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Comparison of the effects of hydroxocobalamin and oxyhaemoglobin on responses to NO, EDRF and the nitrergic transmitter

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1 The effects of ranges of concentrations of oxyhaemoglobin (0.01–30 μM) and hydroxocobalamin (1–100 μM) were compared for their abilities to reduce relaxant responses to EDRF released by acetylcholine in endothelium-intact rat aortic rings, the nitergic transmitter in rat anococcygeus muscles, and NO in aqueous solution in both tissues (aortic rings were denuded of endothelium).

2 The concentrations of oxyhaemoglobin producing 50% reduction of responses to EDRF and NO in rat aorta correspond closely, the IC_{50} values being 0.13 ± 0.02 μM and 0.11 ± 0.02 μM respectively.

3 Oxyhaemoglobin was equally effective in inhibiting responses to NO in anococcygeus muscles and in aortic rings with an IC_{50} of 0.14 ± 0.05 μM. However, responses to the nitrergic transmitter were considerably less sensitive to inhibtion by oxyhaemoglobin, the IC_{50} being 19.7 ± 5.1 μM.

4 The IC_{50} values for hydroxocobalamin in inhibiting responses to EDRF and NO in aorta were 3.4 ± 0.2 μM and 8.4 ± 0.63 μM, respectively, but it was less effective against responses to NO in anococcygeus muscles the IC_{50} being 46 ± 9.6 μM. However, even in the highest concentration used (100 μM), it did not reduce responses to the nitrergic transmitter.

5 The findings are compatible with the views that EDRF is NO, but suggest that the nitergic transmitter in the rat anococcygeus muscle does not behave like free NO.

Keywords: EDRF; hydroxocobalamin; nitrergic transmission; NO; oxyhaemoglobin

Introduction

It is now well established (see Moncada *et al.*, 1991) that nitric oxide (NO) or a closely related substance derived from enzymatic oxidation of L-arginine by a nitric oxide synthase (NOS) is the endothelium-derived relaxing factor (EDRF) first described by Furchgott & Zawadzki (1980). There is also abundant evidence that the acitivty of another NOS is essential for nitrergic neuoeffector transmission in a variety of tissues (Rand & Li, 1995a, b). Although the functional integrity of the respective nitric oxide synthases is necessary for both endothelium-dependent relaxtions and nitrergic nerve stimulation-induced relaxations, controversy still exists regarding the precise nature of the mediators released from the NOS-containing cells. For example, it has been proposed that the stability and potency of EDRF more closely resembles that of S-nitrosothiol rather than that of NO (Myers *et al.*, 1990; Bates *et al.*, 1991; Rubanyi *et al.*, 1991) or that it resembles a dinitrosyl iron cysteine complex (Vedenikov *et al.*, 1992). Doubts have also been cast on the assumption that the nitrergic transmitter is simple free NO because a number of agents that block reponses to exogenously applied NO (in aqueous solution) do not block responses to nitrergic transmission (Rand & Li, 1995a, b). For example, superoxide anion generators such as hydroquinone and pyrogallol inhibit responses to NO-induced relaxations but failed to inhibit nitrergic relaxations in the bovine retractor penis muscles (Gillespie & Sheng, 1990). Similarly, Hobbs *et al.* (1991) reported that hydroquinone failed to inhibit nitrergic mediated relaxation in mouse anococcygeus muscles.

The relaxant actions of NO, NO-donors, EDRF and the nitrergic transmitter in some tissues can be inhibited by oxyhaemoglobin (Bowman & Gillespie, 1982; Bowman *et al.*, 1982; Gillespie & Sheng, 1988; 1989; Li & Rand, 1993). The effect is due to the affinity of the haem group for NO (Gibson & Roughton, 1957) and the subsequent oxidation of the NO/oxyhaemoglobin complex to methaemoglobin and NO_3^+ (Kelm & Schrader, 1990). In the case of NO-donors, it can be assumed that oxyhaemoglobin has a higher affinity for NO than the original ligand.

Hydroxocobalamin (vitamin B_{12a}) has a cobalt-containing corrin core that is structurally analogous to the iron-containing haem core in haemoglobin, and can also bind NO (Rajanayagam *et al.*, 1993), converting hydroxocobalamin to nitrosocobalamin (vitamin B_{12c}; Kaczka *et al.*, 1951). It has been shown that hydroxocobalamin significantly inhibited relaxant responses to NO and acetylcholine in the rat aorta (Rajanayagam *et al.*, 1993) and NO-induced relaxation in the rat anococcygeus muscle, but had little effect on responses to nitrergic nerve stimulation (Li & Rand, 1993).

In the present study, we used oxyhaemoglobin and hydroxocobalamin to compare the nitrergic transmitter in the rat anococcygeus muscle and EDRF in the rat aorta with NO. Although many previous studies have dealt with these two compounds, often only a single concentration was used. Therefore, we investigated the effects of a range of concentrations of hydroxocobalamin and oxyhaemoglobin on EDRF-mediated, acetylcholine-induced relaxations in rat isolated aortic rings, nitrergic nerve stimulation-induced relaxations in the rat isolated anococcygeus muscle, and NO-induced relaxations in both tissues.

Methods

Tissue preparation and experimental protocols

Male Sprague-Dawley rats (300–450 g) were killed by decapitation and the thoracic aorta and anococcygeus muscles were removed.

Rings of aorta about 5 mm wide were cleaned and set up in

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an 8 ml organ bath for the measurement of isometric tension as previously described (Rand & Li, 1993). The rings were mounted under a resting tension of 2 g in physiological salt solution (PSS) and equilibrated for a period of 90 min before experimental intervention started. Rings were contracted with phenylephrine (1 μM) and relaxations were elicited by acetylcholine (0.01–3 μM) in endothelium-intact rings or by NO (0.3–1.3 μM) in endothelium-denuded rings.

Two sets of control relaxant responses to NO or acetylcholine were obtained before the addition of increasing concentrations of either oxyhaemoglobin or hydroxocobalamin. Parallel experiments without oxyhaemoglobin or hydroxocobalamin were carried out on a second aortic ring from the same donor rat to serve as a time control. In all experiments, NO was added in successively increasing concentrations at 1–3 min intervals, during which the relaxant action of NO had completely dissipated.

Anococcygeus muscles were set up as previously described (Gillespie, 1972; Li & Rand, 1989). Muscles were mounted under a resting tension of 1 g and equilibrated for a period of 30 min before experimental intervention started. After the tone had been raised by guanethidine (10–30 μM) and clonidine (0.05 μM), relaxant responses to NO or field stimulation (1–5 Hz, 10 s) were obtained. Only one frequency of stimulation and three increasing concentrations of oxyhaemoglobin or hydroxocobalamin were used in each preparation. Oxyhaemoglobin or hydroxocobalamin were added only after responses to field stimulation had stabilized.

Drugs and solutions

The PSS had the following composition (mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 0.45, KH_2PO_4 1.03, $CaCl_2$ 2.5, D-(+)-glucose, 11.1 and disodium edetate 0.067. The PSS was gassed with 5% CO_2 and 95% O_2 and maintained at 37°C.

A phosphate buffer used for preparing oxyhaemoglobin (see below) had the following composition (mM): Na H_2PO_4 155, Na_2HPO_4 103. The pH of the solution was adjusted to 7.8 by addition of NaOH (1 mM).

The constituents of the PSS and phosphate buffer were of analytical reagent quality.

The drugs used and their sources were: acetylcholine perchlorate (British Drug House Ltd, UK); clonidine hydrochloride, hydroxocobalamin acetate (Sigma, St Louis, MO, USA); guanethidine sulphate (Ciba, Sydney, NSW, Australia); nitric oxide (compressed gas; CIG, Melbourne, Vic, Australia). Saturated aqueous solutions of NO were prepared from NO gas as previously described (Rajanayagam *et al.*, 1993).

Preparation of oxyhaemoglobin

Rats were killed by decapitation and blood was immediately collected into a tube containing 150 units of heparin. Usually, between 3 to 5 ml of blood was collected from each rat. If coagulation had occurred, the sample was disregarded. The blood was centrifuged at 2000 r.p.m. for 10 min at 4°C. The plasma and buffy coat layer were removed, and the remaining blood components were then resuspended in a phosphate buffer solution and centrifuged again at 2000 r.p.m. for 10 min. The supernatant was removed and the remaining erythrocytes were washed three times with phosphate buffer. Erythrocytes were haemolysed by mixing 1 ml of washed erythrocytes to 2 ml of distilled water and the mixture was centrifuged at 16,000 r.p.m. for 30 min at 4°C to deposit the erythrocyte membranes. The oxyhaemoglobin-containing supernatant was carefully removed into vials, the air space was gassed with argon gas to remove oxygen and the vials were sealed. The contents were stored at −4°C and used for up to 7 days after preparation. A Coulter counter model S-Plus (Coulter Electronic, Inc, Hialeah, Florida, USA) was used to determine the haemoglobin concentration. This method involved the conversion of haemoglobin to cyanmethhaemoglobin, which was then measured as total haemoglobin pigments. The purity of this haemoglobin was checked by an absorbance spectrometer, using the two characteristic peaks of oxyhaemoglobin between 520–570 nm and the methaemoglobin peak at 620 nm.

Analysis of results

In rat aortic rings, relaxations elicited by NO and acetylcholine were expressed as a percentage of the phenylephrine-induced contraction. In rat anococcygeus muscle, responses to NO and field stimulation were expressed as reductions of tension. Data are expressed as means ± s.e. means and n indicates the number of animals contributing tissues. Differences between means were assessed by Student's t test (paired or unpaired, as appropriate) or analysis of variance (ANOVA; two-ways or repeated measured). Probability values of $P<0.05$ were considered significant.

The IC_{50} values for oxyhaemoglobin and hydroxocobalamin were calulated as follows. The falls in tension caused by the maximum concentrations used of NO (1.3 μM) or acetylcholine (3 μM) or the maximum frequency of field stimulation (5 Hz) were plotted against increasing concentrations of oxyhaemoglobin or hydroxocobalamin. The IC_{50} values were determined for each experiment and the mean ± s.e. of IC_{50} values were then calculated.

Results

Time control responses to acetylcholine, NO and field stimulation

In rat aortic rings, relaxation responses to acetylcholine and NO remained constant throughout the 180 min duration of the experiments ($P>0.05$; ANOVA, repeated measures).

In rat anococcygeus muscles, relaxation responses to field stimulation (1, 2 and 5 Hz) and NO (0.3, 0.5 and 1.3 μM) gradually became slightly larger with time; however, they did not differ significantly from the initial control responses in the 60 min duration of experiments ($P>0.05$; ANOVA, repeated measures for NO).

Responses to NO and acetylcholine in rat aortic rings

In endothelium-denuded preparations, phenylephrine (1 μM) produced an increase in tension of 1.9 ± 0.13 g ($n=9$). Exogenously applied NO at 0.3, 0.5 and 1.3 μM produced decreases in tension of 1.0 ± 0.2, 1.4 ± 0.1 and 1.7 ± 0.1 g ($n=9$) respectively.

Relaxations elicited by exogenous NO (0.3–1.3 μM) were decreased in a concentration-dependent manner by 0.01–0.3 μM oxyhaemoglobin (Figure 1a) and by 1–100 μM hydroxocobalamin (Figure 1b). The IC_{50} values for the inhibition of NO-induced relaxation for oxyhaemoglobin and hydroxocobalamin were 0.11 ± 0.02 μM ($n=4$) and 8.4 ± 0.63 μM ($n=5$), respectively.

In endothelium-intact preparations, relaxations elicited by acetylcholine (0.01–3 μM) were significantly inhibited in a concentration-dependent manner by 0.03–1 μM oxyhaemoglobin (Figure 1c) and 1–100 μM hydroxocobalamin (Figure 1d). The IC_{50} values for the inhibition of acetylcholine-induced relaxation for oxyhaemoglobin and hydroxocobalamin were 0.13 ± 0.02 μM ($n=6$) and 3.4 ± 0.2 μM ($n=3$), respectively.

In endothelium-intact preparations, the resting tone of the rings was not affected by either hydroxocobalamin or oxyhaemoglobin; however, contractile responses to phenylephrine were enhanced, as shown in Table 1.

Responses to NO and field stimulation in rat anococcygeus muscles

Responses to NO (0.3, 0.5 and 1.3 μM) were inhibited by 0.01–0.3 μM oxyhaemoglobin and 10–100 μM hydroxocobalamin as shown in Figures 2 and 3. Mean data for the inhibitory effects of oxyhaemoglobin and 10–100 μM hydroxocobalamin are shown in Figure 4a and 4b, respectively. The IC_{50} values for inhibition of NO-induced relaxation for oxyhaemoglobin and hydroxocobalamin were 0.14 ± 0.05 μM ($n=5$) and 46 ± 9.6 μM ($n=6$), respectively.

Responses to nitrergic nerve stimulation were more resistant than those to NO to the inhibitory effects of oxyhaemoglobin. Low concentrations of oxyhaemoglobin (0.1–0.3 μM), that reduced responses to NO, were without effect; however, higher concentrations (1–30 μM) inhibited the responses in a concentration-dependent manner (Figure 4c). The IC_{50} value for inhibition of field stimulation induced relaxation was 19.7 ± 5.1 μM ($n=6$).

Hydroxocobalamin at 10 and 30 μM did not reduce relaxations elicited by nitrergic nerve stimulation, and with stimulation at 2 Hz it slightly but significantly ($P=0.02$ and 0.03 for 10 and 30 μM, respectively; Student's paired t test) enhanced them (Figure 4d). The highest concentration of hydroxocobalamin used (100 μM) did not significantly reduce responses to nerve stimulation. Higher concentrations of hydroxocobalamin caused a substantial fall in tone which prevented further testing for inhibitory activity.

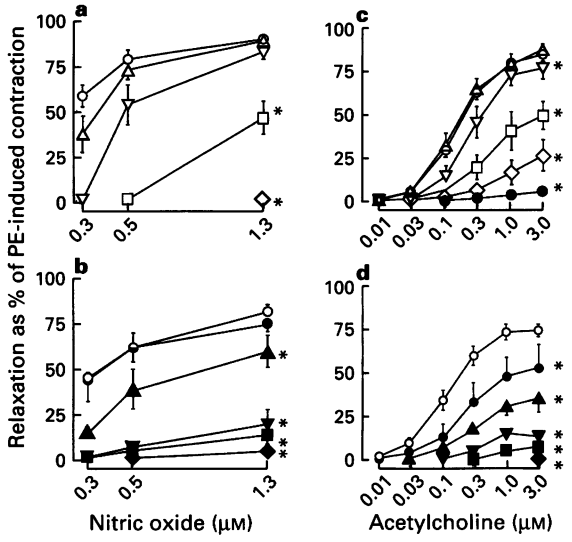

Figure 1 Inhibition by haemoglobin (a and c) and hydroxocobalamin (b and d) of relaxations of rat aortic rings precontracted with phenylephrine induced by NO in aqueous solution (a and b: endothelium-denuded rings) or acetylcholine (c and d; endothelium-intact rings). Control responses are indicated by (○). Concentrations of haemoglobin and hydroxocobalamin in μM are: (△) 0.01; (▽) 0.03; (□) 0.1; (◇) 0.3; (●) 1.0; (▲) 3.0; (▼) 10.0; (■) 30.0; (◆) 100.0. Symbols indicate means with s.e. mean which in some cases were smaller than the size of the symbol. The numbers of observations were a=4; b=5; c=6; and d=3. * indicates $P<0.05$ (ANOVA; repeated measures).

Discussion

Oxyhaemoglobin was about equally effective in blocking EDRF- and NO-induced relaxations in rat aortic rings. The IC_{50} value for inhibition of NO-induced relaxations was 0.11 ± 0.02 μM, and the IC_{50} value for matching EDRF-mediated relaxations elicited by acetylcholine was 0.13 ± 0.02 μM. These values are not significantly different ($P=0.29$; Student's unpaired t test). The conclusions that can be drawn from these findings are that oxyhaemoglobin readily penetrated to the biophase between endothelial cells and the medial smooth muscle and that there is no reason to doubt that EDRF is in

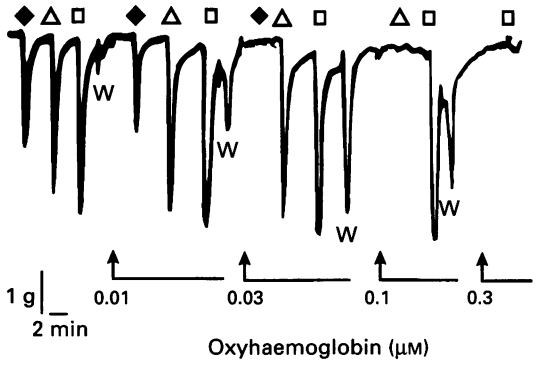

Figure 2 Tracing illustrating relaxations of an anococcygeus muscle induced by NO and their inhibition by haemoglobin. Concentrations of NO in μM are: (◆) 0.3; (△) 0.5; and (□) 1.3. W = wash with fresh PSS.

Table 1 Effects of oxyhaemoglobin and hydroxocobalamin on mean contractile responses in g tension to phenylephrine (1 μM) in endothelium-intact rat aorta

Substance	*Concentration* (μM)	*Mean*	*s.e.mean*	P
Oxyhaemoglobin	0	1.7	0.1	
($n=4$)	0.01	1.8	0.2	0.3
	0.03	1.9	0.2	0.04*
	0.1	1.9	0.2	0.06
	0.3	2.0	0.2	0.02*
	1.0	2.0	0.2	0.02*
Hydroxocobalamin	0	1.6	0.2	
($n=3$)	1	1.8	0.4	0.4
	3	1.9	0.3	0.3
	10	1.9	0.2	0.01*
	30	1.9	0.2	0.02*
	100	2.0	0.2	0.01*

The last column shows the probability levels (P) of the differences between the means of groups, compared to the respective control (Student's paired t tests). *Indicates statistical significance.

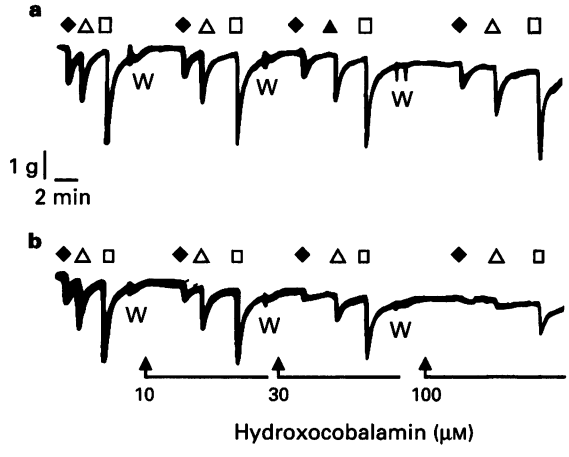

Figure 3 Tracings illustrating relaxations of an anococcygeus muscle induced by NO. Concentrations of NO in μM are: (◆) 0.3; (△) 0.5; and (□) 1.3. (a) Time control experiment. (b) Inhibition by hydroxocobalamin. W = wash with fresh PSS.

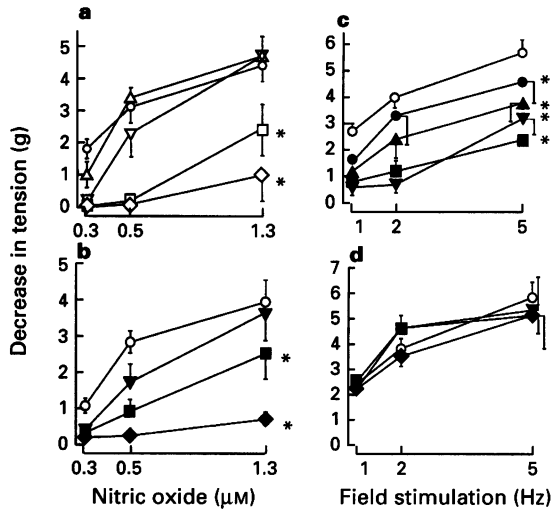

Figure 4 Inhibition by haemoglobin (a and c) and hydroxocobalamin (b and d) of relaxations of rat anococcygeus muscles precontracted with guanethidine and clonidine by NO in aqueous solution (a and b) or field stimulation (c and d). Control responses are indicated by (○). Concentrations of haemoglobin and hydroxocobalamin in μM are: (△) 0.01; (▽) 0.03; (□) 0.1; (◇) 0.3; (●) 1.0; (▲) 3; (▼) 10; (■) 30; (◆) 100. Symbols indicate means with s.e. mean which in some cases were smaller than the size of the symbol. The numbers of observations were: a = 5; b = 6; c = 6; and d = 5. * indicates $P < 0.05$ (ANOVA).

fact NO, as adduced by others (Feelisch *et al.*, 1994; Rand & Li, 1995c).

Oxyhaemoglobin was equally effective in reducing responses to NO in anococcygeus muscles and in aortic rings (cf. Figures 1a and 4a). The IC_{50} values for the inhibition of NO-induced responses was 0.14 ± 0.05 μM as in rat aortic rings. Thus, there was no great difference between the anococcygeus muscles and aortic rings in terms of the NO-scavenging action of oxyhaemoglobin.

In anococcygeus muscles, oxyhaemoglobin was considerably less effective in inhibiting relaxations elicited by nitrergic nerve stimulation ($IC_{50} = 19.7 \pm 5.1$ μM) than by NO. Similarly, in the opossum internal anal sphincter, human recombinant haemoglobin had an IC_{50} value of 3.9 μM against NANC (nitrergic) nerve-mediated relaxations compared to 0.07 μM against exogenous NO (Rattan *et al.*, 1995). Poor penetration of haemoglobin to the biophase between the nitrergic nerve terminals and the smooth muscle could contribute to this difference, as also suggested by Rattan *et al.* (1995). Nevertheless, it apparently did penetrate to a sufficient extent to impair nitrergic transmission when the concentration in the bath fluid was high enough. Another reason, for the difference, based on the assumption that the nitrergic transmitter is in fact NO, is that the rapid rate of diffusion of neurogenic NO would require a reduction of its half-life by oxyhaemoglobin to the sub-millisecond range before its inhibitory effect could be manifested (Wood & Garthwaite, 1995). Alternatively, it may be that the nitrergic transmitter differs from EDRF and is not free radical NO.

Hydroxocobalamin was somewhat more potent in inhibiting responses to EDRF than those to NO in aortic rings. The IC_{50} values for the inhibition of EDRF and NO-induced responses were 3.4 ± 0.2 μM and 8.4 ± 0.6 μM, respectively. These values are significantly different ($P = 0.002$; Student's unpaired *t* test). We have no explanation to offer for this difference. However, as with oxyhaemoglobin, it apparently penetrated readily to the biophase between the sites of release and action of EDRF and there is no reason to doubt that EDRF closely resembles NO.

In anococcygeus muscles, hydroxocobalamin was less effective against NO than in aortic rings, the IC_{50} value being 46 ± 9.6 μM; again, we have no explanation for its lower efficacy. It was, however, completely ineffective in reducing responses to nitrergic nerve stimulation. In fact, in concentrations of 10 and 30 μM, hydroxocobalamin increased responses to nerve stimulation at 2 Hz. One interpretation of the findings is that hydroxocobalamin failed to penetrate readily into the anococcygeus musle, and particularly to the biophase between nitrergic nerve terminals and the smooth muscle. However, as pointed out by Gillespie & Sheng (1989), the gap between nitrergic nerve varicosities and smooth muscle cells in the rat anococcygeus is about 260 nm whereas that between the abluminal surface of endothelium cells and the adjacent muscle cells in 50–100 nm. Since both hydroxocobalamin and oxyhaemoglobin can enter the latter smaller space and inhibit EDRF responses, it would be expected that hydroxocobalamin, like oxyhaemoglobin but with only about one-fifth of the molecular weight, could also reach the larger space of the neuroeffector junctions. There is no obvious physiochemical attribute of hydroxocobalamin that would account for a poorer penetration into tissues than oxyhaemoglobin and, since it has vitamin activity, it probably crosses cell membranes. Another interpretation, which we have advanced elsewhere (Rand & Li, 1995a, b, c), is that the nitrergic transmitter is not free radical NO.

Hydroxocobalamin and oxyhaemoglobin were found to enhance reponses to phenylephrine in endothelium-intact aorta, suggesting that they also inhibited basally released EDRF. The enhanced contraction to phenylephrine is unlikely to contribute to the inhibitory effect of these compounds against acetylcholine-induced relaxations since at some concentrations that had little effect on phenylephrine-induced contractions (oxyhaemoglobin, 0.1 μM and hydrox-ocobalamin 1 and 3 μM), they still significantly inhibited acetylcholine-induced relaxations.

The finding that hydroxocobalamin appears to discriminate between NO and EDRF on the one hand and the nitrergic transmitter on the other hand resembles the findings with another NO-sequestering agent, namely carboxy-PTIO (Rand & Li, 1995c). A further point of similarity is that enhancement of stimulation-induced relaxations was observed with both hydroxocobalamin and carboxy-PTIO. We suggest that this effect may be due to sequestration of NO in the immediate vicinity of NOS which would counteract the feedback inhibition of the enzyme that has been described (Rogers & Ignarro, 1992; Assreuy *et al.*, 1993; Rengasamy & Johns, 1993). If this were so, it would follow that the nature of the NO produced by neuronal NOS changes between its site of formation and its release into the neuroeffector junction.

The strongest evidence for the identity of the nitrergic

transmitter with NO comes from studies with cascade bioassay systems, using the rat gastric fundus, guinea-pig colon or dog ileocolonic junction as donor tissues and relaxations of rabbit aortic rings as detector (Boeckxstaens *et al.*, 1991; 1994; Iversen *et al.*, 1994; De Man *et al.*, 1995). In these experiments, the nitrergic transmitter released from the donor tissues had similar characteristics to NO. The results from these studies do not exclude the possibilities that NO may have been formed from the nitrergic transmitter in the period after it was released from donor tissues. A further consideration is that much of the NO-like mediator in gastrointestinal tissues may have originated from interstitial cells of Cajal, in which endothelial-type NOS is activated by the nitrergic transmitter and serves to amplify the effect of the nitrergic transmitter (Publicover *et al.*, 1994).

In conclusion, the present study demonstrates that oxyhaemoglobin and hydroxocobalamin can discriminate between NO and the nitrergic transmitter, but not between EDRF and NO. This finding, together with previous studies using a variety of tissues and animal species (Jenkinson *et al.*, 1995; Rand and Li, 1995c; and see Rand and Li, 1995a, b for earlier references) suggest either a chemical difference between NO and the nitrergic transmitter or that the milieu of nitrergic neuoeffector junctions differs from that of the space between endothelial cells and medial smooth muscle in such a way that the NO-sequestering action of oxyhaemoglobin and hydroxocobalamin are not readily manifested.

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