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CHARACTERIZATION OF A VASODILATOR FROM THE SALIVARY GLANDS OF THE YELLOW FEVER MOSQUITO *AEDES AEGYPTI*

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Summary

Salivary gland homogenates and oil-induced saliva of the mosquito *Aedes aegypti* dilate the rabbit aortic ring and contract the guinea pig ileum. The vasodilatory activity is endothelium-dependent, heat-stable, sensitive to both trypsin and chymotrypsin treatments, and both smooth muscle activities cross-desensitize to the tachykinin peptide substance P. Both bioactivities co-elute when salivary gland homogenates are fractionated by reversed-phase HPLC. Molecular sieving chromatography indicates a relative molecular mass of 1400. A monoclonal antibody specific to the carboxy terminal region of tachykinins reacts with material in the posterior part of the central lobe of paraformaldehyde-fixed salivary glands. The presence of a vasodilatory peptide of the tachykinin family in the salivary glands of *A. aegypti* is proposed and its role in blood feeding is discussed.

Introduction

During their search for blood, haematophagous arthropods salivate into their host's skin. While their hosts react to such an intrusion with an array of responses that attempt to counteract blood loss or tissue damage, injected saliva neutralizes some of these defences and allows successful feeding. Among the defence mechanisms of the host, blood coagulation, platelet aggregation and vasoconstriction constitute the basis of the haemostatic process. In response, the presence of anticoagulants, antiplatelet-aggregation factors and vasodilators has been reported in the saliva of many bloodsucking animals (Ribeiro, 1987, 1989).

Different types of vasodilators have been reported in the saliva of blood-sucking arthropods. Tick saliva contains vasodilatory prostaglandins (Higgs *et al.* 1976; Dickinson *et al.* 1976; Ribeiro *et al.* 1985), the blood-sucking bug *Rhodnius prolixus* has a salivary nitrovasodilator, in the form of an association between nitric oxide and a peptide (Ribeiro *et al.* 1990), and the saliva of the phlebotomine sand fly *Lutzomyia longipalpis* contains a very powerful vasodilatory peptide (Ribeiro *et al.* 1989; Lerner *et al.* 1991). These substances are thought to help

Key words: tachykinin, substance P, haematophagy, saliva, mosquito, *Aedes aegypti*.

haematophagy by increasing the amount of blood reaching the arthropod mouthparts (Kemp *et al.* 1983) and by shortening the dangerous period of contact between the host and the arthropod (Gillett, 1967).

Increased blood flow was detected at the skin sites where the mosquito *Aedes aegypti* had fed on the arms of human volunteers, suggesting the presence of a salivary vasodilator in this insect (Pappas *et al.* 1986). This postulated vasodilator, however, was never characterized. Accordingly, in this paper, the presence of vasodilatory activity in *A. aegypti* salivary glands was investigated pharmacologically, and a vasodilatory peptide with properties similar to those of neuropeptides of the tachykinin family is described.

Materials and methods

Organic reagents, substance P, histamine and noradrenaline were purchased from Sigma Chemical Co. (USA). Anti-substance P rat monoclonal antibody was catalogue no. YMC1021 and lot no. E9381 from Accurate Chemical and Scientific Corporation, Westbury, NY. According to the manufacturer, this antibody recognizes the C-terminal end of substance P. Goat anti-rat IgG, fluorescein isothiocyanate (FITC)-labelled conjugate, was obtained from Boehringer Mannheim, Indianapolis. Inorganic salts were of American Chemical Society standard or analytical grade. Deionized water was used throughout the work.

Aedes aegypti L. (Diptera:Culicidae) mosquitoes of the Rock strain were reared at 25–27°C on guinea pig food. Adult females, 5–14 days old, that had never fed on blood, but only on a cotton swab containing 10% sucrose solution, were dissected to obtain the salivary glands. These were stored in groups of 10–30 pairs in 25 μl of 5 mmol l^{-1} Tris–HCl buffer, pH 7.5, at −80°C.

Oil-induced saliva (Hurlbut, 1966; Rossignol and Spielman, 1982; Ribeiro *et al.* 1984) was obtained by quickly cold-anaesthetizing mosquitoes (10–15 s at 0°C), taking their legs and wings off, removing the mouthparts from the sheath with the help of fine forceps, and inserting the feeding parts into a 2 cm piece of surgical polyethylene tubing (0.24 mm i.d.) containing light mineral oil previously saturated with water. The procedure can easily be done without injury to the sheath so that there is no haemolymph contamination. Each individual mosquito and collecting tube were deposited on a glass slide and allowed to stay for 30–60 min on a slide warmer at 37°C. The droplets of saliva could easily be seen accumulating in the mineral oil. Tubes not containing saliva, or those in which the mosquitoes had ingested the oil, were discarded and these accounted for about 25% of the samples. The saliva was ejected into 10 μl of 1 mmol l^{-1} HCl with air blown from a 10 μl Hamilton syringe with a 29 gauge needle. The sample was stored at −80°C until needed, when 0.5 μl of 3 mol l^{-1} NaCl was added, plus 10 μl of phosphate-buffered saline, pH 7.4 (10 mmol l^{-1} phosphate). The volume of saliva injected in oil was calculated by Rossignol and Spielman (1982) to average 3788 $\mu m^3 s^{-1}$ per mosquito, but this flow rate does not remain linear over time. Saliva volume is not known precisely. From previous experience, by measuring the apyrase content of

these salivary samples (Ribeiro *et al.* 1984; J. M. C. Ribeiro, unpublished observations), it is estimated that samples of oil-induced saliva from five individual mosquitoes contain about the same amount of apyrase as one pair of homogenized salivary glands. It is not known whether the oil induces the mosquito to salivate, or merely traps the saliva that flows at a basic secretion rate or at a high secretion rate following general stimulation of the animal throughout the procedure.

Vasodilator activity was measured on 3 mm wide rabbit aortic strips or rings in a 5 ml glass bath at 37°C. The preparation was immersed in Tyrode's solution (concentration of salts in $mmol\,l^{-1}$ were: NaCl, 137; KCl, 2.68; $CaCl_2$, 1.8; $MgCl_2$, 0.49; NaH_2PO_4, 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 and 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 was added and, when a plateau had been obtained, the various experiments were performed.

The guinea pig terminal ileum preparation was used and treated as for rabbit 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 out. Substance P additions were made at 5 min intervals and allowed to contact the preparation for 30 s before being washed out, to prevent desensitization. Estimates of the substance P equivalent content of the salivary glands were made by bracketing experimental samples within known doses of substance P.

Molecular sieving HPLC was performed on salivary gland homogenates with a TSK 125 column (300 mm×7.5 mm) and pre-column (75 mm×7.5 mm, obtained through Bio Rad, USA), with $0.2\,mol\,l^{-1}$ ammonium formate, pH 4.0, in 20 % acetonitrile at a flow rate of $0.8\,ml\,min^{-1}$. The absorbance at 280 nm was recorded, and fractions were collected every 0.3 min. The relative molecular mass markers used were adenosine monophosphate, substance P, cyanocobalamine, aprotinin and cytochrome *c*. Reversed-phase chromatography was performed using an Alltech (Deerfield, IL) Macrosphere 300 C18 column (4.6 mm×250 mm). Substances were eluted by a solution of 20 % acetonitrile in water and 0.1 % trifluoroacetic acid, run for 20 min, and the concentration of acetonitrile was then raised to 80 % in the following 60 min. Fractions were collected at 1 min intervals. A 4000 series Milton Roy pump, detector and integrator were utilized. All column fractions were collected into vials containing 10 μl of 1 % bovine serum albumin. The fractions were dried on a Speed Vac (Savant) before bioassay.

Immunohistochemistry was performed on whole salivary glands essentially as described by Goldberg *et al.* (1988). Briefly, glands were fixed for 6–12 h with 4 % paraformaldehyde at 4°C and rinsed six times for the next 6–12 h with phosphate-buffered saline containing 0.3 % Triton X100 (PBS-T). The primary antibody was incubated with PBS-T at a dilution of 1:300 for 18–24 h, rinsed as before, incubated with secondary antibody at a dilution of 1:25 for 18–24 h, rinsed as

before, and briefly washed in 4 mmol l^{-1} Na_2CO3, pH 9.5, before observation in a fluorescence microscope. Controls were made by omitting the primary antibody or by preincubating the primary antibody for 4 h at 4 °C with 0.1 mg ml^{-1} substance P before addition to the fixed salivary glands.

All animals used in these experiments were humanely killed according to approved protocols reviewed by the University of Arizona Institutional Animal Care and Use Committee.

Results

When four pairs of homogenized *Aedes aegypti* salivary glands were added to a rabbit aortic ring pre-constricted with 200 ng ml^{-1} noradrenaline, a reversible relaxation of the preparation was observed, with strong tachyphylaxis, i.e. a reduced response with repeated administration (Fig. 1). The observed vasodilation was endothelium-dependent because when the endothelium was scraped off the aortic ring, the strip lost its sensitivity to acetylcholine (not shown) as well as to *A. aegypti* salivary homogenates, but not to *Lutzomyia longipalpis* (Ribeiro *et al.* 1989) salivary vasodilator (Fig. 1). Further studies indicated that the vasodilatory activity in the mosquito glands was not sensitive to exposure at 100 °C for 2 min, was retained in the supernatant fraction of a 90 % ethanol treatment, and was destroyed by both trypsin and chymotrypsin incubations (0.1 mg ml^{-1} enzyme for 30 min at 37 °C, results not shown). Substance P is a vasodilatory peptide that matches all the properties of the *Aedes aegypti* salivary vasodilator, except for its the trypsin sensitivity (Munekata *et al.* 1987; Franco-Cereceda and Rudehill, 1989). The hypothesis that the vasodilatory activity in *Aedes aegypti* salivary glands was a peptide of the tachykinin family was then pursued.

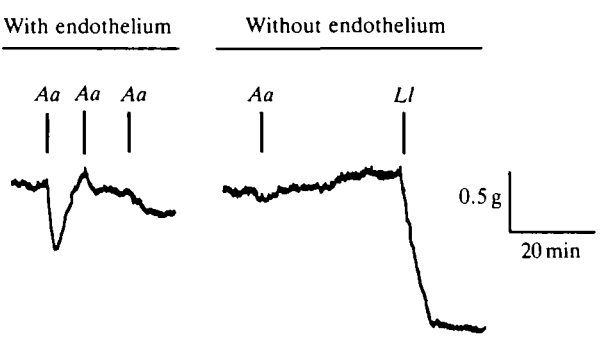


Fig. 1. *Aedes aegypti* salivary gland homogenates induce a tachyphylactic, endothelium-dependent vasodilation in rabbit aortic ring pre-constricted with noradrenaline. Aortic rings, with or without endothelium, had a resting tension of 2 g and were constricted by 200 ng ml^{-1} noradrenaline. *Aa* indicates addition of four salivary gland pairs from *Aedes aegypti*. *Ll* indicates addition of five salivary gland pairs of the sand fly *Lutzomyia longipalpis*. All results were reproduced in at least two other preparations and with different pools of salivary gland homogenates.

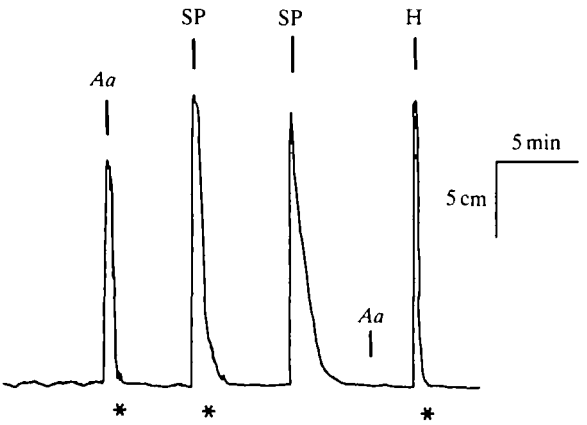


Fig. 2. *Aedes aegypti* salivary gland homogenates induce contraction of the guinea pig ileum preparation, an effect that can be desensitized by substance P. *Aa* indicates addition of five *Aedes aegypti* homogenized salivary gland pairs. SP indicates addition of 0.1 μmol l^{-1} substance P. H indicates addition of 300 ng ml^{-1} of histamine. Asterisks indicate washing of the preparation. Notice that the second addition of substance P is not followed by washing and that the ileum becomes desensitized to substance P and to the effect of *Aedes aegypti* salivary homogenate, but not to histamine. Two other preparations yielded the same results.

Substance P and other tachykinins strongly contract the isolated guinea pig ileum, which also shows desensitization to the tachykinins (Hall and Morton, 1990). When tested on this preparation, salivary gland homogenates of *A. aegypti* induced contractures, and cross-desensitization with substance P was observed (Fig. 2). In three experiments, with three different guinea pig preparations and three different mosquito homogenates, the substance P content per pair of salivary glands was 1.8±0.65 ng equivalents (mean±S.E.). Cross-desensitization was also observed on the aortic ring preparation (Fig. 3). These results further support the hypothesis that the salivary vasodilator of *A. aegypti* is a member of the tachykinin family.

Because the aorta-relaxing and the ileum-contracting activities could be the result of different substances, salivary homogenates were submitted to reversed-

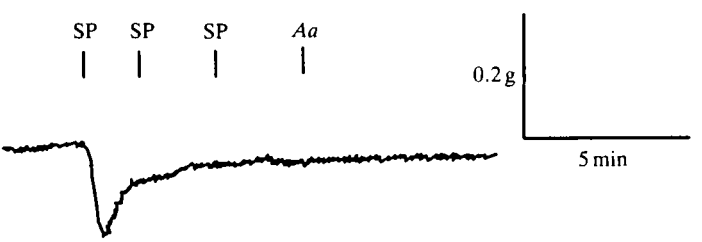


Fig. 3. Cross-desensitization of the rabbit aortic ring between substance P (SP) and *Aedes aegypti* salivary homogenates (*Aa*). Results were reproduced in two other preparations.

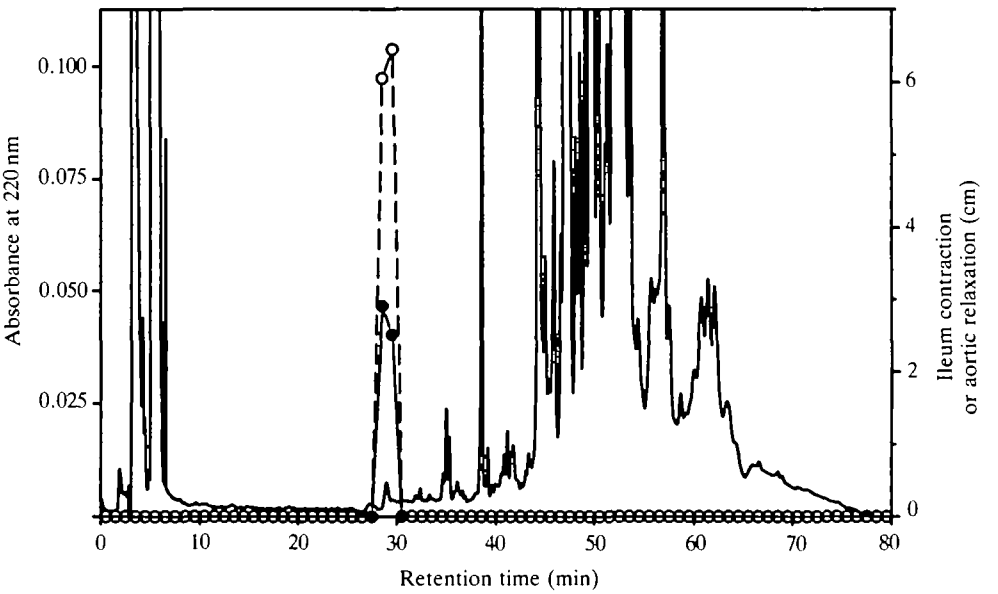


Fig. 4. Reversed-phase chromatography of 100 pairs of salivary glands from *Aedes aegypti* mosquitoes. The continuous line is the absorbance at 220 nm. All 80 fractions were tested both on the rabbit aortic ring pre-constricted by norepinephrine (filled symbols joined by continuous line) and on the guinea pig ileum (open symbols joined by dashed line). Except for the two fractions shown, all other fractions gave no activity in either smooth muscle preparation.

phase chromatography and the resulting fractions were assayed in both preparations. The biological activities co-eluted (Fig. 4), supporting the hypothesis that they reside in the same molecule.

To determine the relative molecular mass of the pharmacologically active substance, the homogenate was submitted to size-exclusion chromatography using a TSK 125 column, and the dried fractions were assayed on the guinea pig ileum preparation. The pharmacologically active component eluted with the same retention time as synthetic substance P and had an apparent relative molecular mass of 1400 as derived by linear regression analysis with the standards indicated in the inset of Fig. 5.

To test further for the possible presence of a tachykinin in the salivary glands of *A. aegypti*, as well as to visualize its location in the tissue, paraformaldehyde-fixed salivary glands were treated with a monoclonal antibody specific for the carboxy terminal region of the tachykinins (Goldberg *et al.* 1988) and with a secondary FITC-labelled antibody. The posterior medial lobe of the salivary glands showed intense reaction with the antibody (Fig. 6A), whereas the controls lacking the monoclonal antibody (Fig. 6B), or where the monoclonal antibody had previously been incubated with substance P, failed to react.

To test whether the pharmacological activities found in the salivary gland homogenates of *A. aegypti* are secreted with saliva, mosquitoes were induced to

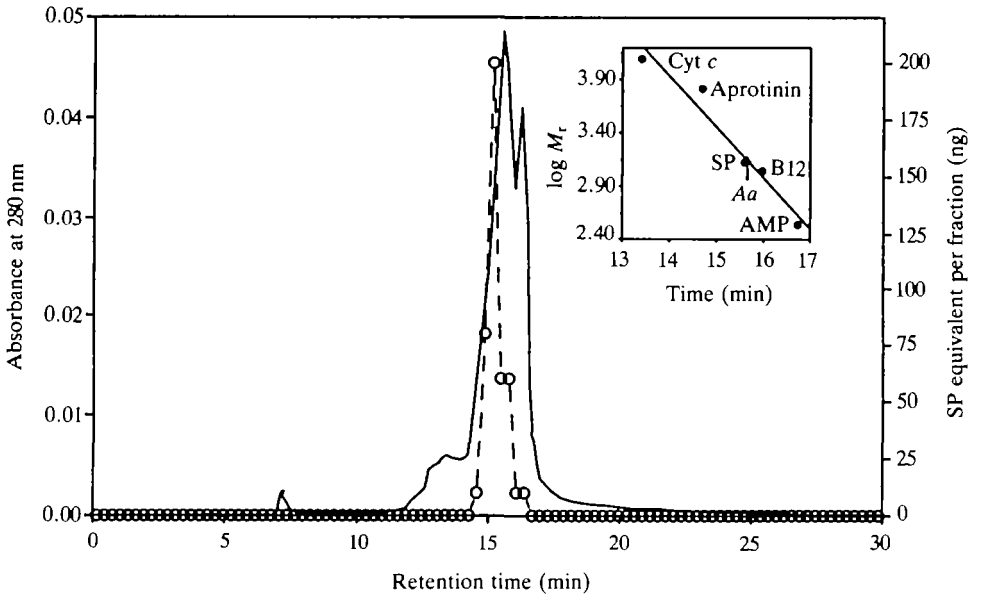


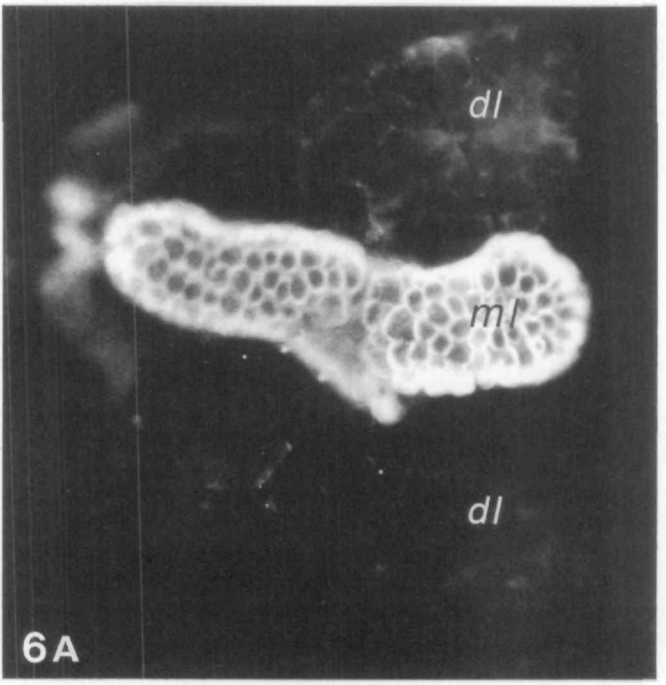
Fig. 5. Molecular sieving chromatography of 100 pairs of salivary glands from *Aedes aegypti* mosquitoes. The continuous line indicates the absorbance at 280 nm. The open circles joined by dashed line indicate the nanogram equivalent of substance P per fraction, determined in the guinea pig ileum assay. The inset shows the calibration markers for cytochrome *c* (Cyt *c*), aprotinin, substance P (SP), cyanocobalamin (B12) and adenosine monophosphate (AMP). The arrow points to the retention time of the smooth muscle contracting activity (*Aa*).

salivate in oil (Hurlbut, 1966; Rossignol and Spielman, 1982; Ribeiro *et al.* 1984), and pools of 15 saliva samples were added to the rabbit aorta pre-constricted with noradrenaline and to the guinea pig ileum. Saliva induced relaxation of the aortic ring and contraction of the ileum (Fig. 7).

Discussion

The results reported in this paper describe for the first time the presence of a vasodilatory substance in the salivary glands of a mosquito. Its pharmacological behaviour in the rabbit aortic strip and in the guinea pig ileum, its chromatographic properties and immunohistochemical evidence indicate that this vasodilator is a peptide of the tachykinin family. The secretory nature of the vasodilator was confirmed by the aorta-relaxing and ileum-contracting activities of saliva collected from mosquito mouthparts immersed in oil. Although vasodilators have been described in the saliva or salivary glands of other blood-sucking arthropods, this is the first description of a salivary vasodilator belonging to the tachykinin family.

Tachykinins are widely distributed in animals, having been found in vertebrate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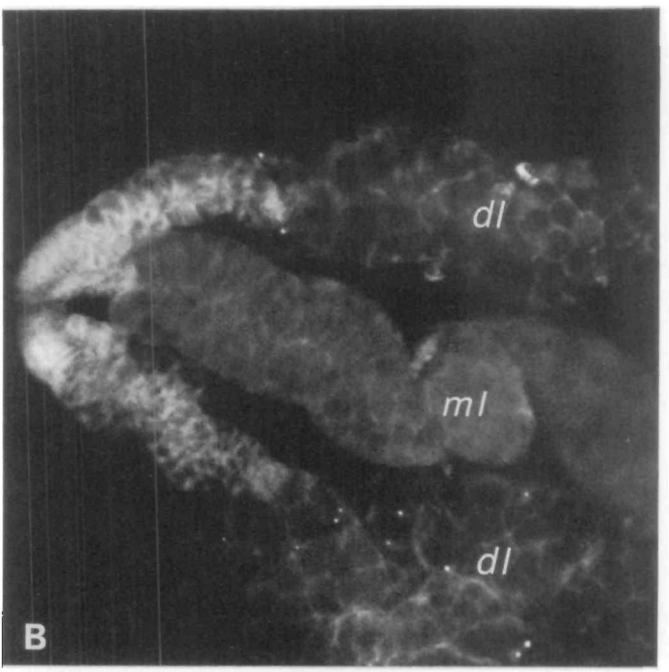
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Fig. 6. Immunostaining of whole paraformaldehyde-fixed *Aedes aegypti* salivary glands using a rat monoclonal antibody specific for the carboxy terminal region of tachykinins and a secondary fluorescein-labelled goat anti-rat antibody. (A) No pretreatment of the monoclonal antibody. (B) Monoclonal antibody preincubated with $0.1\,mg\,ml^{-1}$ substance P prior to incubation with salivary glands. The trilobular gland contains two distal lateral lobes (*dl*) and one medial lobe (*ml*).

Fig. 7. Vasodilatory and ileum-contracting activities of oil-induced saliva of *Aedes aegypti* mosquitoes. (A) Effect of saliva on the pre-constricted rabbit aortic ring. (B) Effect of saliva on the guinea pig ileum preparation. Similar results were obtained in two other experiments. *Aa*, addition of mosquito saliva.

gut and brain, in amphibian skin and in invertebrates (Iversen, 1982; Simmaco *et al.* 1990). All these peptides have a conserved carboxy terminal sequence, Phe-X-Gly-Leu-Met-NH_2, recognized by the monoclonal antibody used in this study. All tachykinins containing this carboxy terminal sequence are endothelium-dependent vasodilators and contract the guinea pig ileum. However, other functions, such as mast cell degranulation induced by substance P, are dependent on the amino terminal amino acid sequence and are not a properties shared by all tachykinins (Foreman, 1987). Ultimately, purification of enough salivary peptide to allow determination of the amino acid sequence of *A. aegypti* salivary vasodilator will be needed to confirm the tachykinin nature of *A. aegypti* salivary vasodilator.

Evolution of blood-sucking behaviour is polyphyletic, having been accomplished independently in many different arthropods, annelids and mammals. In addition to inhibiting blood clotting and platelet aggregation (Ribeiro, 1987, 1989), it appears to be advantageous for such animals to inject vasodilators at the feeding site, either to counteract the vasoconstriction associated with the haemostatic process or to increase the skin blood flow. Considering the diverse background of such animals, it is not surprising that a corresponding diversity of potent salivary vasodilators has been found in only a small number of the haematophagous species. *Aedes aegypti* vasodilator adds to this diversity by being the first tachykinin-like vasodilator described from a blood-sucking arthropod, indicating the potential wealth of novel compounds awaiting discovery in the salivary glands of these blood-suckers.

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