

Platelet-activating-factor-hydrolyzing phospholipase C in the salivary glands and saliva of the mosquito *Culex quinquefasciatus*

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Summary

A phospholipase C activity specific for platelet-activating factor (PAF), named PAF phosphorylcholine hydrolase, was found in the salivary glands and saliva of the human-feeding mosquito *Culex quinquefasciatus*. The enzymatic activity was demonstrated by inhibition of PAF-induced platelet aggregation, and by identification of substrate consumption and production of diacyl glyceride by electrospray-ionisation mass spectrometry. The activity has a neutral optimal pH and an apparent molecular mass

of 40–50 kDa. Two anthropophilic mosquito species, *Aedes aegypti* and *Anopheles gambiae*, do not have this salivary activity. The results are interpreted within the evolutionary context of the genera *Culex*, *Aedes* and *Anopheles*.

Key words: Platelet activating factor, saliva, hematophagy, phospholipase C, mosquito, *Culex quinquefasciatus*.

Introduction

Hematophagous insects face their host's hemostatic response when attempting to obtain a blood meal. Mammalian hemostasis is complex, consisting of platelet aggregation, blood clotting and vasoconstriction; each of these processes is redundant. For example, several pathways can initiate platelet aggregation, including those involving adenosine diphosphate (ADP), collagen, thrombin and the lipid platelet-activating factor (PAF). Additionally, aggregating platelets secrete serotonin and produce thromboxane A₂ (TXA₂), which are both vasoconstrictive substances acting at different receptors in the vascular smooth muscle cell (for a review, see Ribeiro, 1995). In response to these threats, bloodsuckers have evolved a sophisticated mix of salivary compounds that inhibit host platelet aggregation, blood clotting and vasoconstrictive compounds produced during hemostasis (Ribeiro, 1995). For example, the yellow fever mosquito *Aedes aegypti* contains a serpin that inhibits Factor Xa of the blood coagulation cascade (Stark and James, 1998), a vasodilative tachykinin peptide named sialokinin (Champagne and Ribeiro, 1994) and large amounts of the enzyme apyrase, which inhibits ADP-induced platelet aggregation by hydrolyzing ADP to AMP (Ribeiro et al., 1984b; Champagne et al., 1995). The hematophagous bug *Rhodnius prolixus* secretes, in addition to apyrase (Sarkis et al., 1986), nitric oxide (both a vasodilator and platelet inhibitor) (Ribeiro et al., 1993; Ribeiro and Walker, 1994), an adenine nucleotide scavenger (Francischetti et al., 2000) and proteins that scavenge histamine (Ribeiro and Walker, 1994) as well as inhibiting blood-clotting Factor VIII (Ribeiro et al., 1995). This mix of salivary compounds allows the speedy finding and

feeding of blood, as indicated by the poor feeding performance of *Aedes aegypti* or *Rhodnius prolixus* when deprived of salivation by surgical removal of the organs or by section of the salivary duct (Mellink and Van Den Boven Kamp, 1981; Ribeiro and Garcia, 1981; Ribeiro et al., 1984a).

PAF is an ether phospholipid (Fig. 1) involved in inflammatory and allergic reactions (Braquet et al., 1987). It is a potent agonist in rabbit platelets and the responses induced have been shown to be independent of thromboxane A₂ production and secreted ADP (Cazenave et al., 1979; Chignard et al., 1979). Although it has been suggested that platelet responses to PAF were mediated by a third pathway independent of these mechanisms (Vargaftig et al., 1980), studies on the role of thromboxane A₂ and ADP in the responses of human platelets to PAF have yielded controversial conclusions. A few studies have shown that ADP scavengers and indomethacin do not inhibit platelet secretion induced by PAF in human platelets (McManus et al., 1981). In contrast, most studies have shown that PAF-induced full aggregation and secretion in human platelets is dependent on secreted ADP and on thromboxane synthesis (Marcus et al., 1981; Chesney et al., 1982; Rao et al., 1982; Klopogge et al., 1983). In addition, platelet responses to PAF are clearly impaired in patients with congenital secretion defects, providing additional evidence that PAF is a weak platelet agonist in human platelets (Rao et al., 1984). It thus appears that platelet response to PAF is highly dependent on the species in which platelets originate.

In the present work, we investigated whether salivary gland homogenates (SGH) and saliva of mosquitoes inhibit PAF-

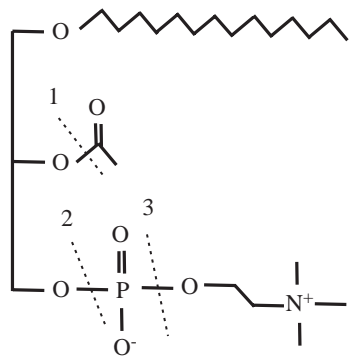


Fig. 1. Structure of platelet-activating factor (PAF) and the possible sites of hydrolysis by PAF acetyl hydrolase (1), phospholipase C (2) and phospholipase D (3). Note the ether group, which can vary in chain length.

induced platelet aggregation. We found that saliva and SGH of *Culex quinquefasciatus*, a mosquito only recently adapted to human blood feeding (Mattingly, 1962; Service, 1968; Bogh, 1998), inhibit PAF-induced platelet aggregation and have a salivary PAF-specific phospholipase C. *Aedes aegypti* and *Anopheles gambiae*, two mosquitoes highly adapted to human blood feeding (Horsfall, 1955), do not display this activity (Ribeiro, 2000).

Materials and methods

Materials

All water used was of 18 M Ω quality and was produced by a MilliQ apparatus (Millipore, Bedford, MA, USA). Organic compounds were obtained from Sigma Chemical Corporation (St Louis, MO, USA), including platelet activating factor PAF(C16) (L- α -phosphatidylcholine, β -acetyl- γ -O-hexadecyl), enantio-PAF (D- α -phosphatidylcholine, β -acetyl- γ -O-hexadecyl), lyso-PAF (L- α -lysophosphatidylcholine, γ -O-hexadecyl), 1-O-hexadecyl-2-acetyl-rac-glycerol, N-palmitoyl-D-sphingomyelin and the indicated phosphatidylcholines with specified acyl chains of equal length.

Insects

Mosquitoes were reared in the Section of Medical Entomology (LPD/NIAID/NIH) under the expert supervision of Mr André Laughinghouse. Insectary rooms were kept at 26 \pm 0.5 $^{\circ}$ C, 70 % relative humidity and a 16h:8h L:D cycle.

The strains of mosquito used were the Liverpool black eye strain of *Aedes aegypti*, the G3 strain of *Anopheles gambiae* and the Vero Beach strain of *Culex quinquefasciatus*. The most recent strain (*Culex quinquefasciatus*) has been in continuous culture for 8 years. Mosquito female adults used in the experiments were between 3 and 7 days old, took no blood meals and were maintained on a diet of 10 % Karo syrup solution.

Salivary glands from adult female mosquitoes were dissected and transferred to 10 or 20 μ l Hepes saline (HS: 0.15 mol l $^{-1}$ NaCl, 10 mmol l $^{-1}$ Hepes, pH 7.0) in 1.5 ml

polypropylene vials, in groups of 20 pairs of glands in 20 μ l of HS or as individual glands in 10 μ l of HS. Salivary glands were kept at -75° C until needed, when they were disrupted by sonication using a Branson Sonifier 450 homogenizer (Branson Ultrasonics, Danbury, CT, USA). Salivary homogenates were centrifuged at 10 000 g for 2 min and the supernatants used for experiments. To obtain saliva, female mosquitoes were lightly anesthetised by exposure to 0 $^{\circ}$ C for 1 min. After removal of their wings and legs, 0.1 μ l of 1 mmol l $^{-1}$ serotonin in HS was injected into the coelomic cavity using a heat-pulled glass capillary micropipette operated by mouth pressure (Ribeiro et al., 1984a). After the injection, the feeding fascicle was exposed from within the sheath and inserted into a 2 cm piece of polyethylene tubing (PE-10, i.d. 0.28 mm, o.d. 0.61 mm; Clay Adams, Parsippany, NJ, USA) containing a column of mineral oil of approximately 0.5 cm. This operation was done under a stereoscope with two fine-pointed tweezers. After salivating in the oil at room temperature for 10 min, the mouthparts were removed from the tubing, a #30 needle was inserted in the end of the tubing not containing oil and the oil was blown into 20 μ l of HS with the help of a 20 μ l syringe (Hamilton, Reno, NV, USA) containing a Luer-lock fitting. After accumulating 10 samples per 20 μ l of solution, the tube was spun at 10 000 g for 5 min and the water solution removed without the small amount of the oil phase.

Platelet aggregation assays

To prepare PAF solutions for platelet aggregation assays, 26 μ l of PAF (1.9 mmol l $^{-1}$ in ethanol) was evaporated under gentle helium atmosphere. PBS (100 μ l, pH 7.4) was added and the sample (500 μ mol l $^{-1}$ PAF) was sonicated at room temperature at a duty cycle of 100 % and output control set at 10 using a Branson Sonifier. Finally, the sample was diluted to 500 nmol l $^{-1}$ in tyrode-BSA (137 mmol l $^{-1}$ NaCl, 27 mmol l $^{-1}$ KCl, 12 mmol l $^{-1}$ NaHCO $_3$, 0.42 mmol l $^{-1}$ NaH $_2$ PO $_4$, 1 mmol l $^{-1}$ MgCl $_2$, 5.55 mmol l $^{-1}$ glucose, 0.25 % bovine serum albumin, pH 7.4).

Fresh platelet-rich plasma (PRP) in acid citric/dextrose (ACD, 1:10) anticoagulant was obtained by plateletpheresis from medication-free donors (Department of Transfusion Medicine/NIH Blood Bank, under the direction of S. Leitman). Platelet aggregation assays were performed with a Thermomax microplate reader (Molecular Devices, Menlo Park, CA, USA). Briefly, in a flat-bottomed 96-well plate, 50 μ l of PAF (250 nmol l $^{-1}$, final concentration) was incubated at 37 $^{\circ}$ C with 0, 0.025, 0.1, 0.25 and 0.5 pairs of *Culex quinquefasciatus* or *Aedes aegypti* SGH per assay, yielding a final volume of 85 μ l. After 15 μ l of PRP was added to start platelet aggregation, the plate was stirred for 5 s in a microplate mixer before being transferred to the microplate reader, where the turbidity change at 650 nm was measured in units of absorbance every 11 s with agitation between readings.

Enzymatic assays

Enzymatic assays were performed using 50 μ l of 0.1 mol l $^{-1}$

ammonium acetate adjusted to pH 7.2 and containing $100\mu\text{mol l}^{-1}$ of the indicated substrate (stock solutions at 10mmol l^{-1} or higher were prepared in ethanol) and the indicated amounts of enzyme, usually one or two pairs of homogenised salivary glands or oil-collected saliva, as indicated. After addition of the substrate but before addition of the enzyme, the mixture was sonicated using a Branson sonicator as described above. The reaction, started by adding the enzyme source, proceeded at 37°C . At the indicated time points, $5\mu\text{l}$ of the reaction mixture was transferred to a tube containing $50\mu\text{l}$ of 80 % methanol plus 0.1 % acetic acid, mixed and injected into the mass spectrometer. Areas corresponding to the substrate masses were integrated using the instrument software (see next paragraph). For pH dependence studies, the 100mmol l^{-1} ammonium acetate buffer was adjusted with NH_4OH or acetic acid to the desired pH. Although most pH values were far from the pK of the buffers, pH did not change by more than 0.1 pH units during the incubation. For these pH measurements, a $50\mu\text{l}$ sample was diluted to $100\mu\text{l}$ with water and the pH measured using a glass electrode.

Mass spectrometric experiments

Mass spectrometry was performed with an LCQ-Duo ion trap mass spectrometer (ThermoFinnegan, San José, CA, USA) equipped with an electron spray interface. A solution of 80 % methanol in water containing 0.1 % acetic acid was pumped into the interface at $50\mu\text{l}$ per minute, using a Spectra System P400 pump from ThermoSeparation Products (Rivera Beach, FL, USA). Samples were injected through a model 7125 loop injector (Rheodyne, Rohnert Park, CA, USA). The instrument was tuned using authentic PAF or other standards, as indicated. These conditions were: sheath gas flow rate, 20 arbitrary units; spray voltage, 4.5 kV; capillary temperature, 200°C ; capillary voltage, 27 V. Samples were injected in volumes of $50\mu\text{l}$. To estimate the amount of particular masses of interest, the area under the curve determined by the desired range of ion intensity over time was estimated using the instrument's XCalibur software, and compared with a standard curve performed under the same conditions. The instrument response to PAF was linear in the range of 30–600 fmol injected. It is important to have the methanol concentration at or higher than 80 % to achieve a good signal intensity. Additionally, to avoid erratic results, which are presumably due to the separation of the lipid micelles from the suspension and/or adsorption to the tube wall, the samples should not be cooled after enzymatic assays or prior to injection on the mass spectrometer.

Although the output of the mass spectrometer reports the intensity of ions with mass m divided by their charge z (m/z), inspection of the m/z ions just above the peak of interest reveals whether the z value is equal to 1, 2 or larger. For example, a singly charged peak at 272 will display a peak at 273, usually with 8–15 % of the intensity of the 272 mass due to heavier isotopes of C, N and H. A higher intensity at 272.5 would be indicative of a doubly charged ion. For this reason, the Results

section refers to masses, as all ions reported in this paper are singly ionized.

Chromatographic experiments

Molecular sieving chromatography was done with a Superdex-75 column ($3.2\text{mm}\times 300\text{mm}$) (Amersham/Pharmacia Biotech, Piscataway, NJ, USA) perfused with 150mmol l^{-1} ammonium acetate, pH 7.2, at $50\mu\text{l min}^{-1}$. A Spectra System P-400 pump (ThermoSeparation Products) was used. The eluate was monitored at 220 nm and samples collected at 1-min intervals. Portions ($5\mu\text{l}$) of these samples were incubated for 30 min at 37°C with $5\mu\text{l}$ of 0.2mmol l^{-1} PAF in 0.1mol l^{-1} ammonium acetate, pH 7.2 (previously sonicated). The reaction was stopped by dilution to $100\mu\text{l}$ with 80 % methanol containing 0.1 % acetic acid. $50\mu\text{l}$ reaction samples were injected into the mass spectrometer to determine substrate disappearance and product formation. The column was calibrated with bovine serum albumin, ovalbumin, carbonic anhydrase, myoglobin and cytochrome *c*.

Results

Inhibition of platelet aggregation by *Culex quinquefasciatus* salivary homogenates

Preincubation of salivary gland homogenates (SGH) of *Culex quinquefasciatus* (0.5 pairs/assay) for 1 min with 250 nM PAF caused a strong inhibition of platelet aggregation (Fig. 2A); however, *Aedes aegypti* SGH at the same dilution had no effect on PAF-induced platelet response (Fig. 2A). Inhibition of PAF-induced platelet aggregation by *Culex* SGH could be due to PAF receptor antagonism, to physiological antagonism, or to breakdown of PAF. To investigate further the nature of the inhibition induced by the SGH, we preincubated PAF with various concentrations of SGH, before adding platelets. Preincubation of PAF for 1 min with *Culex* SGH inhibited PAF-induced platelet aggregation in a dose-dependent manner, with an IC_{50} of approximately 0.05 pairs of glands per assay (Fig. 2B). At longer incubation times (20 min), total inhibition of platelet aggregation was attained at 0.05 pairs of SGH per assay (Fig. 2B). In contrast, there was no effect on PAF-induced platelet responses for *Aedes aegypti* SGH (Fig. 2B). These results suggested that a salivary enzyme in *Culex* SGH could be destroying PAF. *Culex* SGH brought platelet aggregation to near basal levels while *Aedes* SGH did not; we thus concluded that inhibition of PAF-induced platelet aggregation was not due to *Culex* apyrase, because *Aedes* has a much higher concentration of salivary apyrase than *Culex* (Ribeiro, 2000). Additionally, apyrases only partially inhibit platelet aggregation by PAF; primary aggregation is characteristically not affected (Marcus et al., 1981). Because each pair of salivary glands contains approximately $1\mu\text{g}$ of protein and approximately $0.5\mu\text{g}$ are released during the blood meal of approximately $3\mu\text{l}$, the inhibitory dose for PAF-induced platelet aggregation on the order of 0.5 ng of salivary homogenate per μl of platelet-rich plasma (PRP) is considered physiological.

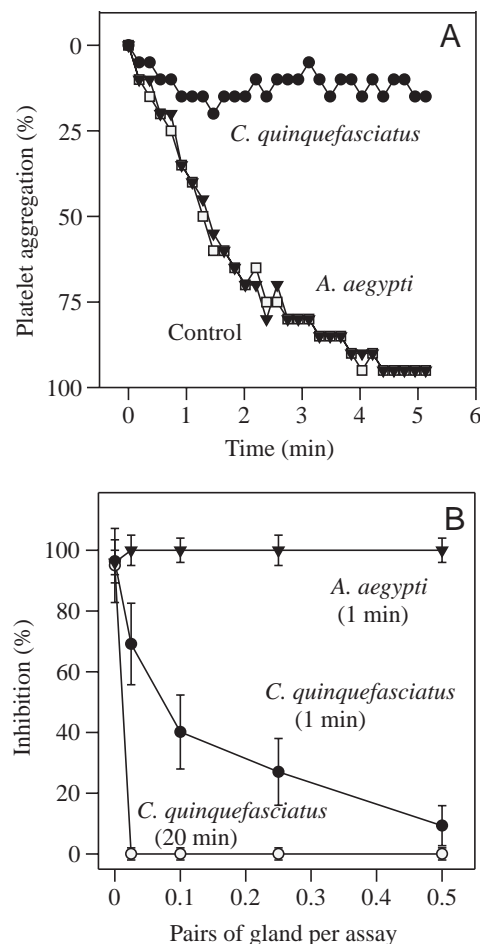


Fig. 2. Effect of mosquito salivary gland homogenates (SGH) on PAF-induced platelet aggregation of human platelets. (A) Platelet aggregation tracings in the absence (control, open squares) or presence of 0.5 pairs of homogenised salivary glands of *Aedes aegypti* (filled triangles) and *Culex quinquefasciatus* (circles). (B) Effect of the amount of *Culex quinquefasciatus* SGH preincubated with PAF for 1 min (filled circles) or 20 min (open circles) and *Aedes aegypti* (filled triangles) preincubated with PAF for 1 min, on PAF-induced platelet aggregation. Inhibition of the aggregation was estimated at 5 min after start of the assay. Values are means \pm S.E.M. of three determinations. For more details, see Materials and methods.

Characterization of a phospholipase C activity from *Culex quinquefasciatus* saliva acting on PAF

To investigate whether salivary homogenates could be destroying PAF, this substance was incubated with *Culex quinquefasciatus* SGH in a volatile buffer; at various time intervals, samples were diluted and injected directly into the mass spectrometer. At time zero of incubation, predominant masses of 524.4 and 546.4 were seen, consistent with the protonated and sodium ion masses of PAF (Jensen et al., 1987; Weintraub et al., 1991) (Fig. 3A). This is consistent with the strong sodium adduct previously found, even when the PAF standard was thoroughly washed and re-extracted into organic

solvent (Weintraub et al., 1991). As time progressed, the combined amounts of the masses 524.4 plus 546.4 decreased and a prominent mass at 739.0 appeared, together with masses at 341.3, 359.2, 376.1, 381.4 and 463.1 (Fig. 3B–H). The masses at 359.2, 376.1 and 381.3 are consistent with the protonated, ammonium and sodium adducts, respectively, of the action of a phospholipase C enzyme on PAF, which would remove the phosphorylcholine moiety (Fig. 1). The 341.3 mass is consistent with the loss of one water molecule from the 359.2 mass, a common occurrence in mass spectrometry measurements. The 739 mass is consistent with the sodium adduct of a dimer of the compound with mass 358.2 ($2M+Na^+$). The minor mass at 463.1, having a mass of 81.8 larger than the 381.4, is consistent with the phosphorylated 381.4 mass ($H_3PO_3=82$). Addition of Ca^{2+} or Mg^{2+} ($100 \mu\text{mol l}^{-1}$) to the reaction mixture did not alter the results (not shown).

To confirm that the majority of the observed masses could result from a phospholipase C action on PAF, we determined whether authentic 1-*O*-hexadecyl-2-acetyl-rac-glycerol (the proper stereoisomer was not commercially available) produced a mass spectrum with a base peak at 738.9 m/z as well as prominent peaks at 359.2 and 376.1. These peaks were indeed observed (Fig. 4). The observed dimer ($2M+Na^+$) formation has been reported for other lipidic compounds (Jensen et al., 1987; Weintraub et al., 1991). The minor mass at 463.1 is also observed in the mass spectrum of 1-*O*-hexadecyl-2-acetyl-rac-glycerol, indicating that it may be formed during electrospray. We thus conclude that there is phospholipase C activity in the SGH of *Culex quinquefasciatus* that is transforming PAF into the corresponding diglyceride.

Substrate specificity

A preliminary analysis of the substrate specificity of the *C. quinquefasciatus* salivary phospholipase C was undertaken. The enzyme was poorly active against lyso-PAF (the product of the enzyme PAF-acetylhydrolase, EC 3.1.1.47, on PAF) or against the enantio-PAF isomer (Fig. 5). When tested with other phosphatidylcholine substrates having different lengths of the acyl groups, the activity was also poor (Fig. 5). No activity was observed when sphingomyelin was used as a substrate (not shown). These results indicate that the salivary phospholipase C here described is highly specific for PAF.

Optimum pH

The enzyme activity had an optimum pH between 7 and 8 (Fig. 6), indicating it is fully active at the physiological extracellular pH.

Chromatographic estimation of molecular mass of *Culex quinquefasciatus* salivary PAF phosphorylcholine hydrolase

To estimate the molecular mass of the salivary phospholipase C active on PAF, SGH from 20 pairs of *Culex quinquefasciatus* salivary glands were applied to a molecular sieving column. The activity, as determined by the decrease in PAF as well as increase in the product and inhibition of PAF-

induced platelet aggregation, eluted at 22–23 min, indicating an apparent molecular mass of between 40 and 50 kDa (Fig. 7).

Identification of PAF phosphorylcholine hydrolase activity in *Culex quinquefasciatus* saliva

The salivary PAF-hydrolyzing activity of *Culex quinquefasciatus* could be a secreted enzyme playing a role in modifying the host site where blood-feeding occurs. Alternatively, it could be a housekeeping enzyme with some other function. To investigate this issue, we incubated 15 µl of ammonium acetate buffer containing 0.1 mmol l⁻¹ PAF with 15 µl of *C. quinquefasciatus* salivary secretion, containing the secretions from six mosquitoes. When this mixture was incubated for 1 h, >95 % of the masses associated with PAF (524–525 plus 546–547) disappeared, while the masses associated with the diacylglycerol were produced (result not shown). A second experiment was performed in which the same amount of salivary secretion was mixed with 50 µl of 0.1 mmol l⁻¹ PAF in ammonium acetate buffer. At different time intervals, 5 µl samples of the mixture were removed, diluted tenfold with 80 % methanol plus 0.1 % acetic acid and analyzed by mass spectrometry. The results showed a progressive decrease in levels of PAF (Fig. 8A) with an increase in the diacylglycerol product (Fig. 8B,C), indicating secretion of the salivary phospholipase C into saliva.

To determine whether other mosquito species have salivary phospholipase C active against PAF, we incubated SGH of *Aedes aegypti* and *Anopheles gambiae* with PAF and investigated the extent of the reaction by mass spectrometry. No hydrolyzing activity could be detected using SGH from either mosquito species (results not shown).

Discussion

In this paper, we report a phospholipase C with a unique specificity for PAF. PAF catabolism in mammals is achieved primarily by the action of PAF acetylhydrolase (Fig. 1), a PAF-

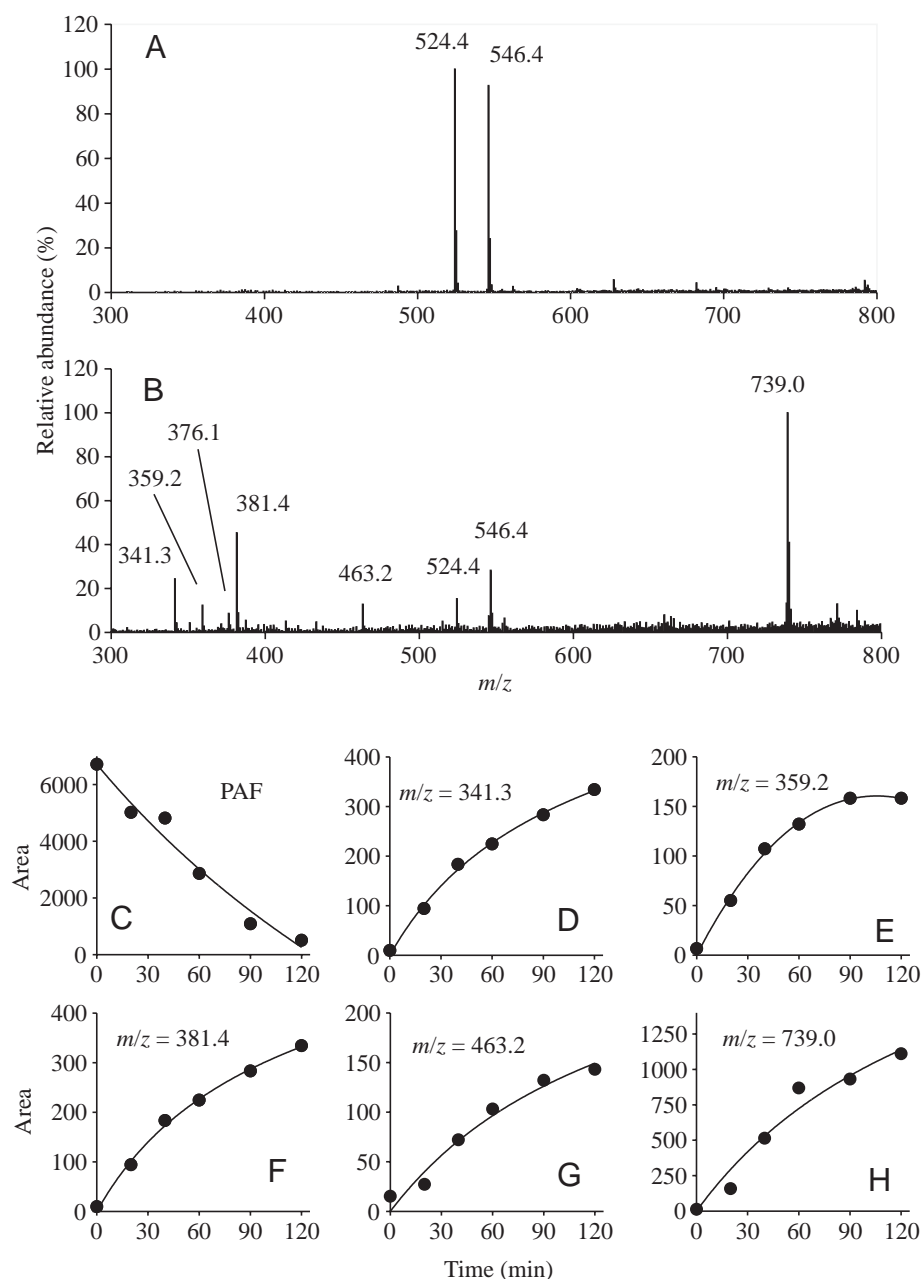


Fig. 3. Hydrolysis of PAF by *Culex quinquefasciatus* SGH. One pair of homogenised salivary glands was incubated with 50 µl of 100 µmol l⁻¹ PAF in 0.1 mol l⁻¹ ammonium acetate, pH 7.2. At the indicated times, 5 µl of the reaction mixture was diluted tenfold with 80 % methanol plus 0.1 % acetic acid and injected into the mass spectrometer. (A) Mass spectrum done immediately after mixing the substrate with the homogenate. (B) Mass spectrum at 120 min. (C–H) Disappearance of PAF-associated masses and appearance of indicated product masses. A control sample without salivary homogenates incubated for 2 h had an identical spectrum to A. The line on C was the best non-linear fit of a two-parameter hyperbolic decay curve. The lines on D–H were the best non-linear fit for a two-parameter hyperbolic curve.

specific enzyme displaying phospholipase A₂ activity (Derewenda and Ho, 1999). To our knowledge, no other phospholipase C enzyme with the unique specificity for PAF has been described. We propose to name this activity PAF phosphorylcholine-hydrolase. The enzyme has a molecular

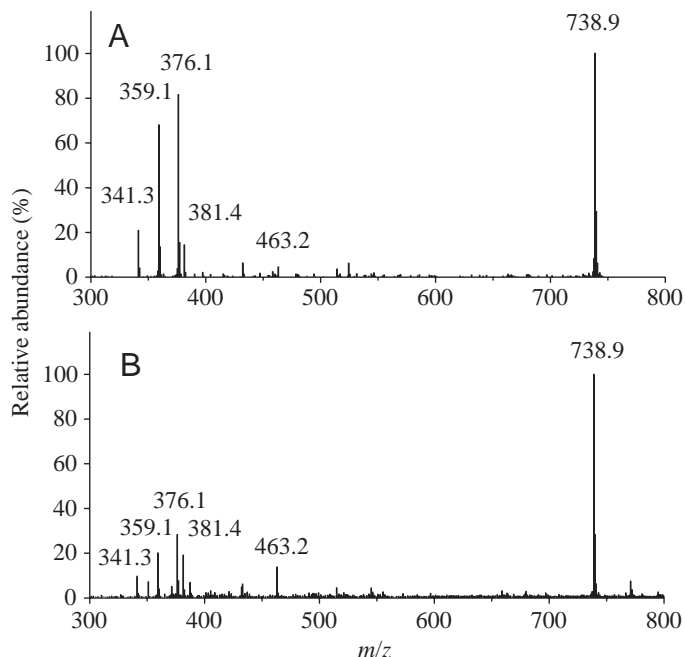


Fig. 4. Mass spectrum of the product of the hydrolysis of PAF by *Culex quinquefasciatus* SGH matches the spectrum of 1-*O*-hexadecyl-2-acetyl-rac-glycerol. (A) Product of the incubation of PAF ($100\mu\text{mol l}^{-1}$ final concentration) with SGH from one pair of glands ($25\mu\text{l}$ final volume) for 2 h, after which $5\mu\text{l}$ were diluted with acid 80% methanol and analyzed by mass spectrometry, as described in Fig. 2. (B) Mass spectrum of authentic of 1-*O*-hexadecyl-2-acetyl-rac-glycerol.

mass of 40–50 kDa, in the range of other phospholipase C enzymes (35–60 kDa), as indicated by the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics (<http://expasy.cbr.nrc.ca/enzyme>). The enzyme is secreted in the insect saliva and has a neutral optimal pH, indicating its adaptation to an extracellular environment.

To our knowledge, this is also the first description of a phospholipase C in the saliva of any blood-sucking arthropod, although phospholipase A₂ and PAF acetylhydrolase have been described in the salivary glands of ticks and fleas, respectively (Bowman et al., 1997; Cheeseman et al., 2001). While abundant in the saliva of the human-feeding *Culex quinquefasciatus* mosquito, such salivary activity is lacking in two other anthropophilic mosquitoes (*Aedes aegypti* and *Anopheles gambiae*). PAF is an important physiologic agonist in aggregation of blood of some mammals such as the rabbit (Cazenave et al., 1979), and also for bird thrombocytes (Cox, 1985). Indeed, PAF is considered a 'strong' agonist in rabbit platelets, where it induces platelet activation at picomolar concentrations, independently of other mediators (Braquet et al., 1984). PAF also participates as a pro-aggregatory molecule after platelet stimulation with collagen, thrombin and other inducers (Braquet et al., 1987). In contrast, the role of PAF as an aggregating agent in human platelets is less remarkable. In fact, platelet aggregation by PAF in these cell types requires

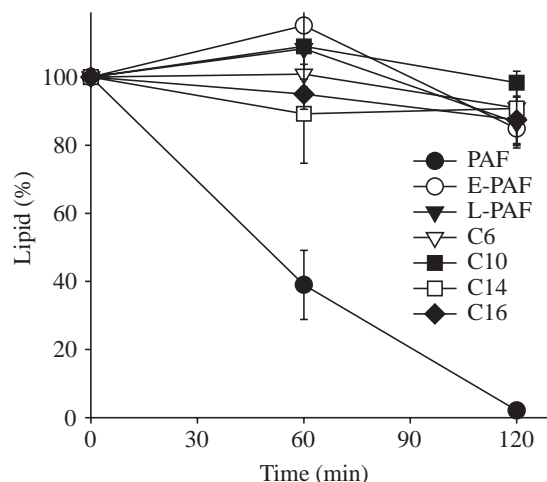


Fig. 5. Specificity for PAF of the salivary phospholipase C activity of *Culex quinquefasciatus* SGH. PAF, enantio-PAF (E-PAF), lyso-PAF (L-PAF) and phosphatidylcholine with acyl chains of the indicated carbon lengths (C6, C10, C14 and C16) served as substrates. Assay conditions were as given in Fig. 2. Values are means \pm S.E.M. of three determinations.

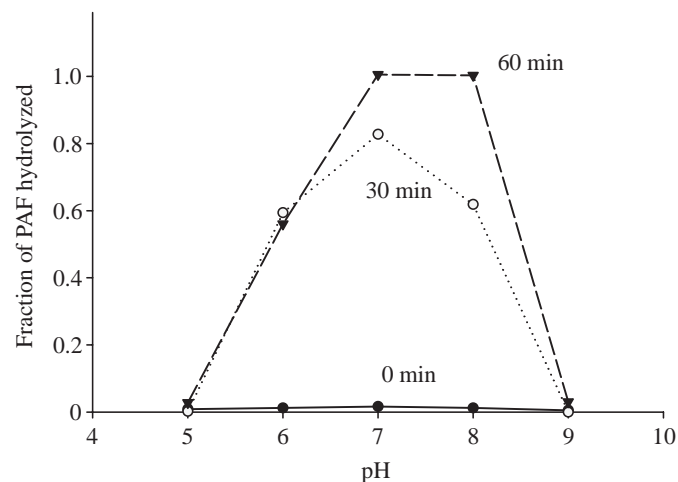


Fig. 6. pH dependence of the PAF phosphorylcholine hydrolase activity of *Culex quinquefasciatus* SGH. The amount of PAF hydrolyzed at the indicated times (0, 30 and 60 min) of incubation and pH are indicated. Other conditions are as in Fig. 2, except that the ammonium acetate buffer was adjusted with acetic acid or ammonia to the indicated pH.

the concomitant production of TXA₂ or secretion of ADP (Marcus et al., 1981; Chesney et al., 1982; Rao et al., 1982; Klopogge et al., 1983; Rao et al., 1984). In addition, the aggregatory response of human platelets activated by other ligands is unaffected by specific PAF receptor antagonists (Ostermann et al., 1990). Moreover, most (87%) of PAF released by collagen and thrombin-stimulated platelets are not in the soluble form, but associated with pro-coagulant microparticles (Iwamoto et al., 1996). However, the participation of PAF in platelet physiology turns out to be

relevant in the context of platelet-neutrophil interactions (Li et al., 2000). At the site of injury where thrombogenic molecules are exposed (such as collagen), platelet-released adenine nucleotides may promote neutrophil activation, leading to the release of superoxide anion (O_2^-), elastase (Si-Tahar et al., 1997), cathepsin G (Si-Tahar et al., 1996) and PAF itself which, in turn, activates platelets and endothelial cells (Braquet et al., 1984). Accordingly, the primary role of PAF in humans has been characterized as a mediator of intercellular interactions (Prescott et al., 2000). We suggest that the presence of a *Culex* PAF-hydrolyzing enzyme, together with other anti-hemostatic *Culex* salivary molecules, is likely to

promote a negative modulation of the inflammatory response in the microenvironments where such interactions occur.

While the genus *Aedes* is mostly associated with primates and *Anopheles* with mammals (Horsfall, 1955), all close relatives of *Culex quinquefasciatus* are bird feeders, indicating that this mosquito is only recently associated with humans (Chevillon et al., 1995). Indeed, *Culex quinquefasciatus* has less salivary antiplatelet activity than either *Aedes aegypti* or *Anopheles albimanus* and takes longer to find blood on mammals than on chicks (Ribeiro, 2000). *Culex quinquefasciatus* salivary PAF phosphorylcholine hydrolase

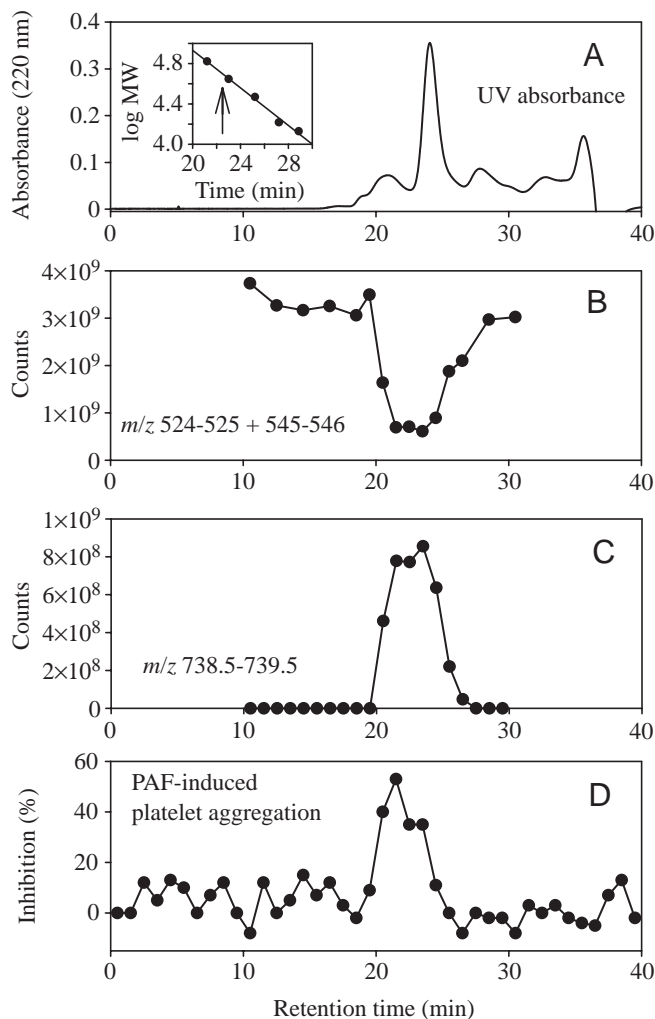


Fig. 7. Molecular sieving chromatography of 25 pairs of homogenised salivary glands from *Culex quinquefasciatus*. (A) Ultraviolet absorbance of the eluate at 220 nm. Inset: Retention time of the enzymatic activity in relation to molecular mass (MW) standards. For more details, see Materials and methods. (B) PAF phosphorylcholine hydrolase activity as determined by disappearance of the masses associated with PAF. (C) PAF phosphorylcholine hydrolase activity as determined by appearance of the mass associated with the diacylglycerol product. (D) Action of the eluate fractions on PAF-induced platelet aggregation.

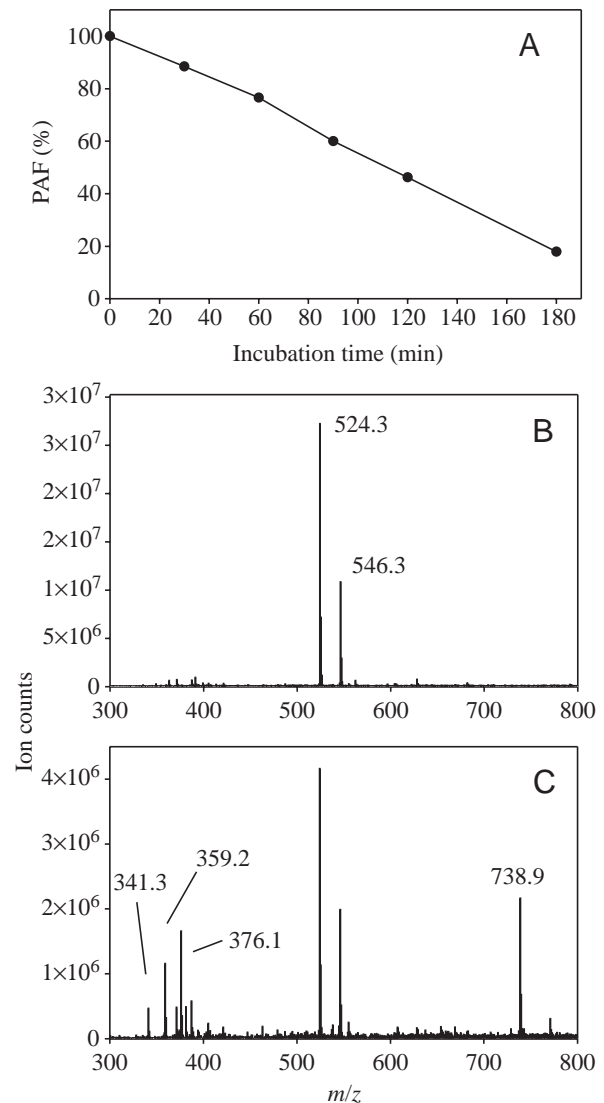


Fig. 8. PAF phosphorylcholine hydrolase activity in saliva of adult female *Culex quinquefasciatus*. Serotonin-induced saliva from six mosquitoes was incubated with 50 μ l PAF (other conditions as for Fig. 2). At various time intervals, a sample was examined by mass spectrometry. (A) Time course of PAF-associated masses (524.3 and 543.3). (B) Mass spectrum obtained immediately after mixing the reaction mixture with the mosquito saliva. (C) Spectrum after incubation for 3 h. A control sample without saliva and incubated for 3 h had a spectrum identical to that in A.

may thus reflect the adaptation to a previous non-human host where PAF is an important physiological agonist of platelet or thrombocyte aggregation.

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