

ACTIONS ON THE CARDIOVASCULAR SYSTEM OF AN INHIBITORY MATERIAL EXTRACTED FROM THE BOVINE RETRACTOR PENIS

ANNE BOWMAN, J.S. GILLESPIE & W. MARTIN¹

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

1 A partially purified material has been isolated from methanol extracts of the bovine retractor penis muscle. This material exerts biological activity only after treatment with acid and subsequent neutralisation. The active principle in this extract, which appears to be no known autacoid, mimics the response to stimulation of the non-adrenergic, non-cholinergic nerves in the bovine isolated retractor penis muscle.

2 This inhibitory extract did not alter the heart rate or blood pressure of the anaesthetized rat when administered either by intravenous or intra-arterial injection, nor did it have any obvious effect on isolated cardiac muscle.

4 The extract produced relaxation of spiral strips of various arteries isolated from ox, cat, rabbit or rat, in which tone was induced by noradrenaline, K^+ or Ba^{2+} .

5 The extract also produced dilatation of the resistance vessels of the rat isolated mesenteric circulation and the rat hindquarters perfused with Krebs solution; tone was induced in these vessels by adrenaline or noradrenaline.

6 Lack of vasodilator activity of the extract in the whole animal appeared to be due to rapid inactivation in the blood, probably by binding to the erythrocytes.

Introduction

The bovine retractor penis muscle is innervated by motor noradrenergic fibres and by inhibitory fibres whose transmitter is unknown (Klinge & Sjöstrand, 1974; Ambache, Killick & Zar, 1975). Ambache *et al.* (1975) extracted an inhibitory material from the tissue which might be the inhibitory transmitter. Further refinement of the technique (Gillespie & Martin, 1978; Bowman, Gillespie & Martin, 1979; Gillespie & Martin, 1980) allowed the extraction of a more purified material, free from protein and from the main pharmacological contaminants, noradrenaline and adenosine 5'-triphosphate (ATP). Whether or not this material is in fact the transmitter, it is worth identifying and studying since it is highly potent pharmacologically. Thus, for example, inhibition of tone in the isolated bovine retractor penis muscle may be produced by about 1 μ l/ml of the partially purified extract, which corresponds to about 1 mg/ml of original tissue. This paper describes experiments designed to study the actions of the inhibitory material on the heart and circulation *in vivo*, and on isolated cardiac tissue and blood vessels. A preliminary account of some of this work has been given to the British Phar-

macological Society (Bowman, Gillespie & Martin, 1980).

Methods

Extraction of inhibitory material

The method of extraction used was based on that described by Ambache *et al.* (1975) as modified by Gillespie & Martin (1980) and Bowman *et al.* (1979). Fresh bovine penises together with the intact retractor penis muscles were collected from the abattoir. Immediately on arrival in the laboratory, the retractor penis muscles were dissected out and chopped into small pieces which were then extracted with cold methanol for several hours. After filtration, the methanol solution was passed over a strong anion exchange resin (Biorad AG1-X8) to which the inhibitory material binds. After washing the column with distilled water, it was eluted with 500 mmol/l NaCl solution. This step eliminates noradrenaline and histamine from the extract. The eluate was then freeze-dried and stored in this form at -20°C for up to 1 week. Immediately before use, the freeze-dried extract was dissolved in cold distilled water. At this stage, the

¹ Present address: Department of Cell Biology, Institute of Animal Physiology, Babraham, Cambridge CB2 4AT.

pH of the solution was about 9. The solution was passed over an alumina column to remove adenine nucleotides which bind to the column (Bowman *et al.*, 1979); successful removal was monitored spectrophotometrically at 259 nm. The solution leaving the column was then acidified to pH 2 with HCl and left at this pH for 10 min. It was then neutralised with NaOH solution and the volume was adjusted with distilled water so that 1 ml of extract contained material from 1 g of retractor penis muscle. The solution was kept on ice and used within 4 h. Activity was checked by testing its effectiveness in producing relaxation of isolated strips of bovine retractor penis muscle. Suitable extracts produced maximal relaxation of the muscle in volumes of 7.5 to 200 μ l added to a 7 ml organ bath.

Anaesthetized rats

Wistar rats of either sex weighing 250 to 350 g were anaesthetized with sodium pentobarbitone injected intraperitoneally (70 mg/kg). Blood pressure was recorded from a common carotid artery by means of a Statham pressure transducer. Heart rate was counted from the ECG which was recorded from needle electrodes placed under the skin and displayed simultaneously with the blood pressure. Injections were made through a cannula in a jugular vein.

In some rats a cannula was inserted into the left atrium allowing injections to be made directly into the systemic circulation. Part of one left rib and the surrounding tissue were removed so that the left atrium was visible through a small hole in the chest wall. The pericardium was opened, and the left atrial appendage secured with Spencer Wells forceps while a fine flexible polythene tube was pushed into the atrium and tied in place. The blood pressure usually fell by 15 to 25 mmHg as a consequence of this procedure, and remained at this lower level throughout the experiment. Artificial respiration was not necessary as the animal could still expand its right lung and appeared to be able to ventilate adequately in this way.

Perfused rat hindquarters

Rat hindquarters were perfused with Krebs solution at 36 to 37°C through a cannula tied into the abdominal aorta. The rate of perfusion (2 ml/min) was maintained constant by a Watson Marlow flow inducer. Perfusion pressure was recorded with a Statham transducer from a side-arm of the inflow cannula. Injections were made in a volume not exceeding 0.25 ml through an injection port on the inflow side. Tone in the blood vessels was induced and maintained by continuously infusing noradrenaline to produce a final concentration of 5×10^{-7} to 10^{-6} mol/l.

Perfused isolated mesenteric blood vessels of the rat

Isolated mesenteric blood vessels were set up as described by McGregor (1965) and perfused with Krebs solution at 36 to 37°C at a constant flow of 2 ml/min. Tone in the blood vessels was induced and maintained by continuously infusing adrenaline (final concentration of 5×10^{-7} to 5×10^{-6} mol/l).

Isolated blood vessels

Spiral strips of bovine penile artery, bovine coronary artery, cat, rabbit and rat aorta, rabbit and cat renal artery, rabbit jugular vein, and a longitudinal section of rabbit and cat portal vein were suspended in Krebs solution at 36 to 37°C. Contractions were recorded isometrically by means of a Grass force transducer (model FT 03), and tone was induced in most experiments by adding noradrenaline to the organ bath to make a final concentration of 5×10^{-8} to 5×10^{-7} mol/l. In some experiments, tone was induced with KCl (final concentration 4 to 8×10^{-2} mol/l) or BaCl₂ (final concentration 5×10^{-3} to 5×10^{-4} mol/l).

Isolated cardiac tissue

Isolated paired or separate left and right atria of guinea-pigs, rats or chickens, and right ventricle strips from rats, were suspended in Krebs solution at 30 to 32°C. Tension was recorded isometrically by means of a Grass (FT 03) force transducer. Paired atria and right atrial preparations were allowed to beat spontaneously; and left atria and the rat ventricle strips were driven to contract by stimulation with rectangular pulses of 0.5 ms duration applied through platinum electrodes at frequencies ranging from 0.125 to 4 Hz.

Isolated strips of bovine retractor penis muscle

Strips of retractor penis muscle about 2 mm wide and 30 to 40 mm long were suspended in Krebs solution at 36 to 37°C. Tension was recorded isometrically with a Grass (FT 03) force transducer. This tissue acquires tone spontaneously within 1 to 2 h and is then very sensitive to the inhibitory material.

Blood, plasma and washed red cells

Rats were anaesthetized with sodium pentobarbitone (70 mg/kg) and a common carotid artery was cannulated with polythene tubing. Blood was collected through this cannula into 3 ml tubes containing 50 iu heparin. Two or three tubes of blood were collected from each rat. The tubes of blood were either stored on ice for experiments with whole blood, or they were

centrifuged for 5 min at 2000 *g*. The plasma was separated and set aside for later use, and the erythrocytes were washed once with 0.9% w/v NaCl solution (saline), and then centrifuged again for 5 min at 2000 *g*: the supernatant was discarded. The erythrocytes were then resuspended in saline so that the final volume approximately equalled the original volume of blood. Whole blood, plasma and erythrocytes reconstituted in saline were stored on ice.

Krebs solution

The Krebs solution used throughout had the following composition (mmol/l): Na⁺ 143, K⁺ 5.0, Ca²⁺ 2.5, Mg²⁺ 2.0, Cl⁻ 126, HCO₃⁻ 25, H₂PO₄⁻ 2.0, SO₄²⁻ 2.0 and glucose 11. It was gassed with 5% CO₂ in O₂.

Recording apparatus

All records were made on a Grass (model 79D) polygraph.

Drugs

The drugs used were: acetylcholine chloride (Koch-Light), adenosine triphosphate disodium salt (Sigma), adrenaline hydrogen tartrate (BDH), atropine sulphate (BDH), cimetidine (Tagamet injection, Smith, Kline & French), heparin (Pularin, Evans Medical), mepyramine maleate (May & Baker), (-)-noradrenaline bitartrate (Sigma), pentobarbitone sodium (Sagatal, May & Baker) and propranolol hydrochloride (ICI).

Results

Anaesthetized rats

In volumes up to 2 ml (the highest used) the extract was without effect on blood pressure and heart rate of anaesthetized rats. Simultaneous application of doses between 50 and 200 µl of the same extract caused complete inhibition of spontaneous tone in the bovine retractor penis muscle. Absence of depressor effect in the anaesthetized rat has been described in an earlier paper (see Bowman *et al.*, 1979, Figure 1).

Isolated cardiac muscle

In volumes up to 0.5 ml (the highest used) added to a 7 ml organ bath, the extract was without effect on the force of driven ventricular strips and on the force of spontaneously beating or driven atria. Slight slowing of spontaneously beating right or paired atria was usually observed when the larger volumes were injected (0.1 to 0.5 ml) but this effect was never more

than could be attributed to the fall in temperature caused by introducing cold solution into the bath, or to the effect of residual ATP to which these tissues are especially sensitive.

Figure 1 illustrates an experiment in which the effects of the inhibitory extract were compared on isolated bovine retractor penis muscle, rat isolated paired atria (spontaneously beating) and rat right ventricle strip stimulated at 1 Hz. In a dose 20 times greater than that causing maximal inhibition of spontaneous tone in the bovine retractor penis muscle, the extract had no effect on contractile force of either atrial or ventricular muscle, and no appreciable effect on atrial rate.

Isolated arteries and veins

Despite the lack of effect of the extract on the blood pressure of the anaesthetized rat, it was considered not unlikely that at least some blood vessels might be affected since Klinge & Sjöstrand (1974) had shown that in the isolated bovine penile artery there is a non-adrenergic, non-cholinergic vasodilator component in the response to field stimulation. For this reason, the effects of the extract were tested on isolated spiral strips of various arteries (bovine penile artery, bovine coronary artery, cat, rabbit and rat aorta, cat and rabbit pulmonary artery, cat and rabbit renal artery) in which tone was first induced with noradrenaline. Without exception, small volumes of the extract (2.5 to 200 µl in a 7 ml bath) produced abrupt, dose-related relaxations of the isolated artery strips. Figure 2a illustrates responses of the isolated aorta of the rabbit which is representative of all the artery strips used.

Experiments on artery strips were carried out simultaneously with experiments on isolated strips of bovine retractor penis muscle in which tone had been allowed to develop spontaneously. The same volumes of the same extract were added to both preparations at the same times. Figure 2 illustrates such an experiment. As described in Methods, the extract requires to be treated with acid and then neutralised before it becomes active on the bovine retractor penis muscle (Figure 2b, first two responses). The same was true for the arterial strips (Figure 2a, first two responses). When the extract is left at room temperature (20°C), activity on the bovine retractor penis muscle slowly disappears at rates that vary from 15 to 30 min for 50% loss of activity. Activity on the arterial strips disappeared at the same rate. This is illustrated for the bovine retractor penis muscle and the rabbit aorta in the fourth responses of Figure 2b and 2a respectively. In this experiment about 80% of activity was lost on both preparations after 60 min at room temperature. Activity on both preparations persisted apparently unchanged in extracts that were kept on

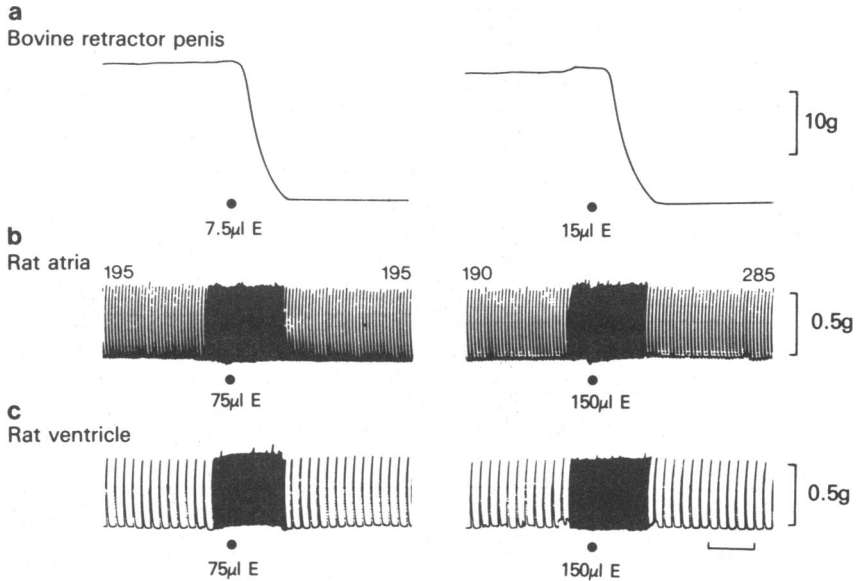


Figure 1 (a) Isolated bovine retractor penis, (b) isolated, paired rat atria, beating spontaneously and (c) isolated rat right ventricle strip, stimulated at 1 Hz. Before injection of the inhibitory extract, and again when the maximum response of the retractor penis to the extract had developed, the paper speed was increased to display individual contractions of the cardiac muscle. In 20 times the volume that caused maximal relaxation of the retractor penis muscle, the inhibitory extract (E) was without effect on force of contraction of rat isolated atria or ventricle, and had no appreciable effect on rate of beating. Rate of beating per min is indicated above the atrial contractions (b). Time mark represents 5 s at fast paper speed and 60 s at slow paper speed.

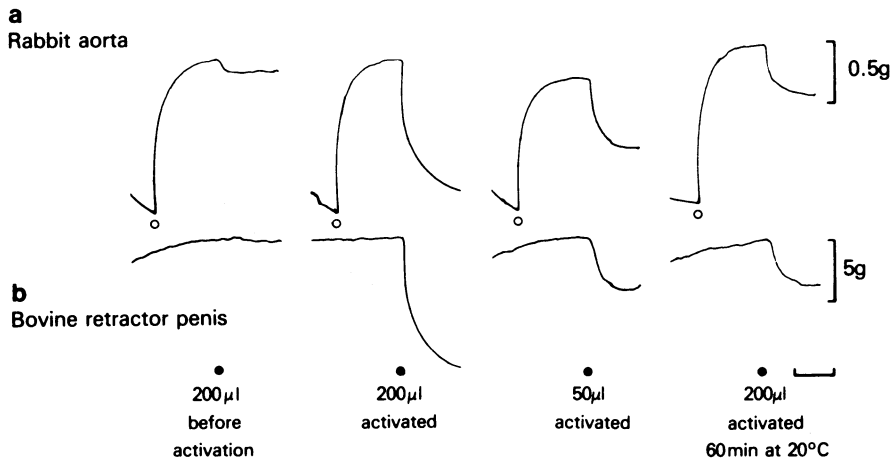


Figure 2 Parallel assay of activity of the inhibitory extract (E) on isolated spiral strip of rabbit aorta (a) and isolated bovine retractor penis (b). Tone was induced in the aortic strip by noradrenaline (added at O, final concentration 10^{-7} mol/l); tone in the bovine retractor penis muscle developed spontaneously. The first panels in (a) and (b) show almost complete lack of activity of the inhibitory extract on either preparation before acid activation. The second and third panels show responses to 200 and 50 μl of activated extract and the last panel shows loss of activity of extract after standing for 60 min at 20°C. Time mark 2 min.

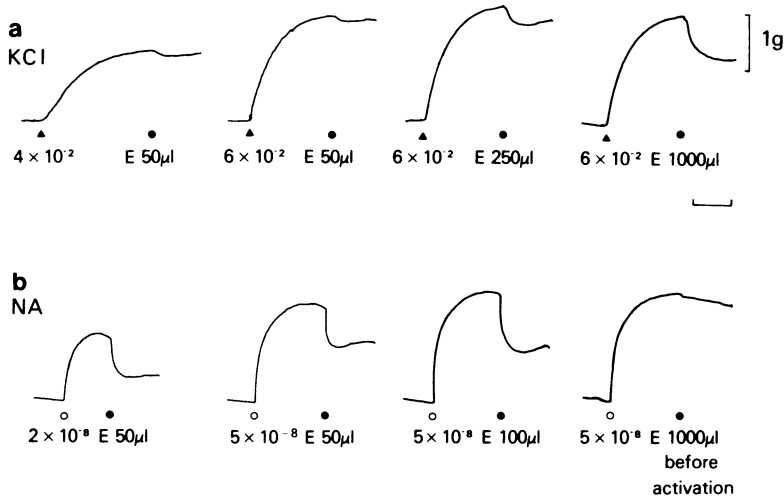


Figure 3 Isolated, spiral strip of rabbit aorta. (a) Contractions elicited in response to KCl (▲), final bath concentration 4×10^{-2} or 6×10^{-2} mol/l. At peak of contraction, inhibitory extract (E, 50 to 1000 µl) was added (●). (b) Contractions elicited in response to noradrenaline (NA) 2×10^{-8} mol/l or 5×10^{-8} mol/l, at (○). Inhibitory extract, 50 and 100 µl, at (●). Inhibitory extract was 10 times more potent in inhibiting noradrenaline-induced contractions than it was in inhibiting contractions induced by KCl. Last tracing in lower panel shows ineffectiveness of extract before acid activation. Time mark 2 min.

ice. After activity had been lost at room temperature, it could be restored, at least partly, by retreatment with acid and subsequent neutralisation, and this occurred to the same extent in isolated artery strips and in the bovine retractor penis muscle. Activity of the extract on both arterial strips and the bovine retractor penis was irreversibly destroyed by heating in a boiling water bath for 2 min. The results of these experiments thus indicated that the active principle responsible for producing relaxation of the arterial strips was the same one that produced relaxation of the bovine retractor penis muscle.

Other spasmogens

In most experiments, noradrenaline was used to induce tone in the isolated arterial preparations. In order to see whether the inhibitory material was equally effective in relaxing tone induced by other spasmogens, its activity against K^+ - and Ba^{2+} -induced tone was also tested. One such experiment with the isolated aorta of the rabbit is illustrated in Figure 3, which shows that tone induced by K^+ (4 and 6×10^{-2} mol/l) is inhibited by the extract. However, the extract was about 10 times more potent in inhibiting noradrenaline-induced tone. Ba^{2+} -induced tone resembled K^+ -induced tone in its lower sensitivity to the extract. The same results were obtained with aortic strips isolated from rats.

Veins

The relaxant activity of the inhibitory extract in isolated veins has not yet been extensively studied. However, it caused inhibition of spontaneous contractions in isolated segments of rabbit and cat portal vein, but was 10 to 20 times less potent on these preparations than on noradrenaline-contracted rabbit aorta. Isolated spiral strips of rabbit jugular vein in which tone was induced by noradrenaline or Ba^{2+} were also relaxed by the extract.

Blocking drugs

So far, no drugs have been found to block extract-induced relaxation of the bovine retractor penis. Relaxation of the rabbit aorta by extract was unchanged in the presence of atropine (10^{-6} mol/l), propranolol (2×10^{-6} mol/l), mepyramine (10^{-6} mol/l) or cimetidine (10^{-4} mol/l).

Perfused rat hindquarters and mesenteric blood vessels

The results obtained with the isolated artery strips gave rise to the apparent paradox that the active principle present in the extract was a powerful inhibitor of arterial muscle tone, yet was without effect on the blood pressure of intact animals. One possibility was that the material was active on the large conducting

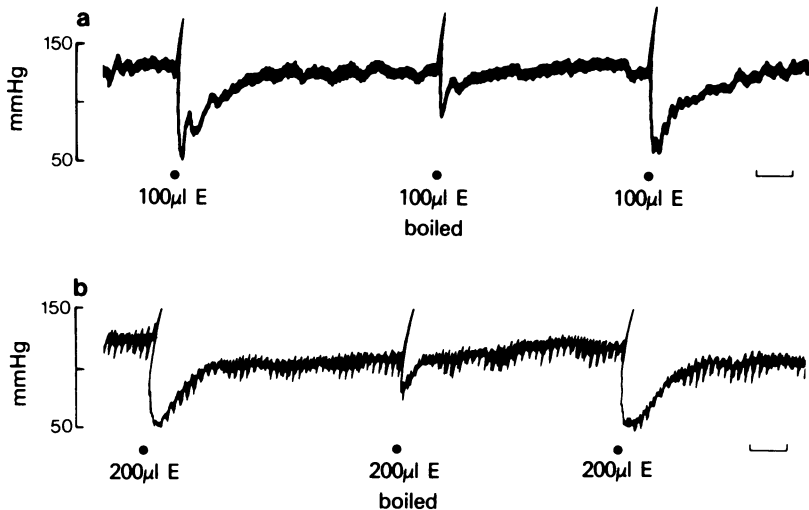


Figure 4 Vasodilator activity of inhibitory extract in isolated, perfused vessels. (a) Pressure in rat isolated mesenteric vessels perfused through the superior mesenteric artery. Tone was induced with adrenaline (10^{-6} mol/l). (b) Pressure in rat hindquarters perfused through the abdominal aorta. Tone was induced with noradrenaline (10^{-6} mol/l). In both preparations, injection of inhibitory extract (E) at (●) caused a fall in perfusion pressure; boiling the extract substantially reduced activity. Time mark in (a) 1 min; in (b) 2 min.

arteries, but not on the resistance vessels, which are the ones responsible for determining the peripheral resistance. For this reason, the effects of the extract were tested on perfused rat hindquarters, and on isolated mesenteric vessels perfused through the superior mesenteric artery. Both preparations include vessels of a wide range of calibres. In these preparations, tone was induced in the resistance vessels by including noradrenaline or adrenaline in the perfusion fluid.

In volumes of 50 μl and above, the extract produced a clear vasodepressor response in both preparations (Figure 4). As with the bovine retractor penis muscle and the artery strips, activity was dependent on previous acid treatment; it slowly but reversibly disappeared on standing at room temperature, and

was irreversibly destroyed by heating in a boiling water bath for 2 min.

Left atrial injection in anaesthetized rats

The experiments described above showed that the extract is in fact a powerful vasodilator in intact vascular beds. Two possibilities to account for the lack of vasodilator activity in the anaesthetized animal were therefore considered. One was that the material is rapidly destroyed on a single passage through the lungs so that it never reaches the systemic arterial vessels; the other was that it is rapidly inactivated on contact with blood.

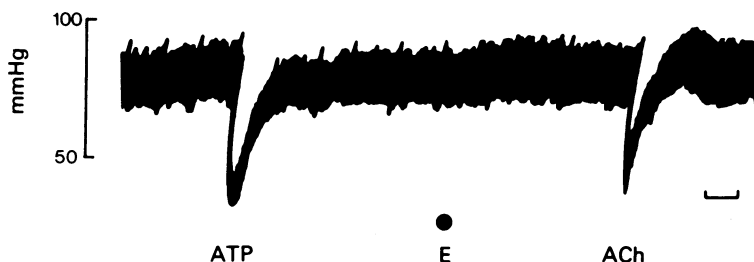


Figure 5 Blood pressure of anaesthetized rat, showing depressor responses to ATP (5.5×10^{-8} mol/kg) and acetylcholine (ACh, 1.5×10^{-8} mol/kg) injected into the left atrium. Inhibitory extract (0.75 ml/kg) was without effect when injected by this route. Time mark 1 min.

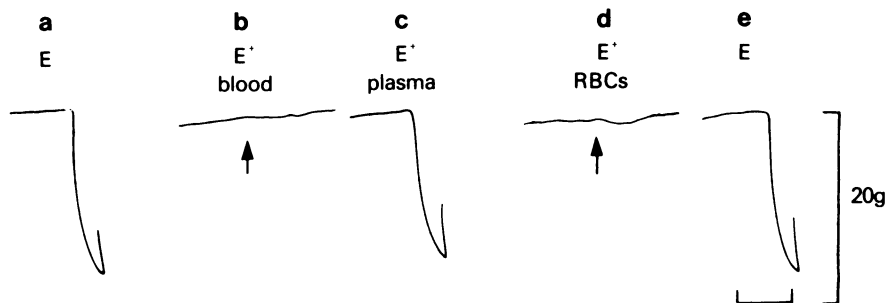


Figure 6 The effect of blood, plasma and washed erythrocytes (RBCs) on the inhibitory activity of extract (E). Activity was assayed in terms of relaxation of spontaneous tone in the bovine retractor penis muscle, and all responses are to injections of 0.1 ml: (a) and (e) are control responses to a mixture of equal volumes of ice-cold extract and saline assayed immediately after mixing. (b) Shows abolition of activity when extract was mixed with cold blood instead of saline. (c) and (d) Show that the site of inactivation is the erythrocytes rather than the plasma. Time marker 2 min.

To test the first possibility, the extract was injected directly into the left atrium of anaesthetized rats. By using this route, the material would reach the systemic circulation without first passing through the lungs. In these experiments, acetylcholine and ATP were injected by the same route for comparison. Both acetylcholine (1.5×10^{-8} mol/kg) and ATP (5.5×10^{-8} mol/kg) produced the expected depressor effect but the extract remained without effect in volumes up to the maximum compatible with the avoidance of a pronounced injection artefact (up to 0.8 ml/kg). Figure 5 illustrates depressor responses to acetylcholine and ATP, and the complete absence of response to the extract. These experiments thus ruled out the lungs as being responsible for the lack of activity of the extract in the intact animal, and pointed to the blood as the probable site of inactivation.

Inactivation by blood

Preliminary experiments in which extract was incubated with rat plasma at 20°C and 30°C did not suggest the presence in plasma of any powerful inactivating enzyme; loss of activity in extract incubated with plasma occurred only slightly more rapidly than spontaneous inactivation at these temperatures.

When whole blood was used instead of plasma, inactivation occurred almost instantaneously. In the experiment illustrated in Figure 6, equal volumes of extract (as always, kept on ice) and whole blood (also kept on ice) were mixed, and assayed on the bovine retractor penis. The injection of the mixture into the organ bath was made within 10 to 15 s of mixing. Complete loss of activity occurred within this time. Under the same conditions, there was no apparent loss of activity when the extract was mixed with an equal volume of plasma, but washed erythrocytes, reconstituted in saline, caused complete inactivation.

Discussion

The origin of these experiments lies in our attempts to isolate the inhibitory transmitter of the non-adrenergic, non-cholinergic nerves in the bovine retractor penis. Vasodilatation of the penile arteries in the bull and in the dog is known to be mediated by nerves that are neither adrenergic nor cholinergic (Dorr & Brody, 1967; Klinge & Sjöstrand, 1974). It is of particular interest therefore that the material extracted from the bovine retractor penis muscle not only inhibits that muscle but is also a powerful vasodilator in a wide range of arterial systems.

The properties of the active material (lipid insolubility, stability to proteases and peptides, requirement for acid activation, instability at room temperature, inactivation by heat, by blood and by ultraviolet light), together with the observations that its effects are not blocked by antagonists of acetylcholine, adrenaline, histamine or 5-hydroxytryptamine, suggest that it does not belong to any known class of autacoids (Ambache, *et al.*, 1975; Gillespie & Martin, 1978; 1980). Although its effects are compatible with the proposition that it is the inhibitory transmitter released from the non-adrenergic, non-cholinergic nerves of the bovine retractor penis muscle, we have no direct evidence that this is so. It seems unlikely that such a powerfully active material should not have a physiological role in the control of penile function and possibly also in controlling the calibre of the blood vessels in some vascular beds. Whatever its role may be, its rapid inactivation in blood would seem to exclude the possibility that it acts as a hormone.

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