of the two compounds.

Firstly, haemoglobin exhibited a higher affinity for exogenous NO than hydroxocobalamin. Furthermore, hydroxocobalamin, but not haemoglobin, actually increased the duration of the responses to NO. A possible explanation is that if NO is sequestered by hydroxocobalamin to form nitrosocobalamin, it may be bound reversibly and can be gradually liberated. In accordance with this suggestion, we found that nitrosocobalamin produced a slowly developing relaxation in the rat gastric fundus that could be inhibited by haemoglobin.

Secondly, hydroxocobalamin enhanced responses to

S-nitrosoglutathione, whereas haemoglobin did not. Pezacka (1993) reported that the cytosolic enzyme cyanocobalamin β-ligand transferase utilises glutathione to metabolise cyanocobalamin to glutathionylcobalamin. It is possible that this enzyme also utilises glutathione to metabolise hydroxocobalamin, removing glutathione from the system, and driving the liberation of NO from *S*-nitrosoglutathione by altering the reaction equilibrium. The concentration of free NO would be increased, and the relaxant response to *S*-nitrosoglutathione enhanced. In support of this hypothesis, we have found that cyanocobalamin (100 μM) also significantly enhanced responses to 1 μM nitrosoglutathione, but had no effect on responses to sodium nitroprusside or NANC nerve stimulation (Jenkinson and Reid, unpublished observations). Cyanocobalamin β-ligand transferase would not metabolism haemoglobin and so the NO-mediated responses to nitrosoglutathione are inhibited rather than enhanced by haemoglobin.

Thirdly, hydroxocobalamin produced concentration-dependent increases in tone of fundus strips, which were reduced in the presence of N^G-nitro-L-arginine, suggesting that they may be partly due to inhibition of the response to tonically produced NO. In contrast, haemoglobin did not increase the tone of fundus strips, however this is in accord with its total lack of effect on responses to NANC nerve stimulation.

The effects of haemoglobin on relaxant responses in the present study agree with those reported by Meulemans et al. (1993), who found that haemoglobin did not affect the relaxations to serotonin-induced release of NO from NANC nerves in the guinea-pig stomach. In contrast, other investigators have reported that haemoglobin inhibited relaxant responses to NANC nerve stimulation (Boeckxstaens et al., 1991) and NO-mediated, stimulation-induced hyperpolarisation (Kitamura et al., 1993) in the rat gastric fundus. However, in the study by Boeckxstaens et al. (1991), the inhibitory effect of haemoglobin was examined in a superfusion bioassay in which rabbit aortic rings were used as the detector tissue. Furthermore, Kitamura et al. (1993) investigated the effect of haemoglobin on membrane hyperpolarisation in the rat gastric fundus, which may not be analogous with the smooth muscle relaxation examined in the present study. Meulemans et al. (1993) suggested that the large molecular size of haemoglobin may prevent its access to the sites of NO release within the gastric wall. However, in experiments using rat gastric fundus strips in which the mucosa had been removed, we found that haemoglobin (10 μM) still had no effect on relaxations to NANC nerve stimulation but abolished the response to sodium nitroprusside (Jenkinson and Reid, unpublished observations). Therefore, the lack of effect is at least not related to the presence of the gastric mucosa. An alternative explanation might be that, if the nitrergic transmitter is an NO-donating pro-drug, then NO may bind to the carrier molecule in preference to haemoglobin in the gastric fundus.

In conclusion, this study has shown that, in the rat gastric fundus, hydroxocobalamin and haemoglobin inhibit relaxations to exogenous NO, but have little effect on relaxations to NO released from nerves. The findings, together with those of previous studies (Gibson et al., 1992; Knudsen et al., 1992; Rajanayagam et al., 1993; Li and Rand, 1993; Rand and Li, 1993), suggest that NO per se may not be the mediator of nitrergic transmission. Furthermore, it is postulated that the precise nature of the mediator involved in nitrergic transmission may vary between different tissues, depending on the presence of appropriate molecules to act as a carrier for NO.

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