A salivary vasodilator in the blood-sucking bug, *Rhodnius prolixus*

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1 Salivary gland homogenates of the blood-sucking bug, *Rhodnius prolixus* induced transient, dose-dependent relaxation of rabbit aortic preparations pre-constricted with 200 ng ml⁻¹ noradrenaline, 1 µg ml⁻¹ histamine or 20 ng ml⁻¹ angiotensin II. Such relaxations were less marked when the aorta was constricted by 60 mM KCl. These effects were observed with as little as 0.2 µg ml⁻¹ of crude salivary gland protein.

2 The vasodilator effect was endothelium-independent, abolished by 50 µM hydroquinone or 50 µM methylene blue, and potentiated by 30 U ml⁻¹ superoxide dismutase.

3 Salivary homogenates generated a coloured compound when reacted with sulfanilic acid in the presence of N-(1-naphthyl)-ethyldiamine, indicating the presence of reactive nitrogen groups, equivalent to 35 ± 3 ng of sodium nitrite per pair of glands.

4 Molecular sieving high performance liquid chromatography of salivary gland homogenates generated a single peak of vasorelaxant activity which coincided with the presence of platelet antiaggregating and spasmolytic (guinea-pig ileum contracted with histamine) activities, as well as with reactive nitrogen groups.

5 It is concluded that a protein of molecular weight 16,500 daltons in the salivary glands of *R. prolixus* contains reactive nitrogen groups which assist the bug during a blood meal. It is suggested that saliva of blood sucking arthropods is a natural resource of novel pharmacological activities.

Introduction

The task of blood-feeding by arthropods is facilitated by saliva which is injected into the host skin during probing and feeding. This role of saliva is mediated by a number of different pharmacologically active compounds, including anticoagulants, anti-platelet activities, anti-inflammatory agents, immunosuppressants and vasodilators that counteract the host's defense mechanisms that could prevent blood finding or blood feeding (Ribeiro, 1987; 1989). Indeed, triatomine bugs or mosquitoes that were surgically deprived of salivary function had a much decreased success in obtaining a blood meal from a living host, but were successful when feeding from an artificial membrane feeder (Ribeiro & Garcia, 1981a; Ribeiro *et al.*, 1984).

The salivary glands of the blood-sucking bug *Rhodnius prolixus* contain a number of different components. An anti factor VIII (Hellmann & Hawkins, 1964; 1965) delays clotting. Anti-platelet activity prevents aggregation of citrated platelet-rich plasma induced by either adenosine diphosphate (ADP), collagen or arachidonic acid (AA) (Ribeiro & Garcia, 1981b; Ribeiro & Sarkis, 1982). A potent salivary apyrase (ATP-diphosphohydrolase) may help to counteract ADP-induced platelet aggregation (Ribeiro & Garcia, 1980; Sarkis *et al.*, 1986), and an anti-thromboxane activity antagonizes aggregation induced by AA and collagen suspensions, as well as the vasoconstrictor effect of thromboxane A₂ (Ribeiro & Sarkis, 1982). Saliva also antagonizes the effects of histamine and 5-hydroxytryptamine on smooth muscle preparations (Ribeiro, 1982). This bug appears to be well equipped to counteract their host's haemostasis.

A very potent salivary vasodilator peptide was described in the sand fly *Lutzomyia longipalpis* (Ribeiro *et al.*, 1989), and

the vasodilator prostaglandin E₂ was found in the saliva or salivary glands of *Boophilus microplus*, *Hyalomma anatolicum* and *Ixodes dammini* ticks (Higgs *et al.*, 1976; Shemesh *et al.*, 1979; Ribeiro *et al.*, 1985). To our knowledge, no vasodilators have been studied from any other of the thousands of haematophagous arthropod species. Yet salivary vasodilators should be common among blood-sucking animals due to the selective advantage these compounds offer. Vasodilators would increase the supply of food at the arthropod's mouthparts and decrease the time necessary for completing the meal (Gillet, 1967; Edman & Kale, 1971; Edman *et al.*, 1974). A fifth instar nymph of *R. prolixus*, weighing about 30 mg, typically takes 300 µl of blood in 15 min from a single cannulated skin venule or arteriole (Lavoipierre *et al.*, 1959). A salivary vasodilator would presumably help the bug to accomplish this feat. In this paper, we investigated and characterized a salivary vasodilator in *R. prolixus*, and discuss the possibility that the saliva of blood sucking arthropods is a rich natural source of pharmacologically active substances.

Methods

Rhodnius prolixus bugs, fed on anaesthetized guinea-pigs, were reared at the Harvard School of Public Health. Salivary glands were removed from adult male and female *Rhodnius prolixus* at one week of age or older (Ribeiro & Garcia, 1980). Groups of 25 pairs of glands were dissected, pooled together in 1.5 ml conical plastic tubes and frozen at -70°C until use. Frozen salivary glands were suspended in 250 µl phosphate buffered saline (150 mM NaCl and 10 mM sodium phosphate, pH 7.4), homogenized in a glass homogenizer and centrifuged at 10,000 g for 5 min. The supernatant, containing 1.8 ± 0.3 mg salivary gland protein (mean ± s.e.mean, n = 3), was collected and used for the experiments. The supernatants could be kept at -70°C and submitted to several freeze and thaw cycles without apparent loss of activity.

Vasodilator activity was measured on 3 mm wide rabbit aortic strips or rings, in a 5 ml glass bath kept at 37°C. The preparation was immersed in Tyrode solution (composition in mM: NaCl 137, KCl 2.68, CaCl₂ 1.8, MgCl₂ 0.49, NaH₂PO₄

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0.362, NaHCO_3 11.9, disodium EDTA 0.03). The pH of the solution, gassed with 95% O_2 and 5% CO_2 , was adjusted to 7.5 (Webster & Prado, 1970). An initial tension of 2 g was applied and the preparation was allowed to rest for 1.5 h with changes in the bathing solution every 30 min. The tension generated was recorded isometrically. After this resting period, 200 ng ml^{-1} noradrenaline (NA) was added and when a plateau was obtained, the various experiments were performed. In certain experiments, the aorta was contracted with 60 mM K^+ instead of NA. When 60 mM K^+ was utilized, the final concentration of Na^+ was reduced 60 mM to maintain osmolality (Karaki, 1987).

For smooth muscle spasmolytic activity a guinea-pig terminal ileum preparation was used and treated as for aortic preparations, except that isotonic contractions against a load of 1.5 g were recorded. Histamine (200 ng ml^{-1}) was added to the bath at 3 min intervals and allowed to act for 20 s before being washed.

Anti-platelet activity was measured in a Born aggregometer (Born & Cross, 1963) by use of $50 \mu\text{l}$ human citrated platelet-rich plasma and $50 \mu\text{l}$ of column eluates in $150 \text{ mM NaCl} + 10 \text{ mM}$ phosphate buffer pH 6.8. Aggregation was triggered by adenosine diphosphate ($10 \mu\text{M}$).

Reactive nitrogen groups (such as NO or NO_2^-) were measured colorimetrically by a slight modification of published techniques (Bell *et al.*, 1963; Ignarro *et al.*, 1987). Briefly, $40 \mu\text{l}$ of 40% trichloroacetic acid was added to $200 \mu\text{l}$ of sample containing one or two salivary glands in phosphate buffered saline. The tube was vortexed and spun at $10,000 g$ for one minute, which produced a visible precipitate containing the salivary pigment. To $200 \mu\text{l}$ of the clear supernatant, $25 \mu\text{l}$ of sulfanilic acid (2 mg ml^{-1}) were added, vortexed, and $25 \mu\text{l}$ of N -(1-naphthyl)-ethylenediamine (NEDA, 1 mg ml^{-1}) were then added. After 30 min, the colour was measured at 560 nm on an ELISA reader. Controls lacking either the sulfanilic acid or NEDA gave the same optical density reading as those lacking the salivary homogenate.

Molecular sieving high performance liquid chromatography (h.p.l.c.) was performed on salivary gland homogenates with a TSK 125 column ($300 \times 7.5 \text{ mm}$) and pre-column ($75 \times 7.5 \text{ mm}$, obtained through Bio-Rad, U.S.A.), with 150 mM NaCl and 10 mM sodium phosphate, pH 6.8. The absorbance at 280 nm was recorded. Molecular weight markers used were human IgG, bovine serum albumin, ovalbumin, myoglobin and cyanocobalamin which were supplied by Bio-Rad (U.S.A.). A 4000 series Milton Roy pump, detector and integrator were utilized.

Organic reagents, histamine, angiotensin II, noradrenaline and superoxide dismutase were purchased from the Sigma Chemical Co. (U.S.A.). Other salts were of A.C.S. or analytical grade. Deionized water was used throughout the work. Protein was determined by the Bio-Rad Commassie-blue kit following the manufacturer's instructions.

Results

To investigate whether salivary glands of *Rhodnius prolixus* contained vasodilator activity, we added different amounts of salivary gland homogenates to the rabbit aortic strip preparation pre-constricted with NA. Addition of 1% of one pair of glands (about $1.8 \mu\text{g}$ of protein) to a 5 ml bath gave a noticeable transient relaxation of the preparation. The phenomenon was dose-dependent (Figure 1a). Repeated additions of the same dose gave reproducible transient relaxations on the same preparation (Figure 1b). Relaxation was also observed when the aorta was contracted with $1 \mu\text{g ml}^{-1}$ histamine or 20 ng ml^{-1} angiotensin II (data not shown).

We tested the effects of the homogenate on aortic strips from which the endothelium was removed by rubbing the intima with filter paper. Addition of salivary homogenates continued to elicit relaxation on endothelium-free preparations (Figure 2).

Figure 1 Transient relaxation of the rabbit aortic strip induced by *Rhodnius prolixus* salivary gland homogenates. The preparation was constricted with 200 ng ml^{-1} noradrenaline and the additions made are of fractions of one pair of glands to a 5 ml organ bath.

The action of the salivary vasodilator was inhibited by $50 \mu\text{M}$ methylene blue added at least 10 min before saliva addition (Figure 3a), and by $50 \mu\text{M}$ hydroquinone, added 3–5 min before saliva addition (Figure 3b). The vasodilator activity was, however, potentiated by superoxide dismutase (30 u ml^{-1}), added 3–5 min before saliva (Figure 3c). Similar results were obtained on 3 different aortic preparations with 3 different batches of salivary glands.

The vasodilator was less effective at relaxing potassium-induced (60 mM) contractions than those induced by 200 ng ml^{-1} NA (Figure 4).

To test whether *R. prolixus* salivary gland homogenates contained reactive nitrogen compounds, we treated the acidified homogenate with sulfanilic acid and NEDA. A coloured product developed indicating the presence of $35 \pm 3 \text{ ng}$ of sodium nitrite equivalents per pair of salivary glands (mean $\pm$ s.e.mean, $n = 4$ in pools of 25 pairs of glands).

To determine the molecular weight of the substance(s) responsible for the effects of saliva, we fractionated *R. prolixus* salivary homogenates on a molecular sieving column and measured its relaxant or inhibitory effects on NA-induced contractions of the rabbit aorta, histamine-induced contractions of guinea-pig ileum and adenosine diphosphate-induced aggregation of human citrated platelet-rich plasma. Additionally, reactive nitrogen groups were measured colorimetrically. Vasodilator, spasmolytic, anti-platelet and reactive nitrogen activities co-eluted with the major 280 nm absorption peak, with an apparent molecular weight of $16,500$ daltons (Figure 5).

Figure 2 Endothelium-independence of the effect of *Rhodnius prolixus* salivary homogenate on the rabbit aortic ring constricted with noradrenaline (200 ng ml^{-1}). The efficiency of removal of the endothelium, accomplished by rubbing the intima with a filter paper, was determined by the failure of acetylcholine ($1 \mu\text{g ml}^{-1}$) to elicit vasodilatation. The numbers indicate the fraction of one salivary gland added to a 5 ml organ bath. Two other preparations yielded similar results.

Figure 3 Sensitivity of *Rhodnius prolixus* salivary vasodilator to oxidants and protection by superoxide dismutase. Rabbit aortic strips were constricted with 200 ng ml^{-1} noradrenaline (NA) and 10% (a and b) or 3% (c) of one pair of glands was then added (indicated by S). (a) Inhibition of the vasodilator effect by $50 \mu\text{M}$ methylene blue (MB) added with noradrenaline. (b) Inhibition of vasodilator effect by $50 \mu\text{M}$ hydroquinone (HQ). (c) Enhancement of the vasodilator effect by superoxide dismutases (SOD, 30 u ml^{-1}). Two other experiments, with different rabbit aortic strips and different batches of salivary gland homogenate, yielded similar results.

Figure 4 Dose-response curves showing the vasodilator activity of *Rhodnius prolixus* salivary gland homogenates on rabbit aortic strips constricted with 200 ng ml^{-1} noradrenaline (○) or with 60 mM KCl (●). Symbols and vertical bars show the mean and s.e.mean of 4 determinations representing 4 different aortic preparations and 4 different pools of salivary glands.

Figure 5 Molecular sieving liquid chromatography of 10 homogenized pairs of salivary glands of *Rhodnius prolixus* in a TSK 125 column, run at 0.8 ml min^{-1} with 150 mM NaCl and 10 mM phosphate buffer, pH 6.8. Results shown are combined from two runs under identical conditions. Fractions were collected at every 0.2 min except for reactive nitrogen group determinations, where it was at 0.3 min intervals. (a) The absorbance (Abs) at 280 nm (optical density). (b) Vasodilatation was measured by adding $10 \mu\text{l}$ of each fraction to a rabbit aortic strip constricted with 200 ng ml^{-1} noradrenaline. (c) Spasmolytic activity was measured by adding $50 \mu\text{l}$ of each fraction to a guinea-pig ileum constricted with 200 ng ml^{-1} histamine. Contractions were elicited before application of each experimental fraction. (d) Anti-platelet activity was determined by mixing $50 \mu\text{l}$ of indicated fractions with $50 \mu\text{l}$ of human citrated platelet-rich plasma, and measuring the resulting aggregation 5 min after addition of $10 \mu\text{M}$ ADP. (e) Reactive nitrogen groups were measured in 0.2 ml samples from each fraction to determine nitrite equivalent.

Discussion

The transient nature of the *Rhodnius prolixus* salivary gland-induced aortic relaxation (Figure 1) is similar to relaxation seen with some nitrovasodilators and nitric oxide (Gruetter *et al.*, 1980). The actions of nitrovasodilators are endothelium-independent and are inhibited by oxidant compounds such as methylene blue (Gruetter *et al.*, 1979; 1980) and hydroquinone (Rapoport & Murad, 1983). In addition, the actions of nitric oxide are enhanced by superoxide dismutase (SOD) (Gryglewski *et al.*, 1986). These same characteristics were observed with the *R. prolixus* salivary vasodilator (Figure 2 and 3).

Further similarities between *R. prolixus* salivary vasodilator activity and nitrovasodilators were obtained when comparisons were made between the relaxation induced in aortic strips precontracted with either NA or KCl. As is the case with nitric oxide (Ignarro *et al.*, 1988), saliva-induced relaxation was more marked in NA pre-constricted aortic strips than in KCl pre-constricted strips (Figure 4).

Nitrovasodilators also relax guinea-pig ileum and inhibit platelet aggregation (Azuma *et al.*, 1986; Moncada *et al.*, 1988; Buga *et al.*, 1989). *R. prolixus* salivary gland homogenates also possess these activities. Additionally, when submitted to molecular sieving fractionation, the spasmolytic and anti-platelet aggregating activities of homogenates co-eluted with the vasodilator activity. Furthermore, reactive nitrogen groups were found in the same fractions. Taken together, these results strongly support the hypothesis that the vasodilator in *R. prolixus* salivary glands is a nitrovasodilator.

Because nitrovasodilators have multiple actions (Moncada *et al.*, 1988), the presence of a salivary nitrovasodilator could explain the inhibitory effects on the actions of histamine, 5-hydroxytryptamine and thromboxane previously obtained in the saliva of *R. prolixus* (Ribeiro, 1982; Ribeiro & Sarkis, 1982), and thus a single substance could account for multiple actions, all of potential benefit for blood feeding.

If it is assumed that there is a nitrovasodilator in *R. prolixus* salivary glands, the nature of the carrier molecule of the reactive nitrogen groups, as well as how these groups are released when in contact with the tissues remain to be investigated. It is possible that a protein with an apparent molecular weight

of 16,500 daltons present in saliva is the parent molecule from which the nitric oxide-like material is released. *R. prolixus* salivary glands have a deep cherry colour, first studied by Wigglesworth (1942–3), who determined that the spectroscopic properties of the pigment were similar to those of haemalbumin (haeme groups bound to human serum albumin). However, Wigglesworth did not measure the molecular weight of the pigments. Indeed all three major 280 nm absorbing peaks shown in Figure 5 absorb at 405 nm (the haeme Soret band) more intensely than at 280 nm, and their spectra is consistent with a haeme group (data not shown). Haeme proteins are known to bind nitric oxide, some with very high affinity (Ignarro & Kadowitz, 1985). It is therefore possible that the 16,500 molecular weight peptide in saliva binds haeme with high affinity and thus is able to bind nitric oxide when the peptide is at a high concentration, such as when stored in the salivary glands. The nitric oxide could then dissociate from the haeme when the concentration of the protein-haeme-nitric oxide complex is lowered upon contact with tissue. Alternatively, a reducing or enzymatic activity of the tissues could lead to the release of the nitric oxide from the haeme. It is also possible that the putative nitrogen groups are bound to a non-haeme group.

Blood feeding evolved independently in several orders of insects and ticks, and also in annelids and mammals. Thus, different genetic backgrounds became the stage for the evolution of pharmacologically active salivary compounds that could help these diverse life-forms to obtain food efficiently. It is perhaps not surprising that prostaglandins, peptides and possibly nitrovasodilators were found as the solution to a common problem (Ribeiro, 1987). There are, however, many as yet unexplored blood-sucking species which may have developed alternative pharmacological mechanisms for feeding success. Thus, it is likely to expect that research in the saliva of blood sucking animals should be productive in yielding novel pharmacologically active substances.

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