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BLOCK OF SOME NON-ADRENERGIC INHIBITORY RESPONSES OF SMOOTH MUSCLE BY A SUBSTANCE FROM HAEMOLYSED ERYTHROCYTES

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SUMMARY

1. A preparation of haemolysed rat erythrocytes (the haemolysate) blocked the relaxations of both the bovine retractor penis and the rat anococcygeus muscles in response to field stimulation of their non-adrenergic inhibitory nerves. The effective concentration range was 5–20 μl./ml. of haemolysate, equivalent to 0·25–1·0 μl./ml. of blood. The active principle in the haemolysate was a non-dialysable, heat-labile material of molecular weight between 50,000 and 100,000 daltons. If, as appeared probable, the active component of the haemolysate was oxyhaemoglobin, its effective blocking concentration was 0·5–2 μM.

2. Haemolysate (5–20 μl./ml.) also blocked the relaxation of both the bovine retractor penis and the rat anococcygeus to the inhibitory factor extracted from the bovine retractor penis, an observation supporting the possibility that this inhibitory factor may be the transmitter released by the inhibitory nerves in these tissues. In the bovine retractor penis, haemolysate was also effective in blocking relaxations in response to sodium nitroprusside, but relaxations produced by prostaglandin E_1 or isobutylmethylxanthine were unchanged or only slightly reduced.

3. In contrast, in the taenia of the guinea-pig caecum, haemolysate did not block the non-adrenergic inhibitory response to field stimulation, nor the relaxation produced by ATP, although it did block the relaxation produced by the inhibitory factor.

4. In spiral strips of isolated rabbit aorta, haemolysate (10 μl./ml.) increased the contraction produced by noradrenaline and blocked the relaxation produced by the inhibitory factor. These were shown to be independent effects.

5. Apamin, which blocked the relaxation of the taenia of the guinea-pig caecum elicited by either ATP or field stimulation of its non-adrenergic nerves, was without effect on relaxations of the bovine retractor penis or rat anococcygeus muscles in response to field stimulation of inhibitory nerves or to inhibitory factor.

6. These differences in the blocking effects of apamin and haemolysate suggest either that the transmitter in the bovine retractor penis and rat anococcygeus differs from that in the guinea-pig taenia, or, if the transmitter is the same, then its mechanism of action differs.

INTRODUCTION

Ambache, Killick & Zar (1975) described the isolation from the bovine retractor penis muscle of a factor that caused relaxation of that muscle, thus mimicking the inhibitory response of the tissue to field stimulation of intrinsic nerves. Further studies from this laboratory (Gillespie & Martin, 1978, 1980; Gillespie, Hunter & Martin, 1981) have provided more information about the nature of this factor: it is a substance of small molecular weight, which can be extracted from the muscle in the form of a pharmacologically inactive precursor that is activated by brief exposure to acid. The inhibitory factor does not appear to be any known autacoid. The elucidation of some of its properties led to modifications of the method of preparation and storage and to additional stages of purification, notably removal of noradrenaline (Gillespie & Martin, 1980) and of ATP (Bowman, Gillespie & Martin, 1979) from the partially purified material.

Improvement in the method of preparation meant that the actions of the inhibitory factor could be investigated on a wider range of systems, including the gastrointestinal (Crossley & Gillespie, 1980) and the cardiovascular systems (Bowman, Gillespie & Martin, 1980, 1981). We found that the inhibitory factor was a potent vasodilator in isolated blood vessels; it caused relaxation of isolated artery strips and a fall in pressure in vascular beds perfused with Krebs solution at constant flow. Despite its vasodilator activity in isolated systems, it was without effect on the blood pressure in the anaesthetized, intact animal, not only when applied by intravenous injection, but also after injection into the left atrium to exclude the pulmonary circulation. These experiments suggested that the lack of depressor activity in the intact animal arose because the inhibitory factor was rapidly inactivated by blood, and led to further study of the mechanisms involved.

The original observation that gave rise to the experiments described in this paper was that mixing inhibitory factor with rat blood immediately caused a complete loss of inhibitory activity when the factor was assayed on the bovine retractor penis muscle (Bowman *et al.* 1981). Sometimes, after exposure of the isolated retractor penis muscle to assay mixtures containing small amounts of blood, the responsiveness of the tissue to the inhibitory factor was diminished for periods of 30–60 min, despite repeated washing. Accordingly, we were interested to see whether rat blood similarly prevented responses of the bovine retractor penis muscle to inhibitory nerve stimulation and this paper is concerned with experiments designed to study this possibility, as well as with other properties of the active blood component, including some of its actions on other smooth muscles. The opportunity was taken to compare the properties of rat blood with those of the polypeptide apamin, a toxin obtained from bee venom, which also blocks certain inhibitory mechanisms in smooth muscle (Baidan, Vladimirova, Miroshnikov & Taran, 1978; Banks, Brown, Burgess, Burnstock, Claret, Cocks & Jenkinson, 1979; Brown & Burnstock, 1981). Some of our results have been communicated to the Physiological Society and to the British Pharmacological Society (Bowman & Gillespie, 1981*a*, *b*).

METHODS

Preparation of the inhibitory factor from the bovine retractor penis muscle

The method of extraction was similar to that previously described (Gillespie *et al.* 1981) but the following modifications were introduced in order to speed up the procedure. Two or three pairs of muscles were treated as a batch. The muscles were passed through a mincer (Moulinex type 243.2.01) instead of being chopped, and the time of extraction in cold methanol was reduced to 30 min. The methanol extract was applied to an anion exchange resin (Bio-Rad AG1-X8) and the inhibitory factor was eluted with 500 mM-NaCl as before. Aliquots of the eluate were then freeze-dried in ampoules. Each ampoule contained the material extracted from 5 to 8 g of muscle. Blue silica gel was placed in the neck of each ampoule, which was then sealed and stored at −20 °C. The lyophilized powder stored in this form appeared to retain full activity for at least a month. Immediately before use, the powder was reconstituted with a volume of distilled water such that the material extracted from 1 g of original muscle was present in 1 ml. of solution. Acid activation was carried out as previously described and the active extract was kept on ice. Extracts prepared in this way are suitable for use on the bovine retractor penis muscle and rabbit aorta, which are relatively insensitive to ATP. However, for experiments on the rat anococcygeus muscle (in which ATP causes contraction) and on the guinea-pig taenia caeci (in which ATP causes relaxation), it was necessary to remove ATP. This was achieved by passing the reconstituted extract at pH 9 through a column of alumina (Bowman *et al.* 1979). Removal of ATP was confirmed by reduction in U.V. absorbance at 260 nm. One, or both, of two control procedures were routinely used to check that the inhibitory activity of the extract was due to the inhibitory factor: firstly, that the inhibitory effect was absent in extracts not activated by acid, and, secondly, that it was abolished by exposure of the extract in a sealed tube to a boiling water bath for 2 min.

Collection of blood and separation of blood components

Male rats (250–350 g) were anaesthetized with sodium pentobarbitone (65 mg/kg I.P.) and a cannula was inserted into a carotid artery. Blood was collected from this cannula into 3 ml. tubes containing heparin (50 i.u.). Two full tubes were collected from each animal. The blood was centrifuged at 1000 *g* for 20 min at 4 °C, and the plasma and buffy coat were removed by aspiration. (In experiments in which plasma was required, the top 0·5 ml. was collected separately, to avoid contamination with the white cells.) The erythrocytes were washed twice and then resuspended in sufficient phosphate-buffered isotonic saline to restore the volume to 3 ml.: this constituted the washed erythrocyte suspension. Haemolysis was effected by pipetting 1 ml. of the washed erythrocyte suspension into 40 ml. polycarbonate centrifuge tubes containing 19 ml. of hypotonic phosphate buffer (20 m-osmoles/l., pH 7·4). The contents were mixed and centrifuged at 20,000 *g* for 30–40 min at 4 °C; the supernatant from this procedure constituted the haemolysate. The erythrocyte membranes, which formed a button on the bottom of the tube, were washed once in hypotonic phosphate buffer (pH 7·4), centrifuged again at 20,000 *g* and resuspended in hypotonic phosphate buffer. This method was based on that described by Dodge, Mitchell & Hanahan (1963) for the preparation of haemoglobin-free ghosts, and the precautions they suggested for the quantitative recovery of the erythrocyte membranes were observed. In all but the first few experiments, the haemolysate (15 ml.) was dialysed overnight at 4 °C with stirring against 5 l. of either distilled water or hypotonic phosphate buffer, pH 7·4, to remove low molecular weight components. In experiments in which non-haemolysed erythrocytes were required for a comparison with the haemolysate, the comparable suspension was made by pipetting 1 ml. of the washed erythrocyte suspension into 19 ml. of isotonic phosphate-buffered saline.

In experiments to estimate the molecular weight of the active component of the blood, dialysed haemolysate was forced through Amicon filtration cells under a pressure of 25 Lb./sq. in. from a nitrogen cylinder. The filters were used sequentially: that retaining particles greater than 300,000 daltons was used first and the ultrafiltrate from this was passed through either a 100,000 or a 50,000 dalton filter.

Haemolysate containing predominantly methaemoglobin instead of oxyhaemoglobin was prepared by the addition of sodium nitrite to make a final concentration of 0·1 %; after 1 hr, the treated haemolysate was then dialysed overnight against distilled water. Haemolysate containing carboxyhaemoglobin instead of oxyhaemoglobin was prepared by bubbling carbon monoxide through the solution for 3 min.

Isolated organs

Strips of bovine retractor penis muscle and rat anococcygeus muscles were set up as previously described for isometric recording of tone (Gillespie, 1972; Klinge & Sjöstrand, 1974; Gillespie *et al.* 1981). Tone developed spontaneously in the bovine retractor penis muscle but it usually did not in the rat anococcygeus muscle; in this muscle tone was induced by the addition of guanethidine (20 μM). Muscles were set up in ring electrodes and field stimulation (1 msec pulses, supramaximal voltage) was applied from a Tektronix (series 160–162) impulse generator. In most of the experiments involving field stimulation of the bovine retractor penis muscle, phentolamine (5 μM) was present throughout to block the motor response elicited by stimulation of the adrenergic nerves. In the rat anococcygeus, the concentration of guanethidine that was used to induce tone was more than sufficient to block the adrenergic component of the response to field stimulation. Spiral strips of rabbit abdominal aorta were set up with a resting tension of 0·5–1 g. Tone was induced by adding noradrenaline. Contractions were recorded isometrically. Taeniae dissected from guinea-pig caeci were set up in ring electrodes for isometric recording, with a resting tension of 1–2 g. More than half of the preparations were discarded either because they did not develop tone or because of large spontaneous contractions. Occasionally tone was induced by intermittent application of carbachol (20–50 nM) but most of the experiments were done with the minority of tissues that happened to develop stable, high tone.

All contractions were recorded on a Grass (model 7) polygraph using Grass FT03 force transducers. The Krebs solution was maintained between 34 and 35 °C, gassed with 5% CO_2 in O_2, and of the following composition (mM): Na^+, 145; K^+, 6·0; Ca^{2+}, 2·5; Mg^{2+}, 1·2; Cl^-, 127; HCO_3^-, 25; $H_2PO_4^-$, 1·2; SO_4^{2-}, 1·2 and dextrose, 11.

Drugs and solutions

Drugs used were: adenosine triphosphate disodium salt (Sigma), apamin (Serva; each milligram of apamin was dissolved in 9·9 ml. of distilled water, and aliquots of this solution were freeze-dried in ampoules that were then sealed and stored at −20 °C. The lyophilized powder was reconstituted in distilled water before use), atropine sulphate (BDH), carbachol (Koch-Light), ferrous sulphate (BDH), guanethidine sulphate (Ciba), haemoglobin (bovine type 1, Sigma), hemin (bovine type 1, Sigma), heparin (Pularin, Evans Medical), indomethacin (Sigma), 3-isobutyl-l-methylxanthine (IBMX, Sigma), isoprenaline sulphate (Burroughs Wellcome), methaemoglobin (bovine grade 1, Sigma), (−)-noradrenaline bitartrate (Sigma), phentolamine (Rogitine, Ciba), prostaglandin E_1 (Upjohn), sodium nitrite (BDH) and sodium nitroprusside (BDH). Phosphate buffers were made in the way described by Dodge *et al.* (1963): stock solutions were sodium monophosphate, monobasic (NaH_2PO_4), 0·155 M and sodium phosphate, dibasic (Na_2HPO_4), 0·103 M. Isotonic phosphate buffer was made by mixing appropriate volumes of the above solutions to give the required pH, i.e. 7·4, in these experiments. Hypotonic phosphate buffer, 20 m-osmole, was made by diluting isotonic phosphate buffer 1 in 15·5. Isotonic phosphate-buffered saline was made by mixing four volumes of 0·9% NaCl and one volume of isotonic phosphate buffer, pH 7·4.

RESULTS

Bovine retractor penis muscle

Effects of rat blood and its components on the response to field stimulation. The effects of blood and its components (plasma, washed erythrocytes, erythrocyte membranes and membrane-free haemolysed erythrocytes) were separately tested against relaxations evoked by transmural stimulation of the bovine retractor penis muscle. Whole blood (1 μl./ml.), and the equivalent quantity of washed erythrocytes suspended in isotonic phosphate-buffered saline, blocked the relaxations and caused a small rise in tone. However, subsequent experiments showed that the blocking action was an effect of the erythrocyte contents rather than of the intact erythrocytes. It arose because haemolysis occurs when erythrocytes are added to an organ bath containing a strip of bovine retractor penis muscle. Previously haemolysed erythrocytes were

at least twice as effective as the equivalent amount of washed erythrocytes suspended (one volume in twenty volumes) in isotonic buffered saline, suggesting that about half of the latter became haemolysed on addition to the organ bath. Plasma was completely without effect even in volumes up to the equivalent of 40 μl./ml. of whole blood. Removal of the erythrocyte membranes, to form what we have termed the

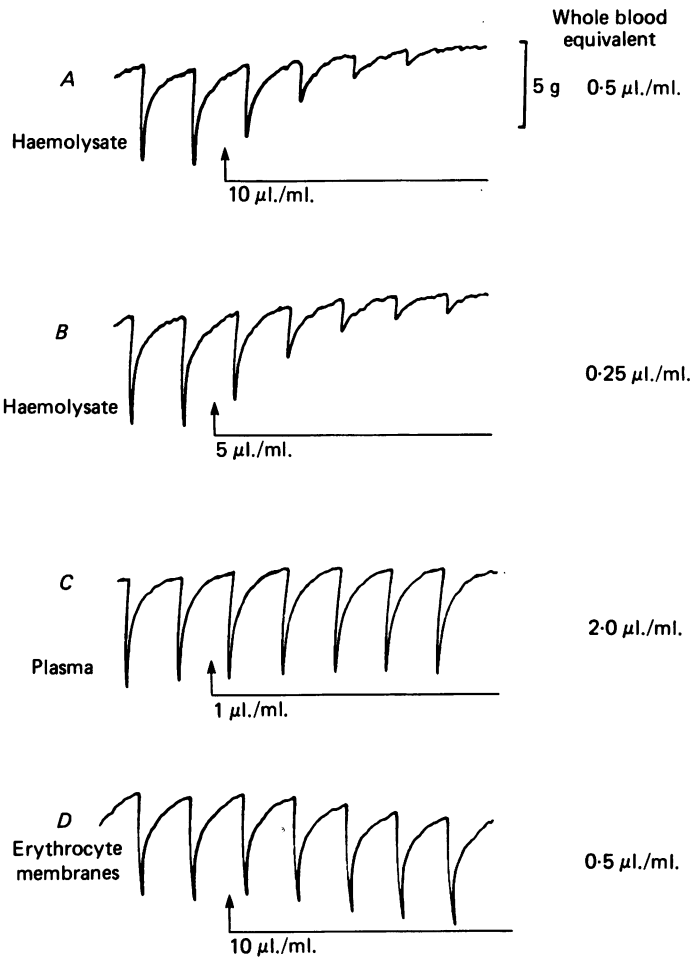

Fig. 1. Relaxations of the bovine retractor penis muscle (1 Hz for 10 sec every 4 min) in the presence of phentolamine (5 μM). Panels *A* and *B* show dose-related inhibitions of the relaxations in the presence of 10 and 5 μl./ml. respectively of haemolysate. Panels *C* and *D* show the absence of effect of plasma and erythrocyte membranes. The equivalent volumes of whole blood are shown on the right.

haemolysate (see Methods), did not affect the potency of the haemolysed erythrocytes, and the erythrocyte membranes themselves were completely ineffective. Fig. 1 shows that the blocking action of the haemolysate was dose-related and that plasma and erythrocyte membranes were without effect.

The nature of the active principle in the haemolysate. Two early observations suggested that the active principle in the haemolysate was of fairly high molecular

weight, or was associated with a high molecular weight component. Firstly, the blocking action of the haemolysate was unaffected by dialysis and, secondly, when the haemolysate was heated, and the protein precipitate that formed was separated off by centrifuging, the supernatant was devoid of activity. Ultrafiltration through Amicon membranes confirmed that the molecular weight was large. The ultrafiltrate from a 300,000 membrane showed no loss of blocking activity when compared with the unfiltered haemolysate. When this ultrafiltrate was passed through the 100,000 membrane, the first 1 or 2 ml. volume that emerged was red and retained most of its blocking activity. Subsequently, however, the fluid that appeared was clear and had no blocking activity. Probably the large amount of protein in the haemolysate quickly blocked the pores in the membrane. No activity was found in the ultrafiltrate from the 50,000 membrane; this ultrafiltrate was completely clear. These experiments suggested that the molecular weight of the active component was between 50,000 and 100,000 daltons, and it seemed likely that it was haemoglobin.

Stability of haemolysate. Haemolysate was stored at 4 °C after preparation. Its pharmacological potency did not deteriorate markedly during the following 2 days but was appreciably reduced after storage for a week in the refrigerator. In an attempt to increase stability on storage, the haemolysate was freeze-dried and stored in sealed ampoules at −20 °C. However, when the lyophilized powder was reconstituted, pharmacological activity was low. In all cases where activity was lost, the colour of the haemolysate had changed from red to brownish red, suggesting the formation of methaemoglobin. Consequently, freeze drying was abandoned and haemolysate was discarded if it had been stored for more than 2 days.

Haemoglobin and its derivatives. The concentration of haemolysate in the organ bath that consistently produced block of the inhibitory response of the bovine retractor penis muscle to field stimulation was 10 μl./ml., which would give rise to 80 μg/ml. (*ca.* 1·2 μM) of haemoglobin. Commercially available bovine so-called haemoglobin in this concentration was ineffective when tested on the bovine retractor penis muscle, but the solution was brownish in colour and, in fact, this purchased 'haemoglobin' consists largely of methaemoglobin, as the manufacturers state on the label. (Haemoglobin is readily oxidized to methaemoglobin on storage.) Purchased methaemoglobin was also without activity in concentrations up to 5 μM. This was the highest concentration tested because so much frothing occurred. Moreover, converting the haemoglobin in the haemolysate to methaemoglobin (by addition of $NaNO_2$) abolished activity. Bovine haemin (as alkaline haematin, up to 30 μM) and ferrous sulphate (up to 30 μM) were without activity. Bubbling CO through the haemolysate to change the haemoglobin to carboxyhaemoglobin did not change its ability to block responses of the bovine retractor penis muscle to inhibitory nerve stimulation.

Effects of haemolysate and apamin on responses to the inhibitory factor and to field stimulation. Fig. 2 illustrates graded relaxations of the bovine retractor penis muscle produced by transmural stimulation (eight and five stimuli at a frequency of 1 Hz) and by two concentrations of the inhibitory factor extracted from bovine retractor penis muscle (10 and 5 μl./ml.). Haemolysate, in a concentration of 10 μl./ml., completely abolished the relaxations produced both by field stimulation and by the inhibitory factor. After repeatedly washing the tissue, both types of inhibitory response returned together.

In contrast to the effect of haemolysate, apamin, in concentrations up to 10^{-6} M (the greatest tested), was without effect on relaxations of the bovine retractor penis produced by field stimulation or by the inhibitory factor. This absence of effect of apamin on the bovine retractor penis is illustrated in Fig. 3.

Specificity of antagonism by haemolysate. The ability of the haemolysate to prevent relaxation produced by the inhibitory factor was compared with its ability to modify

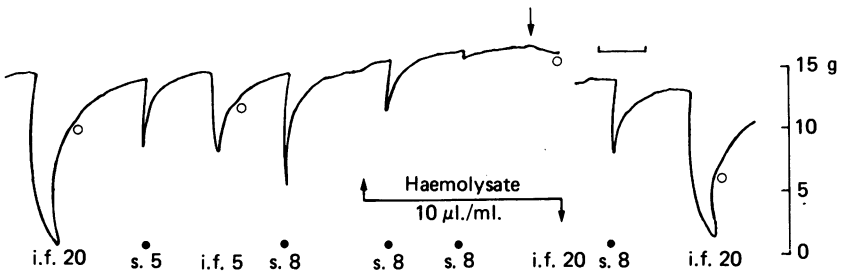

Fig. 2. Relaxations of the isolated bovine retractor penis muscle (spontaneous tone) in response to two concentrations of inhibitory factor (i.f., 20 and 5 µl./ml.) and to field stimulation (s, 1 Hz for 5 or 8 sec at ●). Bath fluid was replaced at ○. Haemolysate (10 µl./ml.) caused a rise in resting tone and blocked the relaxations in response to field stimulation and to 20 µl./ml. of inhibitory factor. There was a gap of 40 min between the first and second panels; during this time the bath fluid was replaced repeatedly until the responses to field stimulation and to inhibitory factor returned towards their original sizes. Time mark, 5 min.

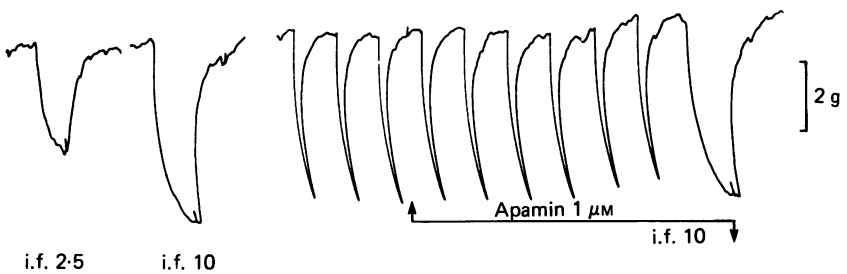

Fig. 3. Relaxations of the isolated bovine retractor penis muscle (spontaneous tone) in response to inhibitory factor (2·5 and 10 µl./ml.) and to field stimulation (2 Hz for 10 sec every 4 min) in the presence of phentolamine (5 µM). Apamin (1 µM) did not alter the responses to field stimulation or to the inhibitory factor.

relaxations of the bovine retractor penis muscle produced by some other drugs, namely, sodium nitroprusside, prostaglandin E_1 (PGE_1) and isobutylmethylxanthine. These experiments were done in two ways. Responses to at least two different submaximal doses of inhibitory factor and of two different submaximal doses of one other agonist were established and the change in sensitivity to each agonist produced by haemolysate (5–20 µl./ml.) was determined. In the other type of experiment, three or four strips cut from the same bovine retractor penis muscle were set up at the same time in separate baths and cumulative dose–response curves were established to each of the agonists, one agonist only being tested on each preparation. Then the same

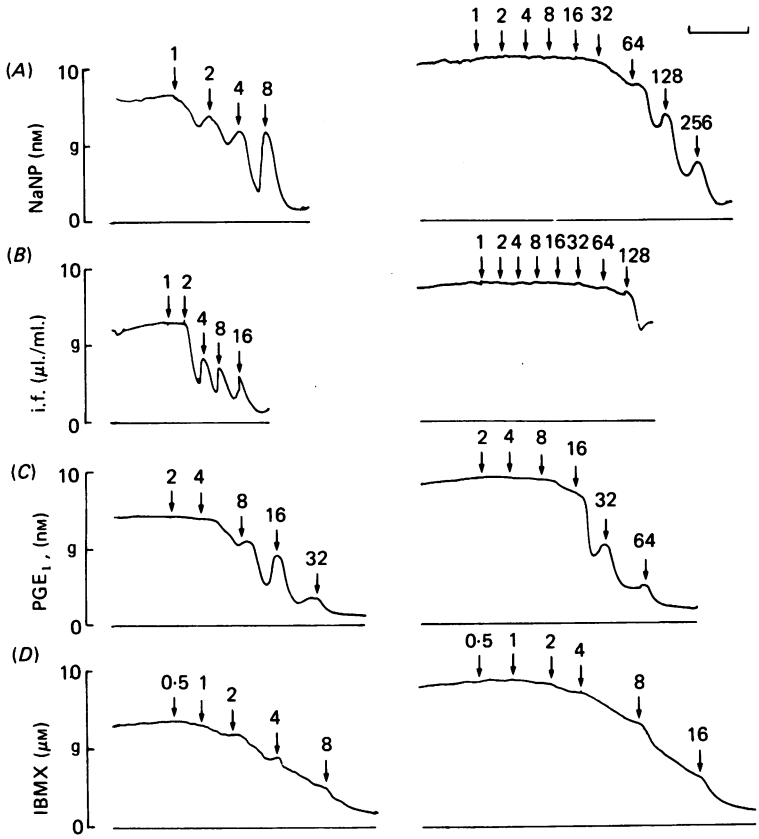

Fig. 4. Cumulative dose–response relationships in the isolated bovine retractor penis muscle to various agonists that cause relaxation: *A*, sodium nitroprusside (NaNP); *B*, inhibitory factor from the bovine retractor penis muscle (i.f.); *C*, prostaglandin E_1 (PGE_1) and *D* isobutylmethylxanthine (IBMX). Each panel is from an experiment on a separate strip but all the strips were cut from the same retractor penis muscle, and all records were made on the same afternoon with the same sample of haemolysate. Panels on the left are control dose–response relationships; then the bath fluid was replaced several times and the muscles allowed to recover their original levels of tone. Then haemolysate (10 μl./ml.) was added, which increased tone in all preparations. Fifteen minutes later, the dose–response relationships shown in the right hand panels were obtained. Haemolysate considerably reduced the sensitivity to sodium nitroprusside and that to inhibitory factor (panels *A* and *B*) but the sensitivities to PGE_1 and IBMX (panels *C* and *D*) were much less affected. Time mark 5 min.

concentration of haemolysate (10 μl./ml.) was added to each bath and, 15 min later, the cumulative dose–response relationship was established again. Relaxations produced by low concentrations of these agonists (with the exception of isobutylmethylxanthine) were brief in duration, the tone beginning to rise again soon after the maximal response to each concentration had been reached (see Fig. 4). However, this did not seriously detract from the validity of the observations, since essentially the same results were obtained from both types of experiment, namely, that haemolysate produced a marked shift in the dose–response curves to inhibitory factor and to nitroprusside but did not much affect those to PGE_1 and isobutylmethylxanthine.

The sensitivity to inhibitory factor and to nitroprusside was decreased to about the same extent in any one experiment, although in different experiments the extent of the decrease ranged from eight-fold to over fifty-fold with 10 μl./ml. of different samples of the haemolysate. With some batches of inhibitory factor, it was not always possible to add big enough volumes in the presence of the haemolysate to determine the exact degree of sensitivity loss. Haemolysate had little or no effect on the relaxations produced by isobutylmethylxanthine or PGE_1; the rightward shift of the dose–response curves recorded for these two substances was usually zero and never greater than two-fold in the presence of 10 μl./ml. of the haemolysate.

The small antagonistic effect sometimes produced against isobutylmethylxanthine and PGE_1 was probably due to physiological antagonism; that is, to the ability of the haemolysate to produce an opposing increase in the tone of the smooth muscle. The increase in tone produced by haemolysate is evident in Fig. 4, which shows cumulative dose–response curves to each of the four agonists before and in the presence of haemolysate. This change in tone makes it difficult to estimate precisely the degree of block by haemolysate, especially with isobutylmethylxanthine and PGE_1 with which the change in small. If relaxations are expressed as a percentage reduction of original tone, then the degree of block of PGE_1 and isobutylmethylxanthine in the experiment illustrated in Fig. 4 (panels *C* and *D*) is only about two-fold.

The unimportance of physiological antagonism in the ability of haemolysate to antagonize inhibitory responses to transmural stimulation is clear from the experiment illustrated in Fig. 5. In this experiment, the increase in tone of the bovine retractor penis muscle produced by haemolysate was counteracted by isobutylmethylxanthine but the responses to transmural stimulation remained blocked. After washing out the bath, the observations were repeated in the opposite order: tone was reduced with isobutylmethylxanthine, so that when haemolysate was added to the bath, it restored tone only to a level equal to that at the beginning of the experiment, yet the responses to field stimulation were still blocked.

The extent of the rise in tone of the bovine retractor penis muscle produced by haemolysate varied from preparation to preparation. It was most clearly seen with large doses of haemolysate in those tissues that did not spontaneously develop a high degree of tone. The rise in tone produced by haemolysate was not reduced in the presence of atropine (0·5 μM), guanethidine (3 μM), phentolamine (2 μM) or indomethacin (20 μM).

Rat anococcygeus muscle

In the rat isolated anococcygeus muscle, in which tone was induced and maintained by the addition of guanethidine (20 μM) to the bathing fluid, haemolysate (5–20 μl./ml.) produced a small rise in tone and blocked inhibitory responses to field stimulation and to the inhibitory factor from the bovine retractor penis muscle. The action of haemolysate in this muscle was in fact closely similar to its action on the bovine retractor penis muscle. However, one minor difference noted was that the blocking effect of haemolysate on both field stimulation and on inhibitory factor was more quickly reversed by washing in the rat anococcygeus muscle than it was in the bovine retractor penis muscle. An experiment with the rat anococcygeus muscle is illustrated in Fig. 6.

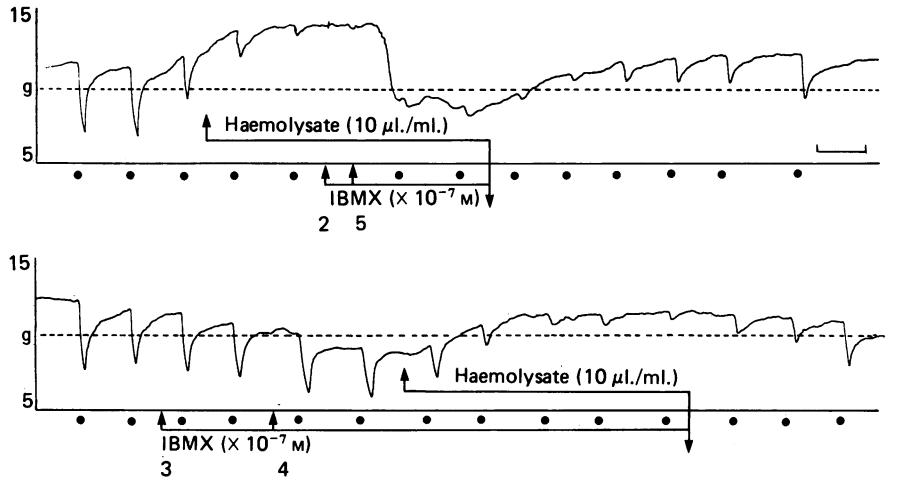

Fig. 5. Relaxations of the bovine retractor penis muscle in response to field stimulation (2 Hz for 10 sec) in the presence of phentolamine (5 μM). In the upper record, haemolysate (10 μl./ml.) increased the tone of the muscle and blocked the relaxations produced by field stimulation. The relaxations remained blocked when tone was restored to the control level by isobutylmethylxanthine (IBMX) and only slowly returned on washing. In the lower record (a continuation of the upper) IBMX was added first and the tone fell. Haemolysate, in the continued presence of IBMX, raised the tone only as far as the control but still blocked the relaxations elicited by field stimulation. The block of the relaxations produced by haemolysate is, therefore, not simply a consequence of the simultaneous rise in tone. Time mark 5 min.

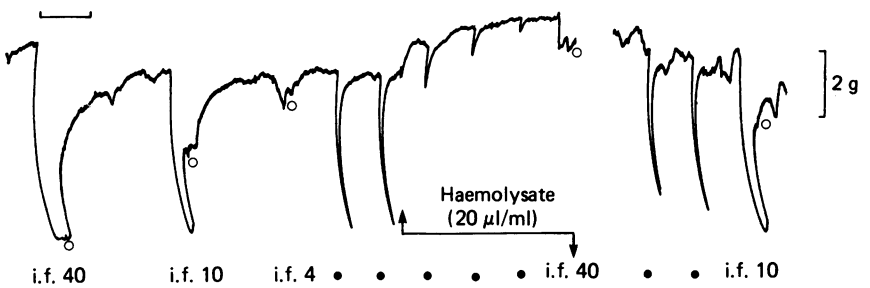

Fig. 6. Relaxations of the rat isolated anococcygeus muscle in response to inhibitory factor obtained from the bovine retractor penis muscle (40, 10 and 4 μl./ml.) and to field stimulation of the inhibitory nerves (5 Hz for 10 sec at ●). Guanethidine (20 μM) was present thoughout the experiment. Haemolysate (20 μl./ml.) blocked the relaxation in response to either field stimulation or inhibitory factor and caused a further rise in tone. The responses recovered when the bath fluid was replaced. Time lapse between the panels was 5 min. Time mark 5 min.

Guinea-pig taenia caeci

The isolated taenia caeci of the guinea-pig, in the presence of guanethidine (3 μM) and atropine (0·5 μM), consistently responded to transmural stimulation (0·5–2 Hz for 10 sec) with an abrupt relaxation, sometimes followed by an after-contraction, as described by many other workers. However, in our hands, it responded somewhat

less consistently to ATP and to the inhibitory factor from the bovine retractor penis muscle, some preparations, despite responding to electrical stimulation, being insensitive even to high concentrations (up to 10 μM) of ATP and (40 μl./ml.) of inhibitory factor. In preparations of taenia caeci that did respond to ATP and to inhibitory factor, as well as to transmural stimulation, it was found that the haemolysate blocked the response to inhibitory factor, but was without effect on the

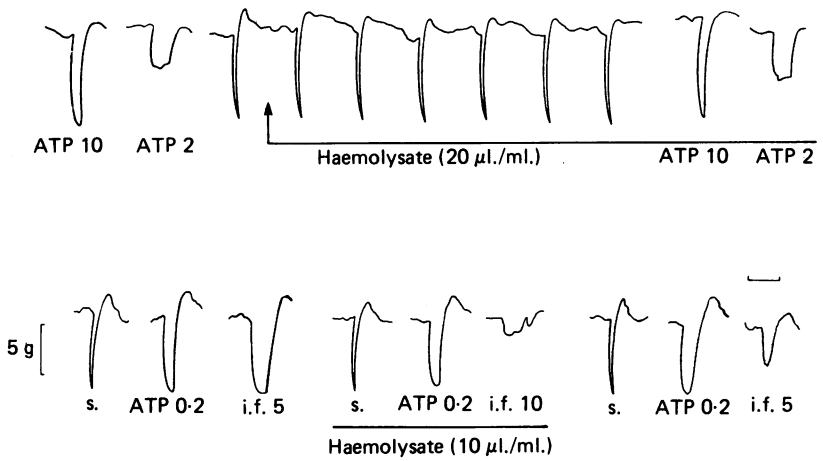

Fig. 7. Relaxations of the isolated taenia of the guinea-pig caecum in response to ATP, inhibitory factor (5 or 10 μl./ml.) or field stimulation (1 Hz for 10 sec) of its inhibitory nerves in the presence of atropine (0·5 μM) and guanethidine (3 μM). Upper panel: haemolysate (20 μl./ml.) did not affect relaxations in response to field stimulation or to ATP (10 or 2 μM). Lower panel (from a different experiment): haemolysate (0 μl./ml.) reversibly blocked the response to inhibitory factor while responses to field stimulation or to ATP (0·2 μM) are unaffected. Time mark 1 min.

relaxations produced by ATP or transmural stimulation. Some of these results are illustrated in Fig. 7.

We confirmed the observations of Banks *et al.* (1979) that apamin blocks the response of the taenia caeci to transmural stimulation and to ATP, and we also showed that apamin was without effect on the relaxation of the taenia caeci produced by the inhibitory factor from the bovine retractor penis muscle.

Rabbit aortic strip

The inhibitory factor extracted from the bovine retractor penis muscle had been previously shown to cause relaxation of isolated blood vessels (Bowman *et al.* 1981). Fig. 8 illustrates an experiment in which tone in a rabbit aortic strip was produced by the addition of noradrenaline (20 or 10 nM). The inhibitory factor in amounts of 0·5, 1 or 2 μl./ml. produced dose-related relaxations. The haemolysate (10 μl./ml.) had two effects. Although it did not alter the basal tone of the smooth muscle in the absence of noradrenaline, it potentiated noradrenaline to the extent that 10 nM-noradrenaline in its presence produced a contraction about equal to that formerly produced by twice that concentration. In addition, the haemolysate reduced the relaxation produced by the inhibitory factor. In the experiment illustrated by Fig. 8,

this occurred to the extent that 2 μl./ml. of inhibitory factor produced a smaller relaxation than did 0·5 μl./ml. in the absence of haemolysate. It is clear from Fig. 8 that the antagonistic effect of the haemolysate against the inhibitory factor from the bovine retractor penis muscle is again not simply the result of a physiological antagonism consequent upon its action in potentiating noradrenaline, since the relaxations produced by 0·5 μl./ml. of inhibitory factor in the absence of haemolysate were about the same whether the tone in the preparation was induced by 10 nM-noradrenaline or by twice that amount.

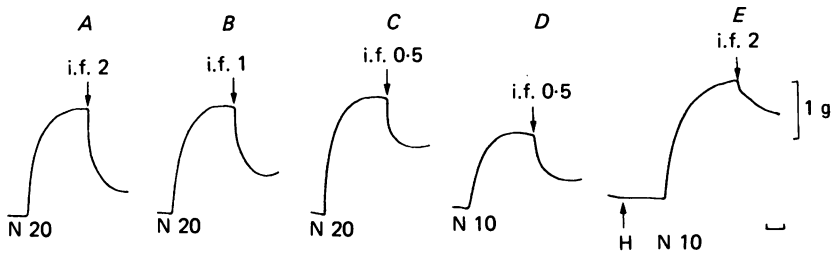

Fig. 8. Tension changes in spiral strip of rabbit aorta. Panels *A*, *B* and *C* show the dose-related inhibitions of noradrenaline (N, 20 nM) induced contractions produced by 2, 1 and 0·5 μl./ml. of inhibitory factor. In panel *D*, the concentration of noradrenaline was halved (10 nM), but 0·5 μl./ml. of inhibitory factor still produced the same amount of relaxation as in panel *C*. Panel *E* shows the effect of haemolysate (5 μl./ml.). The response to noradrenaline (10 nM) was potentiated to about equal to that produced by 20 nM before haemolysate and the relaxation produced by 2 μl./ml. of inhibitory factor was reduced to less than that previously produced by 0·5 μl./ml. (panels *C* and *D*). Between each panel, the bath fluid was replaced, and 4 min elapsed before the next injection. Time mark 1 min.

DISCUSSION

Few substances are known that block responses to stimulation of non-adrenergic, non-cholinergic nerves. One such substance is apamin, a small polypeptide from bee venom, which blocks relaxations of the guinea-pig taenia caeci in response both to non-adrenergic, non-cholinergic nerve stimulation and to ATP (Baidan *et al.* 1978; Banks *et al.* 1979). The action of apamin is, however, non-selective in that responses to non-adrenergic and adrenergic nerve stimulation, and to noradrenaline, are all blocked. Apamin is believed to act by blocking some stage in the opening of Ca^{2+}-operated K^+ channels, thereby preventing the consequent hyperpolarization (Banks *et al.* 1979). However, not all non-adrenergic inhibitory nerves are blocked by apamin; for example, it does not affect the relaxations of the rat anococcygeus produced by stimulation of such nerves (J. S. Gillespie, unpublished) nor, as shown here, of the bovine retractor penis muscle. In haemolysate, we now have another substance with a different spectrum of antagonistic activity towards relaxations induced by non-adrenergic, non-cholinergic nerve stimulation: in contrast to apamin, it blocks in the bovine retractor penis muscle and rat anococcygeus muscles, but does not affect nerve-induced relaxations in the guinea-pig taenia caeci. The haemolysate may, therefore, provide another useful tool for studying inhibitory nerve mechanisms.

Although proof is still lacking, the results obtained with apamin and the haemolysate

are compatible with the idea that the bovine retractor penis and the rat anococcygeus muscles have the same inhibitory transmitter and that this transmitter is identical with, or closely related to, the inhibitory factor extracted from the bovine retractor penis muscle. Thus, whereas the haemolysate blocked inhibitory responses of these smooth muscles evoked either by inhibitory nerve stimulation or by inhibitory factor, apamin was without effect. Furthermore, the results suggest that the inhibitory transmitter of the nerves in the bovine retractor penis and the rat anococcygeus is different from that in the guinea-pig taenia caeci, since haemolysate was without effect on inhibitory mechanisms in this tissue. It cannot be that the inhibitory factor functions as the transmitter in both tissues but through different types of receptor (cf. α- and β-adrenoceptors), since haemolysate blocks the response of the taenia caeci to the inhibitory factor from the bovine retractor penis. These results are not incompatible with Burnstock's evidence (for review, see Burnstock, 1972, 1979) that ATP, or a related nucleotide, is the transmitter in the guinea-pig taenia caeci, since haemolysate was without effect on responses to both ATP and to inhibitory nerve stimulation in this tissue, whereas apamin, albeit non-specifically as others have shown (Banks *et al.* 1979), blocked both. At the same time, the fact that we found ATP to be less consistent than nerve stimulation in producing relaxation of the taenia caeci argues against a transmitter role for ATP, even though such a discrepancy does not constitute conclusive evidence. We are at variance with Burnstock and his co-workers in our proposal that the inhibitory transmitter in the rat anococcygeus is not ATP, since these workers provided suggestive evidence that the non-adrenergic inhibitory nerves in this organ could be purinergic (Burnstock, Cocks & Crowe, 1978).

In some preliminary experiments with Dr D. Pollock, we have found that human haemoglobin, purified by the method of Venuto, Zuck, Zegna & Moores (1977), produces effects on the bovine retractor penis muscle closely resembling those of the haemolysate from rat blood and, in fact, all the evidence to date points to the likelihood that the active principle in the haemolysate is haemoglobin (or, more strictly, oxyhaemoglobin). More work is, however, required to prove beyond question that this is so. Currently, there is considerable interest in the pharmacological properties of haemoglobin (Tanishima, 1980; Wellum, Irvine & Zervas, 1980), triggered by the observation that it may be responsible for the prolonged vasoconstriction of cerebral blood vessels that occurs after cerebral haemorrhage (Echlin, 1971; Osaka, 1977; Boullin, 1980). Studies on a variety of blood vessels (cerebral, coronary, mesenteric and femoral) have shown the cerebral blood vessels to be especially sensitive to the constrictor effect of haemoglobin (Tanishima, 1980). Our own experiments confirmed the lack of sensitivity of a peripheral blood vessel, the rabbit aorta, which did not contract in response to haemolysate. However, the present experiments also show that the high sensitivity of the smooth muscle of cerebral blood vessels is not unique, since both the bovine retractor penis and the rat anococcygeus muscles contract in the presence of haemolysate (haemoglobin). Interestingly, methaemoglobin and alkaline haematin are ineffective in constricting isolated cerebral blood vessels, although carboxyhaemoglobin is effective (Tanishima, 1980), and this pattern of structure–action relations is the same as was found here for blocking responses to inhibitory nerve stimulation in the bovine retractor penis muscle.

The possibility is worth considering that there may be two factors that contribute

to the pronounced vasopasm of cerebral blood vessels produced by haemoglobin *in vivo*. One is the direct constrictor action already mentioned as occurring in isolated cerebral vessels, and, of which, contraction produced in the bovine retractor penis muscle may be a model. The second is related to a possible blockade of non-adrenergic, non-cholinergic nerves to cerebral blood vessels. There is evidence that such nerves exist, in the cat (Lee, Hume, Su & Bevan, 1978), in the monkey, and in man (Toda, 1981). If the transmitter of these nerves is the same as that in the bovine retractor penis muscle, it may play a physiological role in maintaining the patency of the cerebral vasculature; the inhibitory factor from the bovine retractor penis muscle is a powerful vasodilator (Bowman *et al.* 1981). Blockade of this transmitter by haemoglobin would then be another factor leading to cerebral vasospasm.

The haemolysate potentiated the constrictor action of noradrenaline on the rabbit aorta. Potentiation of vasoconstriction by erythrocytes or their contents has been described before (Starling, Boulin, Grahame-Smith, Adams & Gye, 1975), and might further contribute to cerebral vasospasm (Boullin, 1980).

We still have no answer to the question that originally prompted the experiments described in this paper, namely, why is the inhibitory factor rapidly inactivated in the blood after injection into the intact rat? Our earlier experiments in which mixing washed erythrocytes and inhibitory factor caused immediate inactivation (Bowman *et al.* 1981) are probably open to re-interpretation, since we know that even a small amount of haemolysis occurring during handling of the erythrocytes could have accounted for the loss of activity. Estimates of normal values for plasma haemoglobin are prone to exaggeration if any haemolysis occurs when the blood is collected, but the estimates for normal human blood are low, namely, 4 μg./ml. (range 2·5–5·8 μg./ml.) (Hanks & Chaplin, 1959). If there is such an amount of free haemoglobin circulating in rat blood, it is just possible that it is sufficient to abolish the response to inhibitory factor. Certainly amounts not greatly in excess of this (i.e. *ca.* 20 μg./ml.) do cause appreciable reduction of the response to inhibitory factor in the organ bath. In Fig. 1 the substantial degree of block of inhibitory nerve stimulation in panel *B* is in response to an amount of haemolysate that would give a final concentration of about 40 μg./ml. of haemoglobin, but amounts ten times smaller occasionally produce a detectable effect. If there were circulating amounts of free haemoglobin big enough to block the vasodilator effects of injected inhibitory factor, they would not be expected, in health, to impair the inhibitory nerve supply to the retractor penis or anococcygeus muscles, since they would presumably be retained within the blood vessels. However, on the whole, we are disinclined to believe that there is sufficient free haemoglobin in circulating rat blood to produce such a complete abolition of any vasodilator action of the bovine retractor penis inhibitory factor after its intravenous injection *in vivo*.

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