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SALIVARY GLANDS OF THE SAND FLY *PHLEBOTOMUS PAPATASI* CONTAIN PHARMACOLOGICALLY ACTIVE AMOUNTS OF ADENOSINE AND 5′-AMP

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Summary

Salivary gland homogenates of the sand fly *Phlebotomus papatasi* contain large amounts of adenosine and 5′-AMP, of the order of 1 nmol per pair of glands, as demonstrated by liquid chromatography, ultraviolet spectrometry, mass spectrometry and bioassays. These purines, 75–80 % of which are secreted from the glands following a blood meal, have vasodilatory and anti-platelet activities and probably help the fly to obtain a blood meal. Salivary 5′-AMP is also responsible for the previously reported protein phosphatase inhibitor in the salivary glands of *P. papatasi*, which is shown to be artifactual in nature as a result of allosteric modification by AMP of the phosphatase substrate used (phosphorylase a).

Key words: protein phosphatase, phosphorylase a, AMP, adenosine, HPLC, electrospray mass spectrometry, bioassay, platelet, aorta, haemostasis, sand fly, *Phlebotomus papatasi*.

Introduction

Hematophagy evolved independently in several orders of insects and ticks. Perhaps for this reason a variety of salivary anti-hemostatic compounds are found in this diverse group of arthropods. Many different salivary anti-clotting and anti-platelet agents and vasodilators have been described. Salivary vasodilators range from inorganic nitric oxide to prostaglandins, various different peptides and enzymes that destroy norepinephrine. Several anti-clotting peptides, acting against different clotting factors, have been characterised, as have many anti-platelet substances acting on different pathways of platelet aggregation (Champagne, 1994; Ribeiro, 1995).

Many salivary compounds that interfere with vertebrate hemostasis have additional effects on the host immune system, and these may in turn affect the fate of parasites transmitted during the act of blood feeding. For example, the New World sand fly *Lutzomyia longipalpis* has a very potent vasodilator, maxadilan (Lerner et al., 1991), in its saliva, and this also has immunosuppressive activities against macrophages and lymphocytes (Qureshi et al., 1996; Soares et al., 1998). This immunosuppression may be the reason that sand fly saliva potentiates the transmission of *Leishmania* parasites (Theodos et al., 1991; Titus and Ribeiro, 1988; Warburg et al., 1994).

The Old World sand fly *Phlebotomus papatasi* does not have the vasodilator maxadilan, but its saliva nevertheless potentiates *Leishmania* transmission (Theodos et al., 1991). A protein phosphatase inhibitory activity has recently been found in this fly (Waitumbi and Warburg, 1998). *P. papatasi* salivary protein phosphatase inhibitor has been implicated in the down-regulation of nitric oxide production by murine macrophages (Waitumbi and Warburg, 1998). In an attempt to find the salivary protein phosphatase inhibitor, we discovered large amounts (approximately 1 nmol per pair of glands) of 5′-AMP and adenosine in *P. papatasi* salivary glands, two substances with known vasodilatory (Collis, 1989) and anti-platelet (Edlund et al., 1987) functions. In the present study, salivary adenosine monophosphate is shown to be the previously observed salivary protein phosphatase inhibitor of *P. papatasi.*

Materials and methods

Reagents

A protein phosphatase assay kit was obtained from Gibco-BRL, Life Technologies. [γ-^{32}P]ATP (6000 Ci mmol^{-1}) was purchased from Amersham Pharmacia Biotech. Other reagents were purchased from Sigma Chemical Co.

Sand fly rearing and collection of salivary gland lysate

Phlebotomus papatasi (Scopoli) were reared as described previously (Modi and Tesh, 1983). Salivary glands from 3-

to 6-day-old female flies, maintained with a cotton swab containing 10 % sucrose solution, were dissected in phosphate-buffered saline, transferred to conical 1.5 ml plastic tubes (in a final volume no larger than 100 µl) and stored at −70 °C. Before use, the glands were ultrasonically disrupted by immersing the tube, held by forceps, in water under the tip of a Branson Sonifier 450 (Danbury, CT, USA) sonicator with the energy level set to just under cavitation. Approximately 20 intermittent cycles of 50 % duration were sufficient for complete disruption of the glands. The tubes were centrifuged at 14 000 ***g*** for 2 min after sonication, and the supernatant was used for the experiments.

Macrophages

Macrophages for use in protein phosphatase assays were obtained from 8- to 12-week-old C3H/HeN female mice as described previously (Waitumbi and Warburg, 1998). Mice were stimulated with 2.0 ml of 3 % thioglycollate injected intraperitoneally. Four days later, peritoneal exudate cells were collected by peritoneal lavage using 10 ml of RPMI 1640 (Gibco-BRL), and were then washed in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS) before being resuspended in RPMI 1640 containing 10 % fetal bovine serum. 10^7 cells in 5 ml were seeded in a 50 ml culture flask and incubated at 37 °C, 5 % CO_2 and 95 % humidity for 90 min. Non-adherent cells were removed by washing the cells three times with ice-cold HBSS containing 1 mmol l^{-1} sodium vanadate. Adherent cells are hereafter referred to as macrophages.

Preparation of lysates for protein phosphatase assays

Macrophage lysates for use in protein phosphatase assays were prepared as described previously (Dong et al., 1995). Briefly, macrophages were scraped off the plastic culture flask, centrifuged and resuspended in ice-cold lysis buffer containing 20 mmol l^{-1} Tris–HCl, pH 7.0, 250 mmol l^{-1} sucrose, 20 mmol l^{-1} KCl, 2.5 mmol l^{-1} $MgCl_2$, 30 mmol l^{-1} β-mercaptoethanol, 2 mmol l^{-1} EDTA, 1 mmol l^{-1} phenylmethylsulfomyl fluoride, 10 µg ml^{-1} leupeptin, 10 µg ml^{-1} aprotinin, 25 % glycerol and 1 % Triton X-100. The cells were left on ice for 30 min with occasional pipetting. Nuclear and cell debris were removed by centrifugation at 1000 ***g*** for 15 min at 4 °C, and the total protein concentration in the cytosolic fraction was determined using the Bradford method (Bradford, 1976). For preparation of *Leishmania major* lysates, 2×10^7 logarithmic-stage promastigotes were washed three times in HBSS and lysed in 0.5 ml of lysis buffer as described above for macrophages.

Protein phosphatase assays

Macrophages and *Leishmania major* lysates prepared as described above were used for measurements of protein phosphatase activity as described previously (Waitumbi and Warburg, 1998). The assay was carried out using a kit from by Gibco-BRL, which uses radioactive ATP and phosphorylase kinase to transform phosphorylase b to its phosphorylated form, phosphorylase a, which is then used as a substrate for the protein phosphatases 1 (PP1) and 2A (PP2A) contained in the macrophage and *Leishmania major* lysates.

High-performance liquid chromatography

A CM 4100 pump and SM4100 dual-wavelength detector from ThermoSeparation Products were used (Rivera Beach, FL, USA). A diode array detector (model SPD-M10AV, Shimadzu Corp., Columbia, MD, USA) was used to monitor the light absorption of the column effluent in the range 200–350 nm, as indicated. The following columns were used. (1) Molecular sieving Superdex Peptide HR 10/30 from Amersham Pharmacia Biotech (10 mm×300 mm), run with 30 % acetonitrile and 0.1 % trifluoroacetic acid (TFA). This volatile solvent mixture was chosen to allow complete evaporation and increased concentration of the samples for bioassays. This solvent lacks salts that disrupt ionic interactions with the stationary matrix, and this may result in some charged compounds failing to elute in a truly molecular sieving mode. (2) Alltech (Deerfield, IL, USA) C18 macrosphere (4.3 mm×250 mm) run isocratically with 0.1 % TFA in water (prior to each run, the column was washed with 10 vols of 95 % acetonitrile and equilibrated with 10 vols of eluting solution). (3) An anion-exchange TSK-DEAE column (4.5 mm×150 mm) obtained from BioRad (USA), perfused isocratically with 50 mmol l^{-1} sodium phosphate adjusted to pH 3.5 with phosphoric acid. The eluate was monitored either at 220 and 280 nm or at 260 and 280 nm, as indicated. Under these conditions, the adenosine and AMP content of individual salivary gland pairs could be measured. Ratios of absorption at 260 nm and 280 nm were between 6.5 and 8.2, consistent with the absorption ratios of adenosine and AMP standards.

Mass spectrometry measurements

Liquid chromatography mass spectrometry analyses were performed on a Hewlett Packard 1100 electrospray mass spectrometer system. The sample was injected onto a Zorbax SB300 C3 column (2.1 mm×150 mm). The elution was started with 5 % acetic acid with a gradient to 100 % acetonitrile. The mass spectrometer was scanned from *m*/*z* 60 to 1000 (where *m* is mass and *z* is valence) using the fragmentor voltages indicated. Accurate mass measurements were made using fast atom bombardment with a polyethylene glycol matrix.

Platelet aggregation assay

Platelet aggregation assays were performed on a Thermomax microplate reader (Molecular Devices, MenloPark, CA, USA) with a kinetic module (Bednar et al., 1995) with further modifications. Human citrated platelets were obtained by plasmaphoresis and were further diluted with Tyrode's solution without Ca^{2+} containing 1 mg ml^{-1} bovine serum albumin (T-CaBSA) to give a final absorption (turbidity) of 0.1 optical density units at 650 nm. The indicated volumes of dry chromatographic fractions resuspended in T-CaBSA were incubated in a 96-well flat-bottomed plate

(Falcon 3912; Becton Dickinson, Oxnard, CA, USA) in a total volume of 95 µl. The plate was incubated at 37 °C for 2 min, and ADP was added in volumes of 5 µl to give a final concentration of 15 µmol l^{-1}. The plate was shaken for 5 s on a microplate shaker (Cole Parmer series 4732, Vernon Hills, IL, USA), and aggregation was monitored at 11 s intervals with shaking of the plate between readings.

Smooth muscle bioassay

Rabbit aorta was purchased from Spring Valley Labs, Woodbine, MD, USA. Organs were shipped in ice-cold Hepes-buffered Tyrode's solution (see below for details). Rings could be used up to 3 days after organ removal. Rabbit aorta smooth muscle ring bioassays were performed isometrically using Hepes (10 mmol l^{-1}) buffered Tyrode's solution (Webster and Prado, 1970) at 37 °C bubbled continuously with 95 % O_2 and 5 % CO_2. Dexamethasone (0.1 µmol l^{-1}) was added to the bath to prevent induction of inducible NO synthase (Moncada et al., 1991), while EDTA (30 µmol l^{-1}) was added to chelate heavy metal contaminants. Aortic rings (2–3 mm wide) were attached to small (0.5 ml final volume) temperature-controlled (37 °C) cuvettes by means of stainless-steel hooks. Another hook, linked to a polyester thread, connected the ring to an isometric (force) transducer. The isometric force transducer could be moved up and down using a gear mechanism from Harvard Apparatus (MA, USA). This gear mechanism was used to stretch the ring to an initial tension of 3 g. Rings were allowed to stabilise for 90 min, with changes in the saline solution at 15–20 min intervals, when the tension was readjusted if necessary. Experiments were started by the addition of the agonist norepinephrine (1 µmol l^{-1}), which induced a contraction of the ring, measured in units of grams of force. When a stable tension was achieved, samples (25 µl) from the evaporated and reconstituted column fractions were added to the bath to test for vasodilative (tension relaxation) activity. Isometric transducers were obtained from Harvard Apparatus (MA, USA), and their output was recorded in a computer through an analog-to-digital card converter.

Statistical tests

Statistical tests were carried out using Sigmastat Statistical Package version 2.0 (Jandel, San Rafael, CA, USA). When variances were homogeneous between groups, *t*-tests were performed; otherwise, Mann–Whitney rank sum tests were performed. Values for IC_{50}, the dose giving half-maximal inhibition, were calculated from the interpolation of the linear regression lines of the percentage inhibition of phosphorylase a dephosphorylation against the logarithm of AMP concentration.

Results

The salivary protein phosphatase inhibitor of *Phlebotomus papatasi* has been previously characterised as a relatively small molecule because of its capacity to pass through membrane filters with 3 kDa limiting pores. Furthermore, the activity resisted boiling, RNAase, DNAase and proteases and was soluble in ethanol (Waitumbi and Warburg, 1998). To obtain further information on the molecular nature of this activity, we submitted 40 pairs of homogenised salivary glands to molecular sieving chromatography using a 10 kDa exclusion column. The absorbance of the eluate was monitored at both 220 and 280 nm. Samples of the fractions were evaporated and assayed for protein phosphatase inhibitory activity. The chromatogram indicated late elution of two major peaks having a relatively large 280 nm absorption compared with absorption at 220 nm (280 nm absorption being approximately one-third of that at 220 nm). This is atypical of peptides, which have 10–20 times more absorption at 220 nm. Protein phosphatase inhibitory activity co-eluted with the second of the two large 280 nm absorbing, late-eluting, peaks (Fig. 1).

Attempts to use octadecyl reverse-phase protocols to purify the *Phlebotomus papatasi* protein phosphatase inhibitor (PpPPI), using gradients from 10 % to 60 % acetonitrile in 0.1 % TFA in water were unsuccessful, because the activity was eluted in the solvent front (not shown). However, the activity was retained in the same column if elution was performed isocratically using 0.1 % TFA in water. We submitted the homogenate of 40 pairs of salivary glands to ultrafiltration on a Centricon 3 (3 kDa cut-off), and applied the

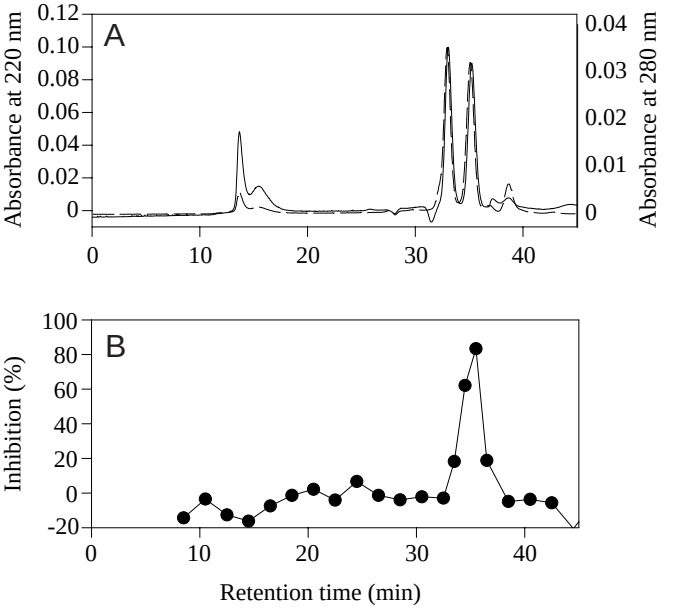

Fig. 1. (A) Size-exclusion chromatography of 40 pairs of homogenised salivary glands from *Phlebotomus papatasi*. The column was eluted with 30 % acetonitrile and 0.1 % trifluoroacetic acid at 0.5 ml min^{-1}. The eluate was monitored at 220 nm (solid line) and 280 nm (broken line). Fractions were collected at 1 min intervals. Samples (50 µl) of each fraction were dried and reconstituted in 50 µl of protein phosphatase buffer, and 20 µl was used to test its effect in the protein phosphatase assay (B). Inhibition of the phosphorylase dephosphorylation is indicated by circles. Another experiment yielded similar results.

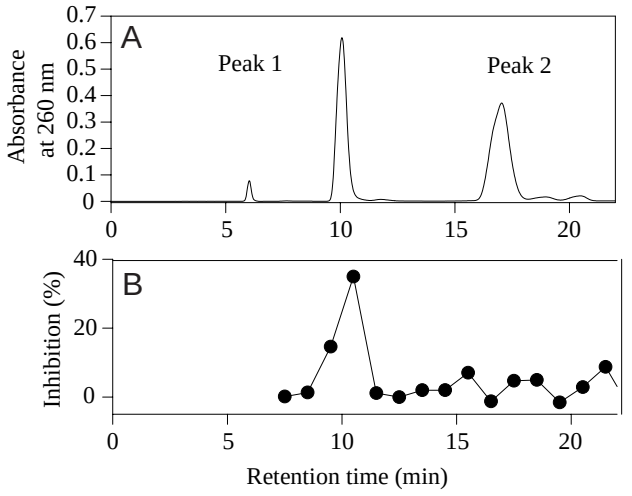

Fig. 2. (A) Reverse-phase high-performance chromatography of the ultrafiltrate from 40 pairs of homogenised salivary glands from *Phlebotomus papatasi* passed through a 3 kDa cut-off ultrafilter. The column was isocratically eluted at 0.5 ml min^{-1} with 0.1 % trifluoroacetic acid in water. Fractions were collected at 1 min intervals, and 20 µl samples were dried and reconstituted as described in Fig. 1 to assay protein phosphatase inhibitory activity (B). Absorption of the eluate at 260 nm is represented by the continuous line. Circles indicate the inhibition of phosphorylase a dephosphorylation.

filtrate to this isocratic reverse-phase protocol. The eluate was monitored in a diode array detector. Two major peaks eluted, both having ultraviolet absorption maxima near 258–260 nm. Protein phosphatase inhibitory activity co-eluted with the first major peak (Fig. 2).

Fractions corresponding to peak 1 and peak 2 were evaporated to dryness and submitted to mass spectrographic analysis. These fractions are named peak 1 and peak 2 throughout this paper. Although only peak 1 showed inhibitory activity in our assay, peak 2 was analysed because it had similar ultraviolet spectral properties to peak 1 and its mass spectrum could help in the identification of peak 1. Peak 1 gave a predominant molecular ion of *m*/*z*=348.1 (Fig. 3A), whereas peak 2 produced a molecular ion of mass 268.1 (Fig. 4A), the difference corresponding to 80 mass units, the same mass as one HPO_3 radical. The observed masses were hypothesised to derive from adenosine monophosphate (protonated mass 348.2) for peak 1 and adenosine (protonated mass 268.2) for peak 2. Accurate mass measurements (fast atom bombardment with polyethylene glycol matrix) of the adenosine phosphate peak confirmed the formula of the peak 1 compound. The protonated mass of the parent ion ($[M\text{-}H]^-$) was 346.0563 (calculated formula $C_{10}H_{13}N_5O_7P$, 346.0553).

To identify the compounds within peaks 1 and 2 as AMP and adenosine, both were submitted to electrospray mass spectrometry at an increased electrospray voltage to produce

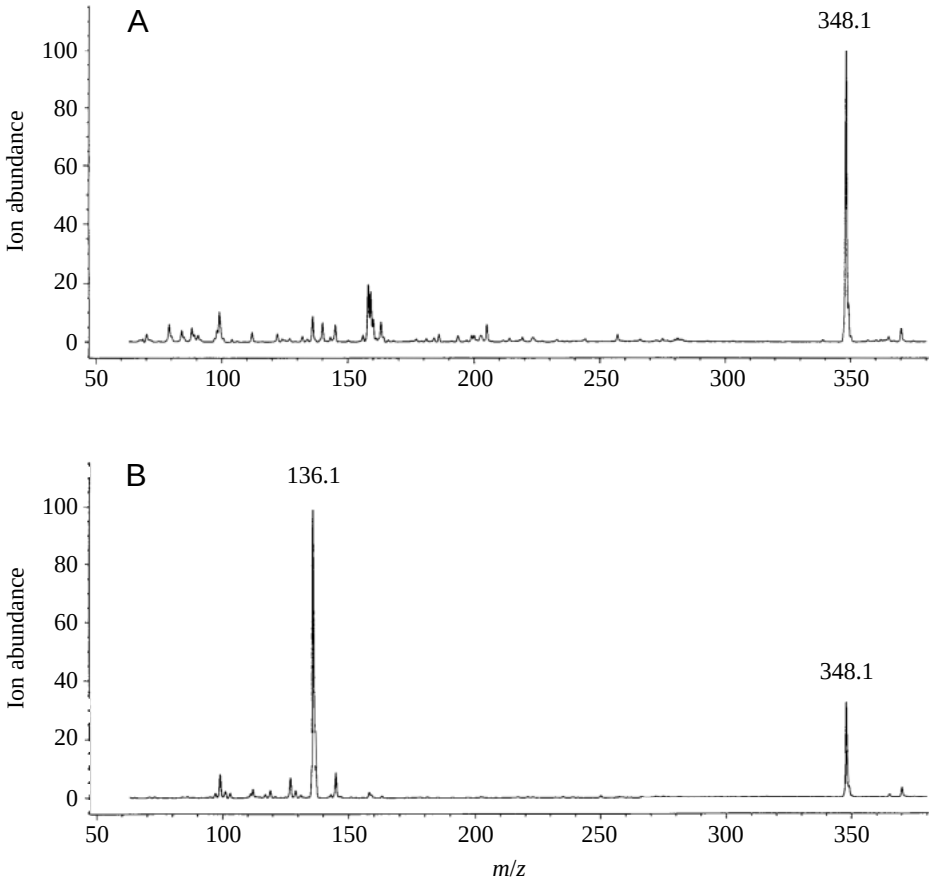

Fig. 3. Direct injection electrospray mass spectrographic analysis of the fraction corresponding to peak 1 from Fig. 2. Fragmentor voltages were 60 V (A) and 100 V (B). *m*, mass; *z*, valence. Ion abundance is expressed relative to that of the major peak.

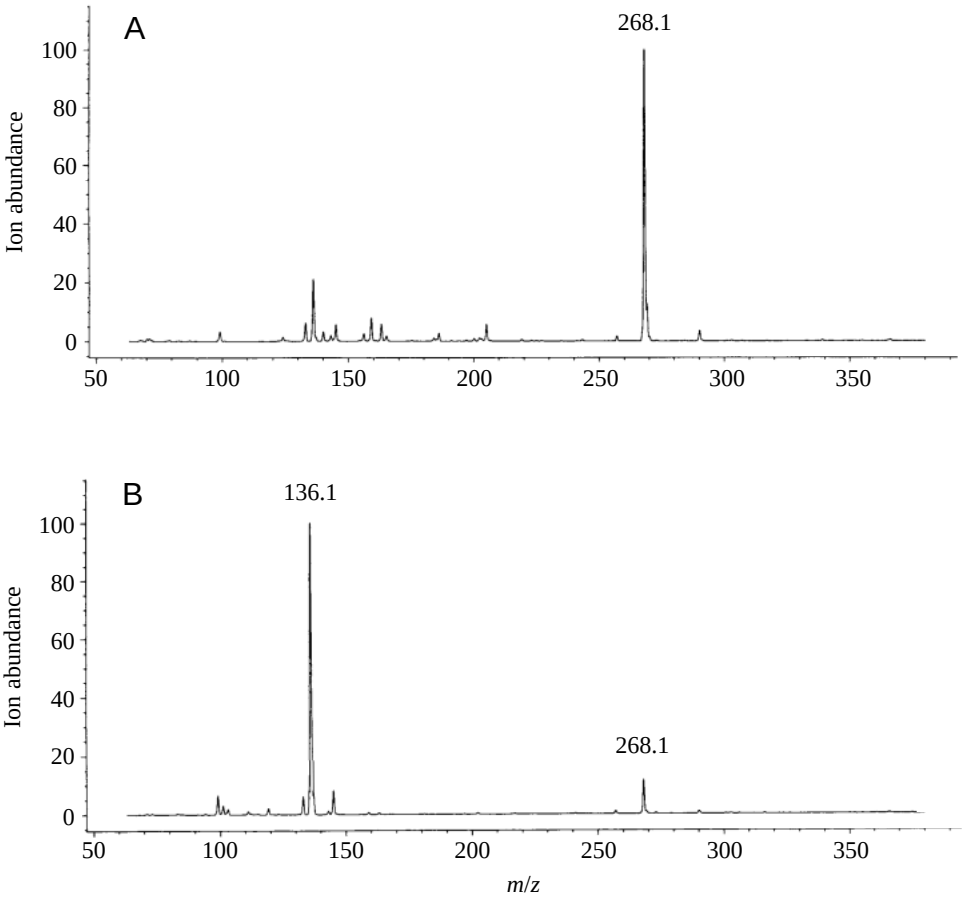

Fig. 4. Direct injection electrospray mass spectrographic analysis of the fraction corresponding to peak 2 from Fig. 2. Fragmentor voltages were 60 V (A) and 100 V (B). *m*, mass; *z*, valence. Ion abundance is expressed relative to that of the major peak.

fragmentation of the compounds. In both cases, the expected protonated adenine mass of 136.1 was obtained as the parental masses of 348.1 and 268.1 were destroyed (Figs 3B, 4B). Reverse-phase chromatography of authentic 5′-AMP and adenosine gave the same retention times (not shown) as peaks 1 and 2, respectively, and the same ultraviolet spectra (Fig. 5). The retention time of 3′-AMP was intermediate between those of AMP and adenosine, and it was therefore was excluded as a candidate for peak 1.

To confirm the role of 5′-AMP as the inhibitor of phosphorylase a dephosphorylation in our assay, we tested the ability of authentic 5′-AMP to inhibit both the *Leishmania major* and murine macrophage protein phosphatase activities, as determined using the phosphorylase a kit. Phosphorylase a dephosphorylation by both enzyme preparations was inhibited, with IC_{50} values of 7.14±0.47 μmol l^{-1} for *Leishmania major* and 8.51±0.27 μmol l^{-1} for murine macrophages (means ± S.E.M., *N*=3) (Fig. 6).

Because approximately one pair of homogenised salivary glands is needed to produce 50 % phosphatase inhibition (Waitumbi and Warburg, 1998), the IC_{50} values measured for AMP suggest that each pair of glands should contain at least 0.6 nmol of AMP to produce a final nucleotide concentration of approximately 10 μmol l^{-1} in the 60 μl reaction volume used in the protein phosphatase inhibition assay. We then dissected 10 pairs of salivary gland from 5-day-old female *P. papatasi* that had never been fed on blood and another 10 pairs from flies that just received a blood meal. The adenosine and AMP contents of individual gland pairs were measured by anion-exchange chromatography. The results indicated the presence of 0.93±0.08 nmol of adenosine and 1.2±0.09 nmol of AMP in the fly salivary glands (means ± S.E.M, *N*=10). These values were significantly reduced (by 75–80 %) following a blood meal ($P<0.001$ in both cases, *t*-test) (Fig. 7). We conclude that a pair of salivary glands possesses the amount of AMP required to produce the observed inhibition of phosphorylase a dephosphorylation in our assay.

Adenosine is a strong inhibitor of platelet aggregation (Edlund et al., 1987), so we tested the ability of peak 1 and peak 2 fractions to inhibit human citrated platelet aggregation induced by 15 μmol l^{-1} ADP. As expected, the peak 2 fraction significantly inhibited platelet aggregation ($P=0.002$, Mann–Whitney rank sum test, *N*=6) (Fig. 8, and a second similar experiment) at all concentrations tested, which included concentrations of the inhibitor equivalent to 1.25, 2.5 and 5 pairs of glands in a 100 μl assay. The same equivalents from the peak 1 fraction gave no significant inhibition of platelet aggregation.

Both AMP and adenosine are known vasodilators (Lewis et al., 1994; Rose'Meyer and Hope, 1990). We therefore tested the effects of the peak 1 and peak 2 fractions on a rabbit aortic ring contracted with norepinephrine. A small but reproducible

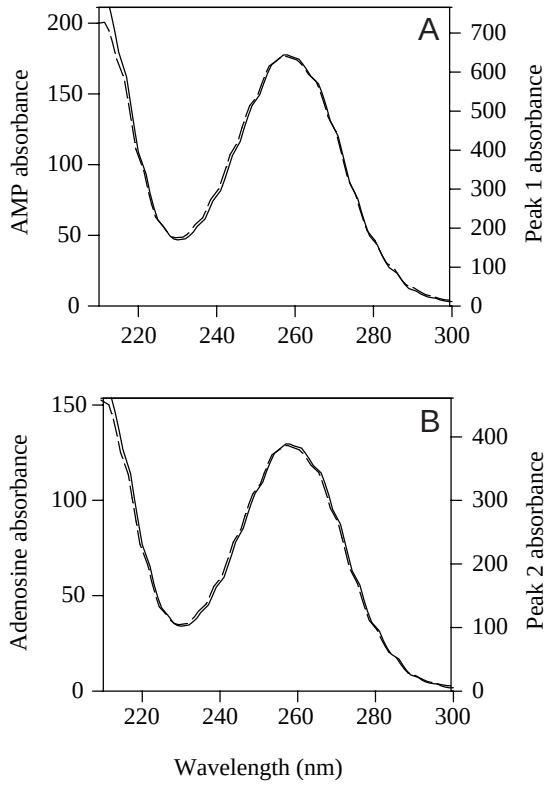

Fig. 5. Ultraviolet spectra of peak 1 (A) and peak 2 (B) compounds, as they eluted from the column chromatography shown in Fig. 2 (continuous line), superimposed onto the spectra of AMP and adenosine (broken lines), respectively. The spectra for AMP and adenosine were obtained using a diode array detector monitoring the eluate of a chromatography column using the same conditions as in Fig. 2.

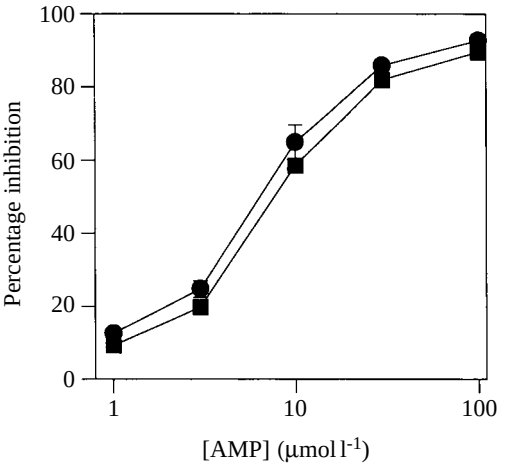

Fig. 6. Inhibition by 5′-AMP of phosphorylase a dephosphorylation by *Leishmania major* (filled circles) and murine macrophage (filled squares) homogenates. Symbols and bars represent the mean ± S.E.M. of an experiment performed in triplicate.

relaxation was observed when either of the two fractions was added to the smooth muscle preparation (Fig. 9). The results were reproduced with material derived from another column and with a second aortic preparation.

Discussion

The results presented in this paper indicate that the previously described protein phosphatase inhibitor of the salivary glands of *Phlebotomus papatasi* (Waitumbi and Warburg, 1998) is 5′-AMP, as identified by chromatographic, ultraviolet spectrographic and mass spectrographic techniques (Figs 2–5). The amounts of 5′-AMP and adenosine in the salivary glands (approximately 1 nmol per pair of glands) are sufficient to account for all the inhibitory activity found in salivary gland homogenates. This is the first time that adenosine and AMP have been found associated with saliva or salivary gland homogenates of blood-sucking arthropods, although fire ant *Pseudomyrmex triplarimus* venom has been shown to have a platelet inhibitor identified as adenosine (Hink et al., 1989). The role of these purines, the levels of which decrease by 75–80 % in the salivary glands during a blood feed, may relate to their inhibition of platelet aggregation and vascular smooth muscle contraction.

The concentrations of adenosine and AMP in *P. papatasi* salivary glands may be calculated assuming that the volume of one pair of salivary glands is 35 nl (Adler and Theodor, 1926) and that the amounts of adenosine and AMP are of the order of 1 nmol per gland pair. This gives a concentration of approximately 30 mmol l^{-1} of each of these purines within the salivary gland. Considering that 80 % of this amount is discharged during a blood meal, and assuming that a volume of 1 mm^3 represents the range of diffusion of the salivary

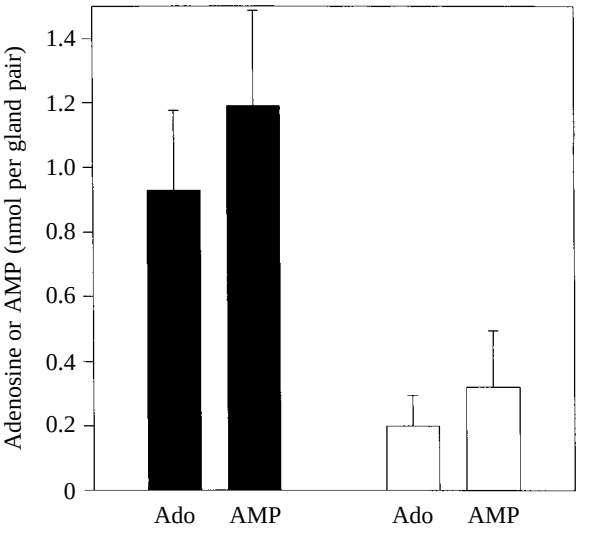

Fig. 7. Amounts of adenosine (Ado) and AMP in the salivary glands of female *Phlebotomus papatasi* before (filled columns) and after (open columns) a blood meal. The bars represent the mean + S.E.M. of 10 determinations. Purine levels were determined by anion-exchange chromatography.

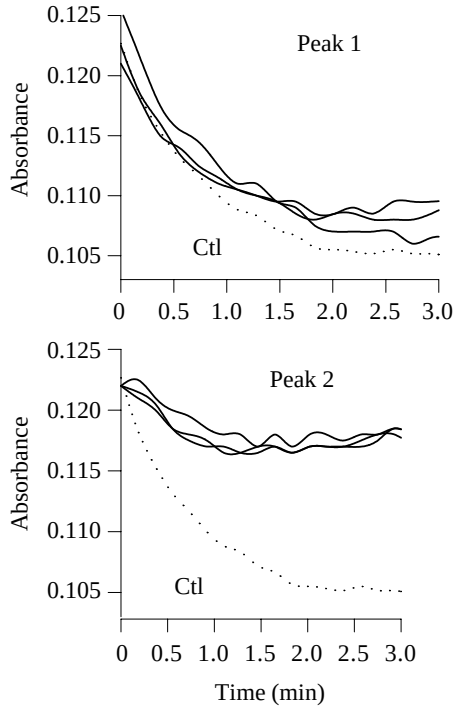

Fig. 8. Effects of peak 1 and peak 2 fractions (from Fig. 2), or of a saline control, on platelet aggregation induced by ADP. Fractions were resuspended in saline solution to a volume equivalent to one pair of glands per microliter. Solid tracings represent additions of 1.25, 2.5 and 5 µl of each fraction. The interrupted line represents the control (Ctl) level of platelet aggregation with 5 µl of saline, which does not differ from the 1.25 µl control (not shown). Absorbance is presented in arbitrary units. This is representative of two experiments.

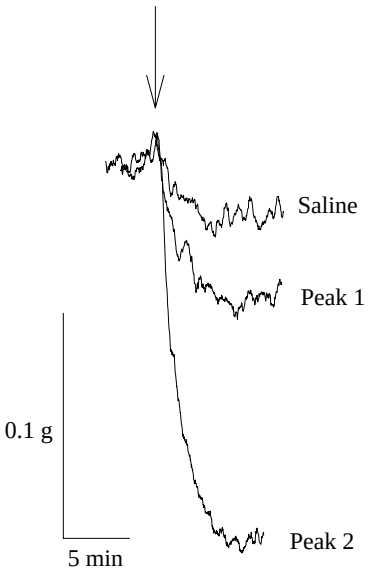

Fig. 9. Effects of peak 1 and peak 2 fractions (from Fig. 2) on rabbit aortic rings preconstricted with norepinephrine. At the arrow, 25 µl of each resuspended fraction (equivalent to one pair of glands per microliter), or of a saline control, was added to the 0.5 ml bioassay cuvette. Another experiment yielded similar results.

compounds into the host skin during feeding, concentrations of approximately 0.8 mmol l^{-1} (0.8 nmol μl^{-1}) would be achieved for adenosine and AMP in the feeding cavity.

The role of AMP and adenosine in helping the sand fly to obtain a blood meal may derive from purinergic receptors found in both blood vessels and platelets. Adenosine is a potent platelet aggregation inhibitor, with a reported IC_{50} value for inhibiting ADP-induced platelet aggregation of 1–2 µmol l^{-1} (Dionisotti et al., 1992). Adenosine increases platelet cyclic AMP concentration and thus prevents platelet aggregation by all agonists, not only by competition with ADP, as is the case for AMP. In addition, both adenosine and AMP are vasodilatory substances, with IC_{50} values of >50 µmol l^{-1} for relaxation of aortic preparations (Gordon and Martin, 1983; Lewis et al., 1994; Rose'Meyer and Hope, 1990; Urquhart and Broadley, 1991). Assuming, from the calculation in the previous paragraph, that the concentrations of secreted purines in the vicinity of the mouthparts are approximately 0.8 mmol l^{-1}, we conclude that pharmacologically active amounts of purines are produced by *P. papatasi* during a blood meal. Indeed, inhibition of platelet aggregation in a 100 µl assay was obtained with the adenosine (peak 2) equivalent of one pair of glands, which should give an adenosine concentration of approximately 10 µmol l^{-1} in the assay shown in Fig. 8. This concentration is 5–10 times larger than the reported IC_{50} for adenosine of 1–2 µmol l^{-1} (Dionisotti et al., 1992), explaining the saturation of the curves for peak 2 shown in Fig. 8. A modest vasodilatory activity was found with both nucleotide peaks when the equivalent of the AMP or adenosine content of 25 gland pairs was added to a 500 µl assay, producing a final concentration of approximately 50 nmol ml^{-1} or 50 µmol l^{-1}.

In addition to being vasodilatory and inhibitory to platelet aggregation, adenosine derivatives are also known to have lymphocyte-immunosuppressive properties (Apasov et al., 1995; Seegmiller et al., 1977; Webster, 1984). These molecules, either alone or in combination with other salivary compounds, could help to establish the *Leishmania major* parasites in the vertebrate host. The effects of adenosine receptor agonists on macrophages are not as clear cut as they are in lymphocytes: adenosine and P2 receptors have been reported either as inactivating (Hasko et al., 1996) or a shaving a stimulatory effect (Hon et al., 1997; Hu et al., 1998) on cultured macrophages. Thus, the role of *P. papatasi* salivary AMP and adenosine in decreasing NO production by murine macrophages (Waitumbi and Warburg, 1998) and in exacerbating *Leishmania* transmission remains to be investigated.

Although *P. papatasi* has pharmacologically active amounts of salivary adenosine and AMP, our unpublished observations indicate that this is not the case with the New World sand fly *Lutzomyia longipalpis*. Instead, *Lutzomyia longipalpis* salivary glands have large amounts of a secretory 5′-nucleotidase (R. Charlab, J. Valenzuela, E. Rowton and J. M. C. Ribeiro, in

preparation) which, acting in concert with the salivary apyrase (Ribeiro et al., 1986), produces vasodilatory and anti-platelet adenosine from ADP and ATP released by the injured skin while the fly is probing for a blood meal. These differences in pharmacological strategies among flies from the same family, but from genera that diverged not earlier than the last separation of the continental plates, stresses the diversity of compounds found in the salivary glands of blood-sucking arthropods.

The observed inhibition of both *Leishmania major* and macrophage protein phosphatases by 5′-AMP, with IC_{50} values of approximately 10 $\mu mol\,l^{-1}$, is somewhat surprising because 5′-AMP was not listed as a protein phosphatase inhibitor in recent reviews on the subject. Perhaps AMP is modifying the substrate used in our assay (phosphorylase a, the enzyme phosphorylating glycogen to produce glucose 1-phosphate) to render it insensitive to protein phosphatases. Indeed, phosphorylase is allosterically regulated by AMP (Gutierrez Merino et al., 1980), and AMP inhibits phosphorylase a dephosphorylation by muscle (Bot and Dosa, 1971) and hepatic PP1A/2A (Farkas et al., 1988), with IC_{50} values of the same magnitude as those found in our assays. Hence, the reported salivary protein phosphatase inhibitory activity of *P. papatasi* may be an artifact of the assay employed. AMP may act on the substrate, rendering it inaccessible to the phosphatase. However, in the process of characterising the activity, we discovered large amounts of pharmacologically active 5′-AMP and adenosine in the salivary glands of this fly.

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References

Adler, S. and Theodor, O. (1926). The mouthparts, alimentary tract and salivary apparatus of the female *Phlebotomus papatasi*. *Ann. Trop. Med. Parasitol.* **20**, 109–142.

Apasov, S., Koshiba, M., Redegeld, F. and Sitkovsky, M. V. (1995). Role of extracellular ATP and P1 and P2 classes of purinergic receptors in T-cell development and cytotoxic T lymphocyte effector functions. *Immunol. Rev.* **146**, 5–19.

Bednar, B. C. C., Gould, R. J. and Connolly, T. M. (1995). Platelet aggregation monitored in a 96 well microplate reader is useful for evaluation of platelet agonists and antagonists. *Thromb. Res.* **77**, 453–463.

Bot, G. and Dosa, I. (1971). The influence of effectors modifying phosphorylase-*a* in the phosphorylase phosphatase reaction. *Acta Biochim. Biophys. Acad. Scient. Hung.* **6**, 73–87.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Analyt. Biochem.* **72**, 248–254.

Champagne, D. E. (1994). The role of salivary vasodilators in bloodfeeding and parasite transmission. *Parasitol. Today* **10**, 430–433.

Collis, M. G. (1989). The vasodilator role of adenosine. *Pharmac. Ther.* **41**, 143–162.

Dionisotti, S., Zocchi, C., Varani, K., Borea, P. A. and Ongini, E. (1992). Effects of adenosine derivatives on human and rabbit platelet aggregation. Correlation of adenosine receptor affinities and antiaggregatory activity. *Naunyn Schmiedebergs Arch. Pharmac.* **346**, 673–676.

Dong, Z. Y. X., Xie, K., Juang, S. H., Liansa, N. and Fidler, I. J. (1995). Activation of inducible nitric oxide synthase gene in murine macrophages requires protein phosphatase 1 and 2A. *J. Leuk. Biol.* **58**, 725–732.

Edlund, A., Siden, A. and Sollevi, A. (1987). Evidence for an anti-aggregatory effect of adenosine at physiological concentrations and for its role in the action of dipyridamole. *Thromb. Res.* **45**, 183–190.

Farkas, I., Toth, B. and Gergely, P. (1988). Regulation of the dephosphorylation of phosphorylase A by glucose, AMP and polyamines. *Int. J. Biochem.* **20**, 197–201.

Gordon, J. L. and Martin, W. (1983). Endothelium-dependent relaxation of the pig aorta: relationship to stimulation of 86Rb efflux from isolated endothelial cells. *Br. J. Pharmac.* **79**, 531–541.

Gutierrez Merino, C., Garcia Blanco, F., Pocovi, M., Menendez, M. and Laynez, J. (1980). Regulation of phosphorylase b by AMP. The effects of enzyme concentration. *J. Biochem.* **87**, 1483–1490.

Hasko, G., Szabo, C., Nemeth, Z. H., Kvetan, V., Pastores, S. M. and Vizi, E. S. (1996). Adenosine receptor agonists differentially regulate IL-10, TNF-alpha and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. *J. Immunol.* **157**, 4634–4640.

Hink, W. F., Romstedt, K. J., Burke, J. W., Doskotch, R. W. and Feller, D. R. (1989). Inhibition of human platelet aggregation and secretion by ant venom and a compound isolated from venom. *Inflammation* **13**, 175–184.

Hon, W. M., Moochhala, S. and Khoo, H. E. (1997). Adenosine and its receptor agonists potentiate nitric oxide synthase expression induced by lipopolysaccharide in RAW 264 murine macrophages. *Life Sci.* **60**, 1327–1235.

Hu, Y., Fisette, P. L., Denlinger, L. C., Guadarrama, A. G., Sommer, J. A., Proctor, R. A. and Bertics, P. J. (1998). Purinergic receptor modulation of lipopolysaccharide signaling and inducible nitric-oxide synthase expression in RAW 26 macrophages. *J. Biol. Chem.* **273**, 27170–27175.

Lerner, E. A., Ribeiro, J. M. C., Nelson, R. J. and Lerner, M. R. (1991). Isolation of maxadilan, a potent vasodilatory peptide from the salivary glands of the sand fly *Lutzomyia longipalpis*. *J. Biol. Chem.* **266**, 11234–11236.

Lewis, C. D., Hourani, S. M., Long, C. J. and Collis, M. G. (1994). Characterization of adenosine receptors in the rat isolated aorta. *Gen. Pharmac.* **25**, 1381–1387.

Modi, G. B. and Tesh, R. B. (1983). A simple technique for mass rearing *Lutzomyia longipalpis* and *Phlebotomus papatasi* (Diptera: Psychodidae) in the laboratory. *J. Med. Ent.* **20**, 568–569.

Moncada, S., Palmer, R. M. J. and Higgs, E. A. (1991). Nitric oxide: Physiology, pathophysiology and pharmacology. *Pharmac. Rev.* **43**, 109–142.

Qureshi, A. A. A. A., Ohnuma, M. T. M., Granstein, R. D. and Lerner, E. A. (1996). Immunomodulatory properties of maxadilan, the vasodilator peptide from sand fly salivary gland extracts. *Am. J. Trop. Med. Hyg.* **54**, 665–671.

Ribeiro, J. M. C. (1995). Blood-feeding arthropods: Live syringes or invertebrate pharmacologists? *Infect. Agents Dis.* **4**, 143–152.

Ribeiro, J. M. C., Rossignol, P. A. and Spielman, A. (1986). Blood finding strategy of a capillary feeding sandfly, *Lutzomyia longipalpis*. *Comp. Biochem. Physiol.* **83**A, 683–686.

Rose'Meyer, R. B. and Hope, W. (1990). Evidence that A2 purinoceptors are involved in endothelium-dependent relaxation of the rat thoracic aorta. *Br. J. Pharmac.* **100**, 576–580.

Seegmiller, J. E., Watanabe, T. and Schreier, M. H. (1977). The effect of adenosine on lymphoid cell proliferation and antibody formation. *Ciba Found. Symp.* **48**, 249–276.

Soares, M. B., Titus, R. G., Shoemaker, C. B., David, J. R. and Bozza, M. (1998). The vasoactive peptide maxadilan from sand fly saliva inhibits TNF-alpha and induces IL-6 by mouse macrophages through interaction with the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor. *J. Immunol.* **160**, 1811–1816.

Theodos, C. M., Ribeiro, J. M. C. and Titus, R. G. (1991). Analyzis of enhancing effect of sand fly saliva on *Leishmania* infection in mice. *Infect. Immun.* **59**, 1592–1598.

Titus, R. G. and Ribeiro, J. M. C. (1988). Salivary gland lysates of the sand fly *Lutzomyia longipalpis* enhance *Leishmania* infectivity. *Science* **239**, 1306–1308.

Urquhart, R. A. and Broadley, K. J. (1991). Comparison of adenosine receptor agonists with other vasodilators on noradrenaline-, potassium- and phorbol ester-contracted rabbit aorta. *Eur. J. Pharmac.* **200**, 35–43.

Waitumbi, J. and Warburg, A. (1998). *Phlebotomus papatasi* saliva inhibits protein phosphatase activity and nitric oxide production by murine macrophages. *Inf. Immun.* **66**, 1534–1537.

Warburg, A., Saraiva, E., Lanzaro, G. C., Titus, R. G. and Neva, F. (1994). Saliva of *Lutzomyia longipalpis* sibling species differs in its composition and capacity to enhance leishmaniasis. *Phil. Trans. R. Soc. Lond. B* **345**, 223–230.

Webster, H. K. (1984). Role of purines in lymphocyte function. *Asian Pac. J. Allergy Immunol.* **2**, 311–317.

Webster, M. and Prado, E. S. (1970). Glandular kallikreins from horse and human urine and from hog pancreas. *Meth. Enzymol.* **19**, 681–699.