Br. J. Pharmacol. (1988), **95**, 1151–1156

Influence of haemoglobin and erythrocytes on the effects of EDRF, a smooth muscle inhibitory factor, and nitric oxide on vascular and non-vascular smooth muscle

John S. Gillespie & Hong Sheng

Department of Pharmacology, University of Glasgow, Glasgow, G12 8QQ, Scotland

1 The relaxant action of endothelium-derived relaxing factor (EDRF), the smooth muscle inhibitory factor (IF) isolated from the bovine retractor penis (BRP), nitric oxide (NO) and sodium nitroprusside (NaNP) on four vascular and non-vascular smooth muscle preparations has been examined. Their sensitivity to EDRF, the IF and NO was the same, suggesting all might be NO. Sodium nitroprusside produced complete relaxation of the rat anococcygeus at low doses, suggesting an action additional to the intracellular release of NO.

2 Haemoglobin added to solutions of EDRF, activated IF or NO completely removed their relaxant properties, consistent with all three acting by virtue of NO.

3 Suspensions of red blood cells with a haemoglobin concentration equivalent to that used in the previous experiments were as effective as haemoglobin in abolishing the relaxant effect of EDRF or NO but were ineffective against the activated IF.

4 The similarity in sensitivity of a series of smooth muscles and the binding by haemoglobin are consistent with NO being the active principle of both EDRF and the acid activated IF. The abolition of the effect of EDRF by red blood cells (RBCs) is further confirmation for this hypothesis, but the ineffectiveness of RBCs against acid-activated IF suggests that either the latter is not NO or that it is bound in a way which makes it unable to diffuse through cell membranes.

Introduction

Drugs such as acetylcholine owe their relaxant effect on vascular smooth muscle to the release of an endothelium-derived relaxant factor (EDRF), which acts on the underlying smooth muscle (Furchgott & Zawadzki, 1980). EDRF is a humoral factor since, after release from a perfused artery or cultured endothelial cells, it can be detected on distant vascular smooth muscle preparations whose own endothelium has been removed (Griffith *et al.*, 1984; Cocks *et al.*, 1985). Recently experimental evidence that EDRF is nitric oxide has been obtained (Palmer *et al.*, 1987). However, only recently has the effect of EDRF on non-vascular smooth muscle been studied (Shikano *et al.*, 1987) and none of the non-vascular muscles tested by these workers (guinea-pig trachea and uterine horn, rabbit taenia coli) responded to EDRF, though the taenia coli was relaxed by nitric oxide which suggests that EDRF is not nitric oxide.

Another naturally-occurring smooth muscle relaxant is the inhibitory factor (IF) isolated from the bovine retractor penis muscle (BRP) (Ambache *et al.*, 1975; Gillespie & Martin, 1980). This IF has several features in common with EDRF; both have short half-lives, both act by stimulating guanylate cyclase and the action of both is abolished by haemoglobin or borohydride (Gillespie *et al.*, 1981; Bowman *et al.*, 1982; Griffith *et al.*, 1984). If both EDRF and the IF act through nitric oxide then the sensitivity of different smooth muscles to these compounds and to nitric oxide should be the same. In this paper we describe such a comparison on four isolated smooth muscle preparations, the rabbit aortic strip, the BRP,

the rat anococcygeus and the guinea-pig tracheal muscle.

If the IF owes its relaxant properties to NO then haemoglobin, which binds NO, should also bind the IF after acid-activation. Furthermore, since NO is a highly permeant molecule passing easily through membranes, red blood cells (RBCs) should be as effective as haemoglobin in binding and removing inhibitory activity. This was tested and it was shown that RBCs bound NO and EDRF, but not IF. Some of these results have been presented to the British Pharmacological Society (Gillespie & Sheng, 1988).

Methods

EDRF was liberated by acetylcholine (ACh) from the endothelium of a 6 cm length of rabbit aorta perfused at 4 ml min^{-1} with oxygenated Krebs solution containing indomethacin (5×10^{-6} M) at 33°C. This donor aorta was contained within the closed cascade system illustrated in Figure 1. The perfusate passed over the test tissues in a 1.6 ml chamber in the same cascade immediately above the donor tissue. The upper chamber, which was oxygenated separately, contained two test tissues one of which was always the highly sensitive endothelium-free spiral strip of rabbit abdominal aorta. Tone was generated in the test tissues by infusions of ACh and 5-hydroxytryptamine (5-HT), each at a concentration of 10^{-5} M, into the upper chamber. Drugs could be injected or infused into either the upper chamber to induce tone (inlet port 1 in Figure 1) or into the flow of Krebs immediately before the donor aorta (inlet port 2). In experiments testing the effect of haemoglobin or RBCs on the activity of EDRF released from the donor aorta, solutions of haemoglobin or suspensions of RBCs were infused into the upper test chamber.

The relaxant effects of NO, IF and sodium nitroprusside were tested more conventionally in 10 ml jacketed organ baths containing Krebs solution at 33°C and oxygenated with 95% O_2/5% CO_2. Tissues were obtained from animals killed by exposure to pure CO_2, except for the BRP muscles which were obtained from the abattoir and used usually within three hours of the animal's death. Lyophilised semi-purified extracts of IF were prepared essentially as previously described (Bowman *et al.*, 1982), but with a NaCl gradient elution from the anion exchange column which separated adenine nucleotides from the IF and made the alumina column unnecessary. Aliquots of a single batch of IF were freeze dried and ampoules from this single extract used for all the experiments described here. Extracts

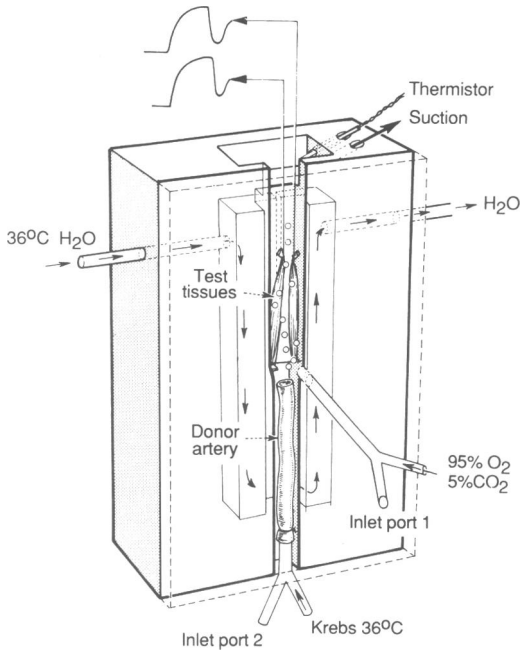

Figure 1 A diagrammatic representation of the closed cascade system used to release EDRF from a donor rabbit aorta and detect this on test tissues in a chamber immediately above the perfused aorta. Drugs can be introduced either into the upper chamber alone through inlet port 1 or into the Krebs solution perfusing the aorta (inlet port 2). The entire cascade is jacketed on three sides with channels through which water at 36°C is pumped to maintain a constant temperature.

were reconstituted in distilled water, activated at pH 2 with 1 N HCl, then neutralised with 1 N NaOH and kept on ice till tested. Gaseous nitric oxide, 99% pure, was obtained from BDH. 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 fine hypodermic needle attached to a gas-tight syringe and 6.5 µl of gas injected into a rubber-sealed 65 ml bottle completely filled with normal saline previously purged for 1 hour with helium. This produced a 4.4×10^{-6} M stock solution of NO which was freshly made each day and protected from light. In some experiments, to extend the dose-response curves, a five times more concentrated stock solution was prepared. Sodium nitroprusside was made up as a 10^{-2} M stock solution in normal saline. Haemoglobin solutions were freshly prepared by haemolysing rat blood as previously described (Bowman *et*

al., 1982). Briefly, blood was collected through a polythene cannula from the carotid artery of an anaesthetized rat into heparin-containing tubes. The RBCs were separated by centrifugation, washed in isotonic phosphate buffer and resuspended in a volume of buffer equal to the original volume of blood. One ml of this suspension was added to 19 ml of a 20 mosmol solution of hypotonic phosphate buffer pH 7.4 to lyse the cells. Cell membranes were removed by centrifugation and the concentration of haemoglobin in the supernatant measured spectrophotometrically as methaemoglobin. In experiments with intact RBCs these were suspended in saline and either added to solutions of relaxants or infused into the upper chamber of the cascade from a syringe driven by a stepping motor.

Results

The effect of EDRF

The effect of EDRF on three of the test smooth muscles is illustrated in Figure 2. The upper trace compares a strip of BRP and the lower a rat anococcygeus with rabbit aortic strips present in the upper chamber. In each pair the EDRF released by ACh produced a dose-related relaxation of the aortic strip. The BRP was also powerfully relaxed but the rat anococcygeus was unaffected. A similar failure to respond was observed in every preparation of the anococcygeus or guinea-pig trachea, whereas every preparation of BRP and aorta relaxed. The results of all experiments are summarized in Figure 3. The maximum relaxation of the most sensitive tissue, the aortic strip, was 45% at a concentration of 5×10^{-6} M ACh. Greater relaxation can be obtained in aortic strips retaining their own endothelium, presumably because the short diffusional distance minimizes delay and dilution. Nevertheless, even in such endothelium-retaining strips, ACh 5×10^{-6} M still produced maximum relaxation suggesting this dose produced maximum release of EDRF. An alternative explanation is that higher concentrations of ACh were producing a motor effect by their direct action on the muscarinic receptors on the smooth muscle of the test tissues which masked the relaxant effect induced by the increased release of EDRF. This would also explain the decline in the relaxant effect of ACh concentrations greater than 5×10^{-6} M which is seen in Figure 3. This possibility was tested in 10 experiments by infusing atropine 10^{-6} M into the upper chamber and at the same time using 5-HT to induce tone in the test tissues. In the presence of

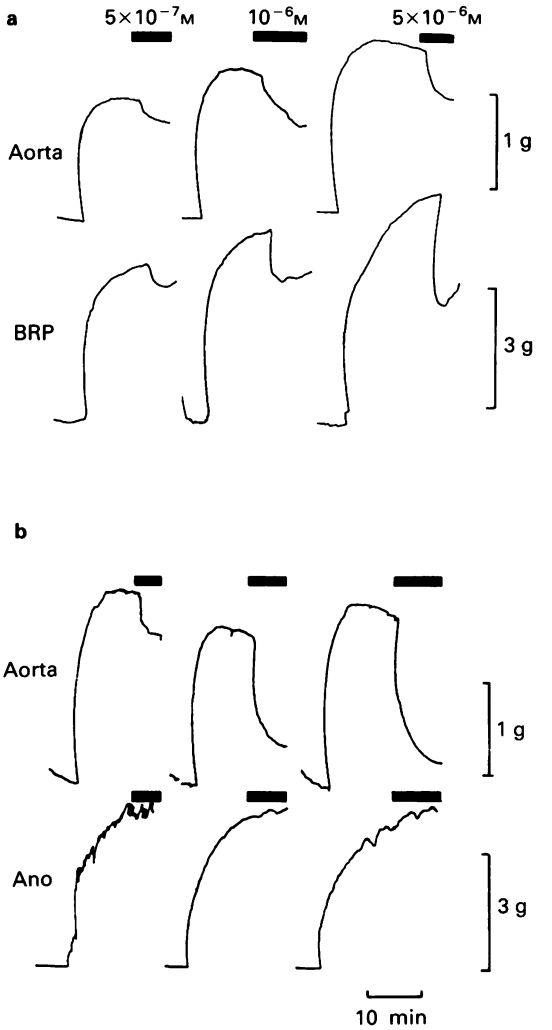

Figure 2 The response of isolated smooth muscle preparations to EDRF liberated by acetylcholine (ACh) from a donor rabbit aorta and superfused over test tissues as shown in Figure 1. The test tissues in (a) are an endothelium-free spiral strip of rabbit aorta and a strip of bovine retractor penis (BRP) muscle; (b) (from another experiment) are a rabbit aortic strip and a rat anococcygeus muscle (Ano). Tone was induced in the test preparations by infusing ACh and 5-hydroxytryptamine (5-HT) into the upper chamber to produce a concentration of 10^{-5} M of each. ACh was infused into the Krebs solution perfusing the donor aorta for the period shown by the black bar to produce concentrations of 5×10^{-7} M, 10^{-6} M and 5×10^{-6} M. EDRF liberated from the donor aorta produced dose-related relaxation of tone in the rabbit aortic strip and the BRP in (a) but, in (b), though the aortic strip relaxed, the rat anococcygeus did not. The Krebs solution contained indomethacin 5×10^{-6} M.

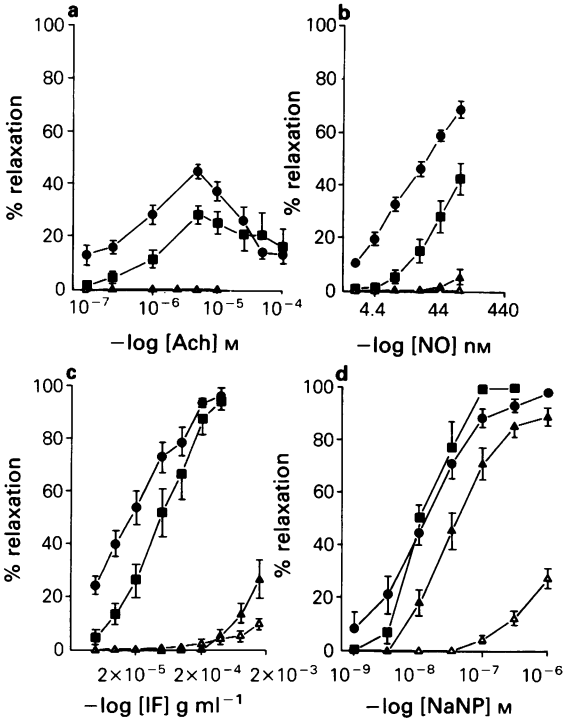

Figure 3 Log dose-response curves for the relaxant effect induced by (a) acetylcholine (ACh) (EDRF), (b) nitric oxide (NO), (c) inhibitory factor (IF) extracted from the bovine retractor penis (BRP) and (d) sodium nitroprusside (NaNP) on spiral strips of endothelium-free rabbit aorta (●), strips of BRP (■), the rat anococcygeus muscle (▲) and the guinea-pig trachea (△). EDRF caused a dose-related relaxation of the rabbit aortic strip and the BRP but had no effect on the rat anococcygeus muscle or guinea-pig trachea. Nitric oxide and the IF also relaxed the rabbit aortic strip and the BRP but had no effect on the rat anococcygeus muscle or the guinea-pig trachea except at high doses. No concentration of NO tested relaxed the guinea-pig trachea. Sodium nitroprusside was equally effective in relaxing the rat anococcygeus muscle, the rabbit aortic strip and the BRP and produced a maximum response of equal magnitude in all three tissues but was almost ineffective on the guinea-pig trachea. Each point is the mean of between 6 and 42 observations and vertical lines indicate s.e.mean. Indomethacin 5×10^{-6} M was present in the Krebs solution throughout.

atropine there was no decline in the response to concentrations of ACh above 5×10^{-6} M, nevertheless maximum relaxation was still observed at this concentration. The dose-response curves for EDRF illustrated in Figure 3 are, therefore, truncated by this upper limit on the amount of EDRF which can be liberated.

The effects of NO, IF and sodium nitroprusside

Dose-response curves for the relaxant effect of each of these three agents on isolated tissues in 10 ml baths is illustrated in Figure 3. Tone was induced in all tissues with the same mixture of 10^{-5} M ACh and 10^{-5} M 5-HT used in the cascade experiments with EDRF. All three stimuli produced graded relaxation of the rabbit aortic strip and the BRP which reached a maximum at concentrations which had little or no effect on the anococcygeus or trachealis muscle. Further increases in the concentration of NO or IF did produce small relaxations in the rat anococcygeus but even the highest concentration of nitric oxide tested did not relax the guinea-pig trachea. The pattern of response to sodium nitroprusside was different. The aortic strip, the BRP and the rat anococcygeus were readily relaxed by it but the trachealis remained relatively insensitive.

The effect of haemoglobin and RBCs

As Figure 3 illustrates, the sensitivity of the four muscle preparations to EDRF, NO and IF was the same, consistent with nitric oxide being the final mediator of relaxation by both EDRF and IF as suggested by Furchgott (1988) and Martin *et al.* (1988). If this hypothesis is true, haemoglobin, which binds NO, should not only abolish the responses to these agents when added to the organ bath but, if added to the solutions of these relaxants and then removed, it should abolish their relaxant effects when subsequently added to the organ bath. We tested this for the IF and nitric oxide by adding haemoglobin 10^{-5} M to solutions of NO, unactivated IF and acid-activated IF and allowing 10 min for binding. The haemoglobin was then removed by filtration through an Amicon YM10 ultrafiltration membrane with a retention value of 10,000 Daltons. The unactivated extract after separation was activated and all three solutions assayed on the rabbit aortic strip. In these experiments with NO care was taken to prevent loss of NO other than by binding to haemoglobin. Two problems in particular caused difficulties. Firstly, in spite of deoxygenating solutions with helium, the stirrer in the Amicon cell caused a near complete loss of activity. The solutions were, therefore, filtered without stirring. Secondly, the membrane of these filters can bind NO. This was controlled by filtering a solution of NO which had not been mixed with Hb and using this as the control against which the NO/Hb mixture was compared. A result, typical of the five experiments carried out, is shown in the upper records of Figure 4. The relaxant effect of nitric oxide was almost completely removed by haemoglobin, and that of activa-

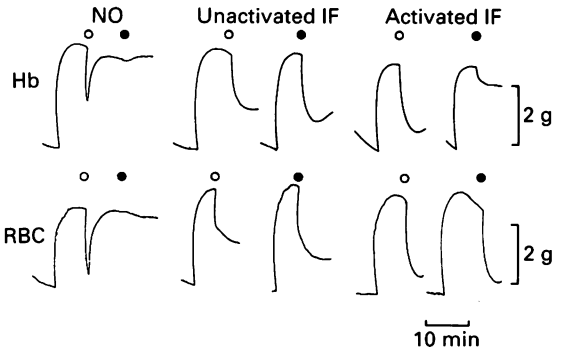

Figure 4 The binding of nitric oxide (NO) and inhibitory factor (IF) extracted from the bovine retractor penis (BRP) by 10^{-5} M haemoglobin (Hb, upper records) or an equivalent concentration of red blood cells (RBC, lower records). In each pair of responses the first (○) is the control to either NO or IF and the second (●) the response to an aliquot of the same solution of nitric oxide or IF mixed either with Hb or RBCs, left for ten minutes when separated either by ultrafiltration (Hb) or centrifugation (RBCs). Where unactivated IF was exposed to Hb or RBCs it was activated with acid after separation and before assay. Haemoglobin completely removed the relaxant activity from a solution of NO, greatly reduced the activity of acid-activated IF but had no effect on the unactivated IF. RBCs abolished the relaxant effect of a solution of NO but had no effect on the relaxant action of either activated or unactivated IF.

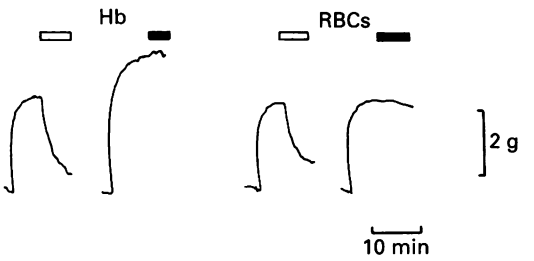

Figure 5 The effect of infusing haemoglobin (Hb), 10^{-6} M, or an equivalent concentration of rat red blood cells (RBCs) over an endothelium-free rabbit aortic strip on the response to EDRF released by infusing acetylcholine (ACh) 5×10^{-6} M through the donor aorta. The open bars show the control responses to ACh infused in the absence of Hb or RBC; the solid bars show the responses in the presence of either Hb or RBC; the RBCs were as effective as Hb in abolishing the relaxant effect of EDRF.

ted IF greatly reduced, but there was no loss of activity in the unactivated extract. Since nitric oxide can easily pass through cell membranes, the haemoglobin in intact RBCs should be as effective as a solution of haemoglobin. RBCs equivalent to a 10^{-5} M solution of haemoglobin were added to nitric oxide solutions and to activated and unactivated IF. The cells were removed by centrifugation after 10 min, and the activity of the supernatant assayed. The results are shown in the lower records of Figure 4. As expected, RBCs were as effective as haemoglobin in abolishing the relaxant effect of nitric oxide solutions but were without effect on either the unactivated or activated IF.

Comparable experiments with EDRF were also done. However, as EDRF has a very short half-life of about 6 s, it was not possible to expose solutions of EDRF to haemoglobin and RBCs. Instead haemoglobin or RBCs equivalent to a 10^{-6} M solution of haemoglobin were infused into the upper chamber of the cascade at the same time as 5×10^{-6} M ACh was added to the Krebs solution perfusing the aorta to release EDRF. When RBCs were used the solution leaving the cascade was collected, centrifuged and examined spectrophotometrically to detect any haemolysis and release of haemoglobin which might have taken place. There was none. In three experiments, one of which is illustrated in Figure 5, RBCs were as effective as haemoglobin in abolishing the responses to EDRF.

Discussion

The recent paper by Shikano *et al.* (1987) suggested that only vascular smooth muscle might be sensitive to EDRF. The present results show this is not so; the BRP is almost as sensitive as the aortic strip. The similar sensitivity of the four muscles tested to EDRF, IF and NO is consistent with all three stimuli acting through nitric oxide. This nitric oxide may be released from IF only after acid activation which would explain the inability of haemoglobin to abolish the relaxant properties of inactive IF. However, if nitric oxide is the active principle of IF it cannot be free in solution since it is unable to diffuse through the membrane of the red blood cell as nitric oxide or EDRF do. Martin *et al.* (1988) have suggested that the IF is NO and that this is liberated from nitrite by acid activation. The released NO is then stabilized by some unidentified component of the semipurified extract. If so, the nitric oxide complex is too large to diffuse through the RBC membrane. An alternative suggestion is that the precursor is some other compound which can liberate NO or has effects similar to nitric oxide. Higher concentrations of IF in the high-speed sediment from

homogenised BRP muscle (Gillespie & Hunter, 1982), a molecular weight between 500 and 1,000, whether activated or unactivated; irreversible destruction by boiling, retention on an anion column even when activated, and the ability to isolate a material with similar properties from connective tissue and the vitreous of the bovine eye (Gillespie, 1987) are properties difficult to reconcile with nitrite as precursor.

Sodium nitroprusside like other nitrodilators is believed to owe its relaxant properties to the liberation of NO within the smooth muscle cell. If so, the sensitivity of different muscles to this agent should parallel that to NO. This is not so, as illustrated in Figure 3. The rat anococcygeus is almost insensitive to NO but highly sensitive to sodium nitroprusside. We have no explanation for this, though it may indicate a relaxant action independent of the release of nitric oxide.

H.S. is a University of Glasgow Postgraduate Scholar. The authors gratefully acknowledge support provided by the Medical Research Funds of the University of Glasgow and the ORS Award to H.S.

References

AMBACHE, N., KILLICK, S.W. & ZAR, M.A. (1975). Extraction from ox retractor penis of an inhibitory substance which mimics its atropine-resistant neurogenic relaxation. *Br. J. Pharmacol.*, **54**, 409–410.

BOWMAN, A., GILLESPIE, J.S. & POLLOCK, D. (1982). Oxyhaemoglobin blocks non-adrenergic non-cholinergic inhibition in the bovine retractor penis muscle. *Eur. J. Pharmacol.*, **85**, 221–224.

COCKS, T.M., ANGUS, J.A., CAMPBELL, J.H. & CAMPBELL, G.R. (1985). Release and properties of endothelium-derived relaxing factor (EDRF) from cells in culture. *J. Cell. Physiol.*, **123**, 310–320.

FURCHGOTT, R.F. (1988). Studies on the relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activatable inhibitory factor from bovine retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. In *Mechanisms of Vasodilatation*. ed. Vanhoutte, P.M. New York: Raven Press (in press).

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GILLESPIE, J.S. (1987). Searching for the non-adrenergic non-cholinergic autonomic transmitter. In *Plenary Lecture Xth International Congress of Pharmacology*. ed. Rand, M.J. & Raper, C. pp. 161–170. Amsterdam: Elsevier Science Publishers B.V.

GILLESPIE, J.S. & HUNTER, J.C. (1982). Subcellular localisation of smooth muscle inhibitory factor isolated from the bovine retractor penis muscle. *Br. J. Pharmacol.*, **76**, 189P.

GILLESPIE, J.S., HUNTER, J.C. & MARTIN, W. (1981). Some physical and chemical properties of the smooth muscle inhibitory factor in extracts of the bovine retractor penis muscle. *J. Physiol.*, **315**, 111–125.

GILLESPIE, J.S. & MARTIN, W. (1980). A smooth muscle inhibitory material from the bovine retractor penis and rat anococcygeus muscles. *J. Physiol.*, **309**, 55–64.

GILLESPIE, J.S. & SHENG, H. (1988). The relationship between the EDRF, the smooth muscle inhibitory factor from the BRP and nitric oxide. *Br. J. Pharmacol.*, **94**, 338P.

GRIFFITH, T.M., EDWARDS, D.H., LEWIS, M.J., NEWBY, A.C. & HENDERSON, A.H. (1984). The nature of endothelium-derived vascular relaxant factor. *Nature*, **308**, 645–647.

MARTIN, W., SMITH, J.A., LEWIS, M.J. & HENDERSON, A.H. (1988). Evidence that inhibitory factor extracted from bovine retractor penis is nitrite, whose acid-activated derivative is stabilized nitric oxide. *Br. J. Pharmacol.*, **93**, 579–586.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium derived relaxing factor. *Nature*, **327**, 524–526.

SHIKANO, K., OHLSTEIN, E.H. & BERKOWITZ, B.A. (1987). Differential selectivity of endothelium-derived relaxing factor and nitric oxide in smooth muscle. *Br. J. Pharmacol.*, **92**, 483–485.

(Received April 30, 1988
Revised July 7, 1988
Accepted July 20, 1988)