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A comparison of haemoglobin and erythrocytes as inhibitors of smooth muscle relaxation by the NANC transmitter in the BRP and rat anococcygeus and by EDRF in the rabbit aortic strip

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1 The inhibitory effect of erythrocyte suspensions and haemoglobin solutions on the response of the bovine retractor penis muscle (BRP) and the rat anococcygeus to field stimulation of their non-adrenergic non-cholinergic (NANC) nerves has been compared. Haemoglobin 3 μM greatly reduced the relaxant response in both tissues whereas a haemoglobin-equivalent suspension of erythrocytes was without effect.

2 A similar comparison of erythrocytes and haemoglobin on the response of the rabbit aortic strip to EDRF liberated by acetylcholine (ACh) showed that both reduced EDRF-mediated relaxation, though haemoglobin was significantly more effective.

3 These results suggest that the NANC transmitter may not be as freely diffusible through the erythrocyte membrane as EDRF and may therefore not be nitric oxide.

Introduction

Haemoglobin is known to block smooth muscle relaxation by the endothelium-derived relaxant factor (EDRF) in the rabbit aorta and the non-adrenergic non-cholinergic (NANC) inhibitory neurotransmitter in both the bovine retractor penis muscle (BRP) and the rat anococcygeus (Bowman & Gillespie, 1982; Bowman *et al.*, 1982; Martin *et al.*, 1985). Recently we have shown that erythrocytes are as effective as haemoglobin in blocking the relaxant effect of EDRF released into the superfusing fluid in a cascade system (Gillespie & Sheng, 1988a,b). Erythrocytes also blocked the effect of nitric oxide. These results are consistent with the suggestion that EDRF is nitric oxide (Palmer *et al.*, 1987) and that the mechanism of action of either haemoglobin or erythrocytes is physically to bind the nitric oxide, either directly or after the nitric oxide has diffused through the erythrocyte membrane.

The identity of the neurotransmitter of the NANC inhibitor nerves in the BRP and anococcygeus is unknown. It has, however, several properties in common with EDRF. For example, both relax the BRP (Gillespie & Sheng, 1988a,b), both increase cyclic GMP (Rapoport & Murad, 1983; Diamond & Chu, 1983; Bowman & Drummond, 1984), both are abolished by anoxia (Furchgott & Zawadzki, 1980; Bowman & McGrath, 1985) and by borohydride (Griffith *et al.*, 1984; Gillespie & Sheng, unpublished observation), as well as by haemoglobin. If EDRF is nitric oxide, it is possible that the NANC transmitter is also nitric oxide. If this is so, then not only haemoglobin but also erythrocytes might block the response to nerve stimulation. A possible error in this argument lies in the short diffusion distance between varicosities and the smooth muscle cells and the likely inability of erythrocytes to penetrate to any significant extent into the tissue. In such a system the erythrocytes would be at a considerable disadvantage in binding the neurotransmitter, in comparison with a cascade system in which EDRF is released into the superfusion fluid and has several seconds for binding before this fluid reaches the test tissue. Aortic strips retaining their endothelium and directly relaxed by EDRF, released from that endothelium by acetylcholine, represent a situation closer to the conditions in the BRP and anococcygeus. We have, therefore, compared the relative abilities of haemoglobin and erythrocytes to abolish the NANC

nerve response in the BRP and rat anococcygeus with their effect on the relaxant action of acetylcholine in the rabbit aortic strip.

Methods

New Zealand rabbits (2–3 kg) were killed by CO_2 and bled. The abdominal aorta was removed, cleaned and spiral strips cut at an angle of 30° to the line of the artery to produce strips 2–3 mm wide and 2 cm long. These strips were prepared under microscopic observation and care was taken to avoid damaging the endothelium. Strips were suspended in 10 ml organ baths under a resting tension of 1 g measured by Grass FTD3 tension transducers. Responses were displayed on a Grass polygraph. The organ bath contained Krebs solution at 36°C and was bubbled with 95% O_2 + 5% CO_2. Tone was induced by adding 5-hydroxytryptamine (5-HT, 10^{-5} M). Acetylcholine 10^{-6} M was added to each preparation at the beginning of the experiment, to check that relaxation was produced and the endothelium was present. All preparations produced 90% or more relaxation of the 5-HT tone in response to acetylcholine.

BRP muscles were obtained from the local abattoir and normally used within 3 h of the animals' death. Muscles were cleaned of connective tissue and thin strips, 2–3 mm in diameter and 2 cm in length, were cut and removed. The rat anococcygeus muscles were isolated as previously described (Gillespie, 1972). Both the BRP preparation and the rat anococcygeus were suspended in pairs of Ag-AgCl ring electrodes under 1 g tension. Tone was raised by adding guanethidine 3×10^{-5} M. Field stimulation through the ring electrodes was applied from a Grass S44 stimulator at supramaximal voltage, 1 ms pulses and trains of stimuli lasting 10 s. The composition of the Krebs solution was (in mM) Na^+ 145, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 127, HCO_3^- 25, HPO_4^{2-} 1.2, SO_4^{2-} 1.2 and dextrose 11.

Preparation of erythrocytes and haemoglobin solutions

The technique for preparing fresh haemoglobin solution from rat blood has already been described (Bowman *et al.*, 1982). Briefly, blood was collected through a polythene cannula in one carotid artery of an anaesthetised rat into heparin containing tubes. The erythrocytes were separated by centrifugation, washed in isotonic phosphate buffer and resuspended after further centrifugation in a volume of buffer equal to the original volume of blood. One ml of this suspension was added to 19 ml of 20 mosmol 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. To prepare a suspension of erythrocytes, the same first stage of centrifuging the heparinised blood, decanting the plasma and resuspending the erythrocytes in isotonic phosphate buffer was used. The resuspended erythrocytes were again centrifuged, the supernatant decanted and the erythrocytes again suspended in isotonic buffer solution. Such washed erythrocytes proved more fragile than unwashed cells, so that when they were added to an oxygenated organ bath the turbulence caused a variable degree of lysis. This was tested at the end of each experiment by removing the bath fluid, centrifuging to remove unlysed cells, then assaying spectrophotometrically the haemoglobin content of the supernatant. Nevertheless, the blocking effect of the released haemoglobin interfered with several experiments and limited the concentrations of erythrocytes which could be used. Whole blood added to the organ bath was much more stable and, since previous experiments (Bowman & Gillespie, 1982) showed plasma to be quite ineffective against the NANC relaxation, in the present experiments suspensions of erythrocytes were produced by adding an appropriate volume of heparinised whole blood to the bath. The concentration of free haemoglobin in the bath fluid was still measured at the end of each experiment and on all occasions was below the level of detection (10^{-7} M).

Drugs

The following drugs were used: acetylcholine (Sigma), 5-HT (Sigma), guanethidine (CIBA).

Results

The effect of haemoglobin or erythrocytes on NANC relaxation

The effect of haemoglobin 3 μM and a suspension of erythrocytes with an equivalent concentration of haemoglobin on NANC relaxation is illustrated in Figure 1. Haemoglobin measurement at the end of this experiment demonstrated the absence of lysis of the erythrocytes. In these circumstances, while haemoglobin produced a 50–60% reduction in relaxation in response to NANC nerve stimulation in both the BRP and the rat anococcygeus, erythrocytes were without effect. In some experiments, particularly with washed erythrocytes or higher concentrations of erythrocytes, a variable degree of inhibition of the NANC response was observed, apparently as a result of haemolysis. The degree of

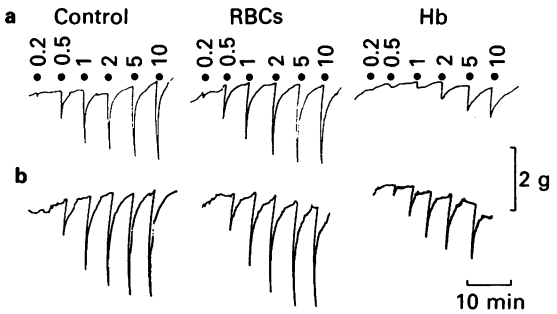

Figure 1 The response of the bovine retractor penis (a) and the rat anococcygeus (b) to field stimulation of their non-adrenergic non-cholinergic (NANC) nerves. The nerves were stimulated for 10 s at the marker (●) at the frequencies shown. Guanethedine 30 μM was present throughout to block the motor adrenergic nerves and raise tone. Haemoglobin (Hb) 3 μM in the last panel reduces responses at all frequencies and in both preparations by more than 50% whereas a comparable concentration of haemoglobin but held within erythrocytes (RBCs) is without effect.

haemolysis depended, among other things, on the time the erythrocytes spent in the bath, the vigour of oxygenation and the gravitational forces used for centrifugation of the bath fluid. Figure 2 compares the free haemoglobin concentration at the end of the experiment with the inhibition of the nerve response and shows a resonable correlation. In some experiments there was no inhibition in spite of free haemoglobin concentrations which would be expected to produce inhibition. In these experiments further haemolysis presumably occurred during centrifugation of the bath fluid. Where whole blood was used to produce the erythrocyte suspension, it was possible to compare the inhibitory effect of haemoglobin (Hb) and erythrocytes both at a concentration equivalent to 3×10^{-6} M Hb without significant haemolysis. The results are shown in Figure 3. Whereas haemoglobin produced about 50% inhibition of nerve-induced relaxation in both the anococcygeus and the BRP, suspensions of erythrocytes were completely ineffective. As the next section of results will show these results are quite different from the effects on EDRF where both erythrocyte suspensions and haemoglobin inhibited the response. The sensitivity of EDRF to inhibition by haemoglobin was higher than that of the NANC response and it was possible that the apparent resistance of the nerve responses was simply a concentration effect. If this were true, then higher concentrations of erythrocytes, with a haemoglobin content which if free would produce an inhibition equivalent to that of EDRF, would be effective against NANC nerve stimulation. Experimentally this was difficult to test because of the

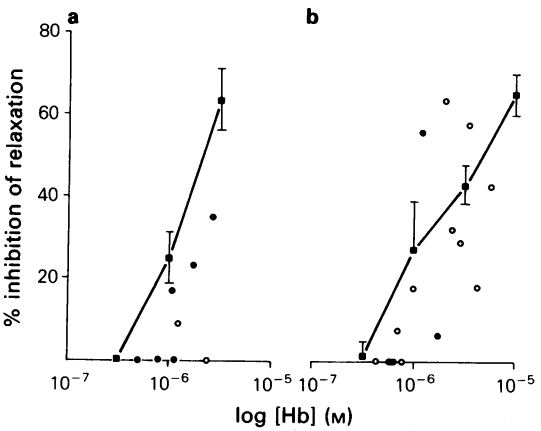

Figure 2 Comparison of the inhibitory effect of haemoglobin solutions on the responses of the (a) bovine retractor penis muscle (BRP) and (b) rat anococcygeus muscle to field stimulation of their NANC nerves, with the inhibition produced by suspensions of erythrocytes which have undergone a variable degree of haemolysis. The solid line represents the inhibitory effect of free haemoglobin at the concentrations shown on the BRP (a) and rat anococcygeus (b). The individual values show the degree of haemolysis of erythrocyte suspensions in terms of the free haemoglobin they gave rise to in comparison, with the inhibitory effect such suspensions had on the response. The erythrocyte suspensions had a haemoglobin-equivalent of either 3 μM (●) or 10 μM (○). The inhibition by the erythrocyte suspensions correlates reasonably well with the free haemoglobin they give rise to, though most values lie below the haemoglobin dose-response curve presumably because some additional cell rupture takes place during centrifugation. The NANC nerves in these experiments were stimulated at 5 Hz for 10 s.

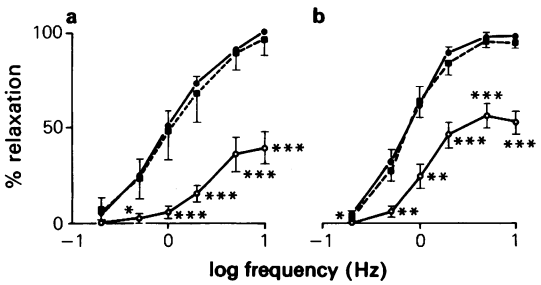

Figure 3 Frequency-response curves to field stimulation of NANC nerves at frequencies between 0.2 and 10 Hz in the (a) bovine retractor penis muscle (BRP) and (b) the rat anococcygeus. Control responses (●——●) and responses in the presence of erythrocytes with a haemoglobin concentration equal to 3 μM (■- - -■ were indistinguishable whereas haemoglobin solutions of 3 μM (○——○) produced a significant reduction at all frequencies. Vertical lines represent s.e.mean, $n = 5$; *P < 0.05, **$P < 0.01$, ***$P < 0.001$.

problem of haemolysis of higher erythrocyte concentrations. By reducing the range of frequencies tested, so as to shorten the time the erythrocytes were in the bath, and reducing the vigour of oxygenation it was possible to compare the effect of 10^{-5} M free haemoglobin with an equivalent suspension of erythrocytes without haemolysis. The results are shown in Figure 5 for the rat anococcygeus. The free haemoglobin solution produced a degree of inhibition similar to that shown for EDRF. However, the suspension of erythrocytes remained ineffective.

The effects of haemoglobin and erythrocytes on EDRF-mediated relaxation

The effect of haemoglobin solutions or erythrocyte suspensions, both at a concentration equivalent to 3×10^{-6} M haemoglobin, on the relaxation of the rabbit aortic strip to acetycholine 10^{-7} and 10^{-6} M is illustrated in Figure 4. The relaxation to the lower dose of acetylcholine was completely abolished by

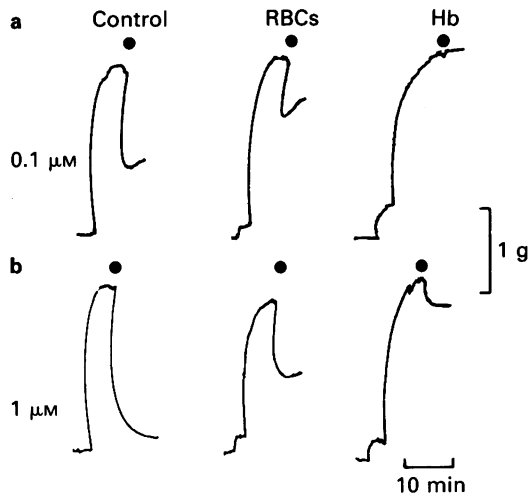

Figure 4 The response of a rabbit aortic strip to endothelium-derived relaxant factor (EDRF) liberated by two dose levels of acetylcholine, (a) 0.1 and (b) 1.0 μM, and the effect on this of haemoglobin (Hb) 3 μM or a suspension of erythrocytes (RBCs) with an equivalent haemoglobin content. Haemoglobin abolished the response to the low concentration of EDRF and greatly reduced that to the high concentration (end panels). Erythrocytes also reduced the response at both concentrations (middle panels) though the reduction was less than with haemoglobin. Tone was raised with 5-hydroxytryptamine (5-HT). In the presence of erythrocytes and haemoglobin the sensitivity to 5-HT was increased. To produce contractions of comparable magnitude the 5-HT concentration in controls, 10 μM, was reduced to 1 μM in the presence of erythrocytes and 0.1 μM in the presence of haemoglobin solution.

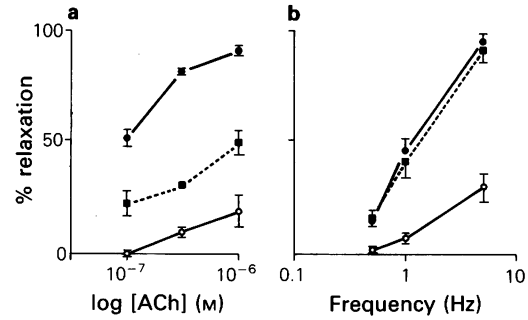

Figure 5 A comparison of the effects of free haemoglobin and a suspension of erythrocytes with an equivalent haemoglobin content on the response of the rabbit aortic strip to endothelium-derived relaxant factor (EDRF) (a) and the rat anococcygeus to NANC nerve stimulation (b). EDRF was liberated by acetylcholine (ACh) at the concentrations shown; the NANC nerves were supramaximally stimulated at 0.5, 1 and 10 Hz for 10 s. For the rabbit aortic strip the concentration of haemoglobin was 3 μM and for the rat anococcygeus 10 μM. These concentrations were chosen to produce similar inhibition of the responses to EDRF and nerve stimulation. However, the corresponding suspension of erythrocytes reduced only the response to EDRF and had no effect on the nerve response. Tone was raised with 5-hydroxytryptamine (5-HT) in the aortic strip and with guanethidine in the anococcygeus. In the presence of erythrocytes and haemoglobin the sensitivity to 5-HT was increased. To produce contractions of comparable magnitude the 5-HT concentration in controls, 10 μM, was reduced to 1 μM in the presence of erythrocytes and 0.1 μM in the presence of haemoglobin solution. Vertical lines show s.e.mean. $n = 6$ for the anococcygeus and 9–15 for the aorta. (●——●) Control responses; (■- - -■) responses in the presence of erythrocyte suspension and (○——○) those in the presence of haemoglobin.

haemoglobin but only reduced by a haemoglobin-equivalent suspension of erythrocytes. The higher concentrations of acetylcholine produced a greater relaxation in the control which, though greatly reduced, could not be completely blocked even by the solution of haemoglobin and again was much less affected by the suspension of erythrocytes. The results of all experiments with EDRF are shown in Figure 5. Both erythrocytes and haemoglobin significantly reduced the EDRF-mediated relaxation at all concentrations, but the haemoglobin solution was significantly more effective than its equivalent in erythrocytes.

Discussion

We have previously shown that haemoglobin or erythrocytes are equally effective in abolishing the

response to EDRF when the two are exposed to one another in the transfer fluid in a cascade-type experiment. The present experiments show that erythrocytes are significantly less effective than haemoglobin in abolishing the response of an aortic strip to the release of EDRF from its own intact endothelium. The most obvious explanation is the inability of the erythrocytes to bring their haemoglobin content into as close contact to the sites of release of the EDRF, particularly on the deep surface of the endothelium facing the smooth muscle. Nevertheless, erythrocytes did reduce by more than 50% the response to even the higher concentrations of acetylcholine. In contrast, erythrocytes were completely ineffective in reducing the response to NANC nerve stimulation in either the BRP or the rat anococcygeus. Does this mean the transmitter, while able to react with haemoglobin, is unable to penetrate the erythrocyte membrane, or is it simply that the sites of release are too deeply buried within the muscle preparation for erythrocytes to be effective? Two factors should be relevant. First, the diffusional distance between the endothelium or nerve varicosity and the muscle layer and, secondly, the distance between the endothelial cell or varicosity and the bathing fluid. The mean gap between nerve varicosities and muscle cells in the rat anococcygeus is about 260 nm (Gillespie & Lullmann-Rauch, 1974) and between the deep surface of the endothelium cell and the underlying muscle is between 50–100 nm (Simionescu & Simionescu, 1977). In terms of the immediate diffusional distance, therefore, the endothelium would be the more difficult site to block. On the other hand, the total distance between erythrocytes and the luminal surface of the endothelium is negligible, whereas there is a substantial distance between the muscle surface and the nerve varicosities in the BRP and anococcygeus muscles. Nevertheless, the rat anococcygeus is a flat relatively thin tissue averaging 250–300 μm in thickness and the nerves are uniformly distributed through the tissue. Some reduction, at least in superficial muscle bundles, might have been expected. The results may indicate either that the NANC transmitter is not nitric oxide or that the nitric oxide is bound in a form not easily diffusible through the erythrocyte membrane.

The ability of erythrocytes with a haemoglobin equivalent of 3 μM, i.e. about 600 times less than whole blood, to reduce the response to EDRF raises a question mark against the physiological role of EDRF. To test this, in some experiments much higher concentrations of haemoglobin equivalent (500 μM) were used. These completely abolished the EDRF response. Unfortunately, some haemolysis always occurred as measured by the haemoglobin in solution at the end of the experiment. Nevertheless, in some preparations that free concentration was less than the 3 μM haemoglobin used in the other experiments, yet the inhibition of the EDRF response was much greater. Further experiments are needed to clarify this observation.

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