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Vasotab, a vasoactive peptide from horse fly *Hybomitra bimaculata* (Diptera, Tabanidae) salivary glands

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Summary

Horse flies feed from superficial haematomas and probably rely heavily on the pharmacological properties of their saliva to find blood. Here we describe the first evidence of vasodilators in horse fly *Hybomitra bimaculata* (Diptera, Tabanidae) salivary gland extract and clone and express one of the active peptides (termed vasotab). Physiological tests using crude salivary gland extracts and reverse-phase HPLC fractions demonstrated positive inotropism in isolated rat hearts, vasodilatation of coronary and peripheral vessels, and Na, K-ATPase inhibition. One of the vasoactive fractions was analysed by N-terminal Edman degradation and a 47-amino-acid sequence obtained. A full-length cDNA encoding the peptide was cloned from a phage library using degenerate primer PCR and the peptide expressed in insect cells. A 20-amino-acid signal sequence precedes the mature 56-amino-acid vasotab peptide, which is a member of the Kazal-type protease inhibitor family. The peptide has a unique 7-amino-acid insertion between the third and fourth cysteine residues. The recombinant peptide prolonged the action potential and caused positive inotropism of isolated rat heart myocytes, and may be an ion channel modulator.

Key words: vasodilator, salivary gland, horse fly, *Hybomitra bimaculata.*

Introduction

Blood-feeding arthropods have developed effective mechanisms to inhibit haemostatic responses (blood clotting, platelet aggregation, vasoconstriction) of their vertebrate hosts. Antihaemostatic factors are contained in saliva and injected into the vertebrate hosts during blood-feeding (Ribeiro, 1995). The ability to feed on blood has evolved independently many times, consequently a wide variety of substances are responsible for antihaemostatic effects (Law et al., 1992; Champagne and Valenzuela, 1996).

Platelet aggregation and vasoconstriction are key haemostatic responses, particularly in small wounds. Aggregating platelets form a clump at the site of vessel injury, which is consolidated by the fibrin network. Venules and arterioles are surrounded by smooth muscle, and the release of serotonin and thromboxane A_2 by platelets leads to vessel contraction, further tightening the contact between the vessel wall and platelet plug. Every haematophagous arthropod examined to date has been found to secrete some type of vasodilator. Ticks use prostaglandins PGE_2, $PGF_{2\alpha}$ and PGI_2 (Bowman et al., 1996), triatomine bugs secrete nitrophorins (Ribeiro et al., 1990, 1993; Ribeiro and Walker, 1994), sand flies *Lutzomyia longipalpus* produce the peptide maxadilan (Ribeiro et al., 1989; Lerner et al., 1991), black flies *Simulium vittatum* produce a peptidic erythema-inducing factor (Cupp et al., 1994), *Aedes* mosquitoes secrete tachykinin peptides (Ribeiro, 1992; Champagne and Ribeiro, 1994), and *Anopheles* mosquitoes use catechol oxidase/peroxidase (Ribeiro and Nussenzveig, 1993). Vasodilators help to maintain blood flow during feeding and increase the probability of finding blood by increasing the size of the target venules and arterioles (Champagne, 1994; Ribeiro, 1995). The vasodilators in blood-feeding arthropod saliva are generally thought to cause the erythema (reflecting increased capillary permeability) observed following the bite of an arthropod. Vasodilation is most significant in vectors that have short feeding stylets (e.g. sand flies) or chewing mouthparts (e.g. biting flies).

Little information is available on antihaemostatic substances in horse fly salivary glands, although anticoagulant activity has been reported (Kazimirova et al., 2001). Female horse flies (Diptera, Tabanidae) require substantial amounts of blood (up to 0.5 ml) for egg production. More than one feeding episode is needed for most tabanids to become fully engorged. Hollander and Wright (1980) estimated that tabanids needed approximately ten landings on a host to complete one blood meal. Tabanids feed predominantly on large mammals. The females of three genera, *Chrysops, Haematopota* and *Tabanus*, commonly attack humans.

Horse flies have chewing and biting mouthparts, which restrict their feeding to superficial haematomas that form in the lacerated tissue. They probably rely heavily on the pharmacological properties of their saliva to find blood. Salivary gland extracts of the deerfly (genus *Chrysops*) contain a potent inhibitor of platelet aggregation (Grevelink et al., 1993). We recently reported that horse fly saliva or salivary glands extracts contain potent vasodilator activity (Rajská et al., 2003). Here we describe the isolation, cloning and expression of an active peptide from the horse fly *Hybomitra bimaculata*.

Materials and methods

Horse fly collection

Horse flies *Hybomitra bimaculata* Macquart 1826 were collected using Manitoba traps with CO_2 at selected sites of south-western and western Slovakia in 1999. Collections were performed between 09:00 h and 17:00 h during optimal weather (sunny, 24–28°C, no wind) in May. Flies (1300 in total) were transported to the laboratory alive and processed immediately.

Salivary gland sample preparation and purification

Horse flies were immobilized by placing them at 4°C and then dissected under a microscope. Salivary glands, 100 pairs (Fig. 1), were placed in 200 μl ice-cold PBS (0.01 mol l^{-1} phosphate buffer and 0.15 mol l^{-1} NaCl, pH 7.2), heated at 80°C for 5 min, then homogenized and centrifuged at 2500 ***g*** for 10 min. The supernatant, termed salivary gland extract (SGE), was stored at –70°C or filtered (Millex-LG, Millipore, Bedford, MA, USA; 0.20 μm, 4 mm syringe unit) and processed by reverse-phase high performance liquid chromatography (RP-HPLC).

RP-HPLC

SGE (300 pairs) were dried in Savant Instruments Speed-Vac (Holbrook, NY, USA) and then diluted in 500 μl of 10% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA; buffer A) and loaded onto a Beckman Instruments (Fullerton, CA, USA) 126/168 DAD HPLC system. In the first purification, SGE was applied to a Vydac C-4 (Hesperia, CA, USA), 250 mm×4.6 mm i.d., 5 μm particle size column, UV monitored at 210 nm and 220 nm. A gradient of 10%–100% ACN with 0.1% TFA, flow rate 1 ml min^{-1}, 1% ACN min^{-1} increments, was used (Fig. 2A). Active fractions were then purified using a Beckman Ultrasphere C-18, 250 mm×4.6 mm i.d., 5 μm particle size column and a gradient of 10%–40% ACN with 0.1% TFA, flow rate 1 ml min^{-1}, 0.5% ACN min^{-1} increments, monitored at 210 and 220 nm (Fig. 2B). The final purification step used a Vydac C 18, 250 mm×4.6 mm i.d., 5 μm particle size column, and the same conditions as the second purification (Fig. 2C). Fractions were collected and dried by Speed-Vac and N-terminal sequencing by Edman degradation was performed by Eurosequence (Groningen, The Netherlands).

Fig. 1. Head showing salivary glands of horse fly *Hybomitra bimaculata.* Bar, 1 mm.

Mass spectrum analysis

Mass spectrum was measured on matrix-assisted laser desorption/ionisation reflectron time-of-flight MALDI-TOF mass spectrometer BIFLEX (Bruker-Franzen, Bremen, Germany) equipped with a nitrogen laser (337 nm) and gridless delayed extraction ion source.

Isolated rat femoral artery preparation

Wistar rats (12 weeks old, both sexes) were killed humanely and two 10 mm long segments of femoral artery placed in a Krebs–Ringer bicarbonate solution comprising 118 mmol l^{-1}

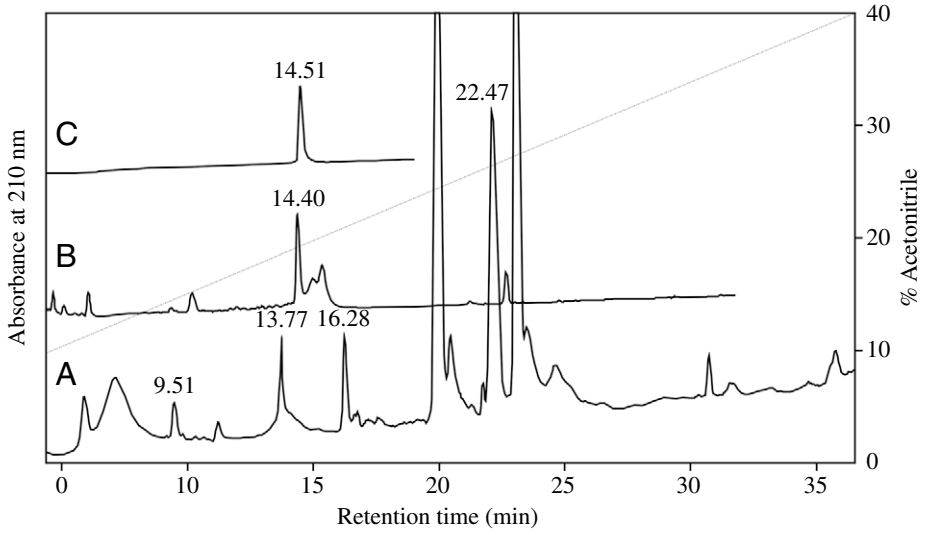

Fig. 2. Reverse-phase HPLC of crude salivary gland extract (SGE) of horse fly *H. bimaculata.* The peaks containing vasodilator activity are indicated by their retention times. The purification procedures are described in the text. (A) Crude SGE purified on a Vydac C4 column; (B) active fraction from A with retention time 13.77 min., second purification on the Beckman Ultrasphere C-18 column; (C) active fraction from B with retention time 14.40 min, third purification on the Vydac C18 column.

NaCl, 5 mmol l^{-1} KCl, 25 mmol l^{-1} $NaHCO_3$, 1.2 mmol l^{-1} $MgSO_4.7H_2O$, 1.2 mmol l^{-1} KH_2PO_4, 2.5 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} EDTA, 1.1 mmol l^{-1} ascorbic acid, and 11 mmol l^{-1} glucose. Endothelium was removed from one half of each segment by gently rubbing the intimal surface. The arteries were cleaned of adherent connective tissue and cut into 3 mm ring segments. Two stainless steel wires were passed through the lumen and the rings were mounted on a myograph capable of measuring the isometric wall tension in a bath of Krebs–Ringer solution at 37°C, pH 7.4, gassed with 95% O_2 and 5% CO_2. The effectiveness of endothelium removal was demonstrated by the failure of acetylcholine (5×10^{-6} mol l^{-1}) to relax a contraction induced by phenylephrine (5×10^{-6} mol l^{-1}). The plateau of the contractile response induced by phenylephrine (5×10^{-6} mol l^{-1}) was taken as a measure of 100% contraction.

Isolated heart preparation and perfusion technique

Rat hearts were excised, placed in ice-cold perfusion buffer, cannulated *via* the aorta and perfused in the Langendorff (1895) mode at a constant perfusion pressure of 70 mmHg (1 mmHg=133.3 Pa) and at 37°C. Perfusion solution, filtered through a 5 μm porosity filter (Millipore), was a Krebs–Henseleit buffer gassed with 95% O_2 and 5% CO_2 (pH 7.4) containing (in mmol l^{-1}): NaCl 118.0, KCl 4.7, $MgSO_4$ 1.66, $CaCl_2$ 2.52, $NaHCO_3$ 24.88, KH_2PO_4 1.18 and glucose 5.55.

An epicardial electrogram (EG) was registered by two stainless steel electrodes attached to the apex of the heart and the aortic cannula and continuously recorded (Miograph ELEMA-Siemens, Solna Sweden). Heart rate (HR) was calculated from the EG. Coronary flow (CF) was measured by timed (10s intervals) collection of coronary effluent weighed on an electronic balance (AND HF 200 G, A&D Company Ltd, Bradford, MA, USA). Left ventricular pressure (LVP) was measured by means of a latex water-filled balloon inserted into the left ventricle *via* the left atrium (adjusted to obtain end-diastolic pressure of 5–10 mmHg) and connected to a pressure transducer (P23 Db Pressure Transducer, Gould Statham Instruments, Inc., El Segundo, CA, USA)

Crude SGE of *H. bimaculata* was investigated. SGE from 8–20 salivary glands made up to 200 μl was injected through a syringe directly into the aortic cannula with continuous measurement of CF, LVP, EG and HR.

Effect on sarcolemmal Na,K-ATPase

Hearts from Wistar Kyoto rats were quickly excised and immediately frozen in liquid nitrogen. Cardiac sarcolemma was prepared from pooled samples of two hearts by the hypotonic shock-NaI treatment method as described previously (Vrbjar et al., 1984). The protein content was assayed by the procedure of Lowry et al. (1951).

The substrate kinetics of Na,K-ATPase were estimated measuring the hydrolysis of ATP by 30 μg sarcolemmal proteins at 37°C in the presence of increasing concentrations of ATP (0.08–4.0 mmol l^{-1}). Assays were undertaken in 0.5 ml of medium containing 50 mmol l^{-1} imidazole (pH 7.4), 4 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} KCl and 100 mmol l^{-1} NaCl. After 15 min of preincubation in the substrate-free medium, the reaction was started by addition of ATP; 15 min later it was terminated by 1 ml of 12% w/v trichloroacetic acid. ATP hydrolysis was shown to be linear throughout the ATP concentration range for up to 20 min. The inorganic phosphorus liberated was determined according to Taussky and Shorr (1953). In order to determine Na,K-ATPase activity, ATP hydrolysis that occurred in the presence of Mg^{2+} only was subtracted. From each sarcolemmal preparation, three individual K_m and V_{max} values were obtained. The influence of crude SGE from *H. bimaculata* on the function of the Na,K-ATPase was tested in *vitro* by addition of 3 μg of SGE-proteins to 30 μg of sarcolemmal proteins. Kinetic parameters were evaluated by direct non-linear regression. All results were expressed as mean ± s.e.m. Significant differences between groups were determined by analysis of variance (ANOVA), Bonferroni test.

Construction of H. bimaculata cDNA library

mRNA was isolated from 100 pairs of *H. bimaculata* salivary glands using the FastTrack™ 2.0 mRNA isolation kit (Invitrogen, Paisley, UK) and cDNA synthesised using a cDNA synthesis kit (Stratagene, La Jolla, CA, USA; Catalog no. 200401-5). The cDNA was ligated into the Stratagene UniZAP XR phage vector (Catalog No. 237211) and packaged with Gigapack® III Gold packaging extract (Stratagene).

Cloning cDNA of vasotab

The N-terminal sequence of the HPLC fraction collected at 14.51 min (designated vasotab) was used to design degenerate primers to amplify the cDNA encoding the peptide. Each 100 μl polymerase chain reaction (PCR) comprised 3 μl cDNA library, 3 μl 10 mmol l^{-1} dNTPs, 2 μl T7 and 4 μl degenerate primer (from stocks of 0.5 μg ml^{-1}), 10 μl 10× REDTaq PCR reaction buffer, 3 μl REDTaq DNA polymerase and 75 μl dH_2O. Thermal cycling parameters were 1× 94°C, 4 min; 30× 94°C, 1 min; 48.5°C, 45 s; 72°C, 90 s; and 1× 72°C, 5 min. Five PCR products were purified from agarose gels using a Qiaex II gel extraction kit (Qiagen, Hamburg, Germany) and sequenced with an ABI PRISM™ dye terminator cycle sequencing ready reaction kit and ABI sequencer (Perkin Elmer, Wellesley, MA, USA). Conceptual translation of one PCR product, derived using primer HF2 (5′ GAR TGY CCN MGN ATN TGY AC 3′) with T7, gave an exact match with the N-terminal sequence of vasotab. The sequence extended beyond the stop codon of the cDNA encoding the peptide. A primer (HR1 5′ AAT ACA ACA TAT TCA AGT GG 3′) matching the region beyond the stop codon was used with the T3 primer to obtain the 5′ end of the cDNA. The cloned and sequenced PCR product revealed a full-length cDNA encoding vasotab.

Sequence analysis

Analyses were carried out using the ExPASy (Expert Protein Analysis System, Basel, Switzerland) proteomics server of the Swiss Institute of Bioinformatics (http://expasy.hcuge.ch/). Sequences were compared with the GenBank non-redundant (NR) protein database using the BlastX program (Altschul et al., 1997) and searched against the Pfam protein family database (Bateman et al., 2000). Multiple sequence alignments were performed with Clustal X (Jeanmougin et al., 1998).

BAC-BAC® Baculovirus expression and purification of vasotab

The vasotab coding region was PCR-amplified (1× 94°C, 4 min; 20× 94°C, 1 min; 48.5°C, 45 s; 72°C, 90 s; and 1× 72°C, 5 min) using the forward primer HF6 (5′ GTA *CGG ATC C*AT GAA ATT TGC CTT GTT CAG T 3′) and reverse primer HR3 (5′ CAT G*CT GCA G***TT A**GT GAT GGT GAT GGT GAT GAC C**CT TGC ACT CGC CAT CATG 3′). HF6 matches the signal sequence of vasotab and has a *Bam*HI restriction enzyme site (in italic type). HR3 matches the sequence encoding the carboxy-terminal end of the protein and includes a codon for a glycine followed by six histidine residues (underlined) then a stop codon (bold) and *Pst*I restriction enzyme site (in italic type). The product was ligated between the *Bam*HI and *Pst*I in the pFastBac1 plasmid (Gibco-BRL®) and the sequence of the construct verified by sequencing with primers pFastBac1 primers PFBR and PFBF. Transformation of the DH10α bacteria carrying the baculovirus DNA, generation of high titre stock and expression in Sf9 cells were performed in accordance with the instructions accompanying the BAC-BAC® expression system (Gibco-BRL®).

For purification, 60 h old cultures were centrifuged (3000 ***g***, 10 min) and 30% (w/v) polyethylene glycol (3350 kDa) added to the supernatant which was stirred for 1 h. After centrifugation (8000 ***g***, 20 min) the protein pellet was resuspended in 20 ml binding buffer (50 mmol l^{-1} Na_2HPO_4/NaH_2PO_4 pH 8, 500 mmol l^{-1} NaCl, 10% glycerol) per gram of wet paste. TALON Metal Affinity Resin was used to purify the histidine tagged peptide in accordance with the manufacturers' instructions (Clontech, Mountain View, CA, USA). Bound proteins were eluted with imidazole and concentrated using Centricon 3 centrifugal filter devices (Amicon, Millipore). Vasotab was purified further by cation exchange chromatography. Concentrated vasotab at 200-fold dilution in running buffer (50 mmol l^{-1} Na_2HPO_4/NaH_2PO_4, pH 6.8) was applied to the SP Sepharose column (Pharmacia, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and eluted using a 30 min 0–0.75 mol l^{-1} NaCl gradient at a flow rate of 0.5 ml min^{-1}.

Measurement of the whole-cell L-type calcium current (I_{Ca})

To measure whole-cell L-type calcium current (I_{Ca}) the pipette was filled with a solution containing (mol l^{-1}): CsCl, 120; $MgCl_2$, 1.0; Mg-ATP, 4.0; disodium-phosphocreatine, 5.0; EGTA, 10; Hepes, 5 (at pH 7.2, adjusted with CsOH).

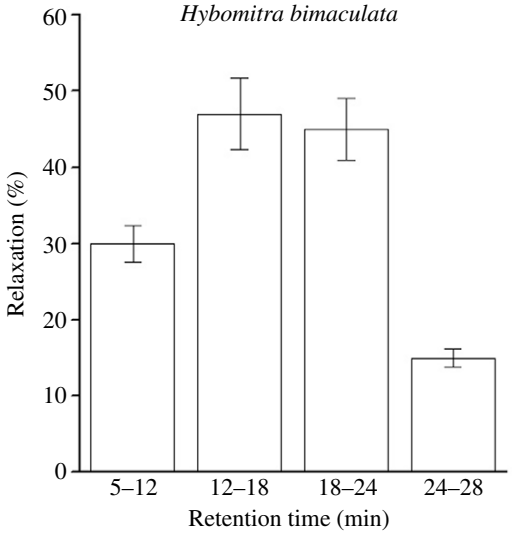

Fig. 3. Vasodilating activity of protein HPLC fractions (purification A, Fig. 1) in the retention time range 5–28 min of *H. bimaculata* SGE on rat arterial rings without endothelium.

Cells were superfused with a solution containing (mol l^{-1}): tetraethylammonium-Cl, 140; $CaCl_2$, 1.8; $MgCl_2$, 1.0; glucose, 10; Hepes, 10; 4-aminopyridine 3 (at pH 7.4, adjusted with tetraethylammonium hydroxide). I_{Ca} was elicited by applying 200 ms depolarizing voltage pulses from a holding potential of –45 mV up to –5 mV (Mészáros et al., 1997). The interval between pulses was 5 s, and the superfusate temperature was 35±1°C.

Results

Vasodilating activity of SGE and identification of active fractions

Application of 1.0 μl (one salivary gland equivalent; 3.6 μg of protein) *H. bimaculata* SGE induced 29±2.0% relaxation of rat femoral artery with intact endothelium. The same amount of SGE induced even greater relaxation of artery (45±3.2%; P<0.05, N=5) from which endothelium had been removed. Subsequent experiments therefore used endothelium denuded rings.

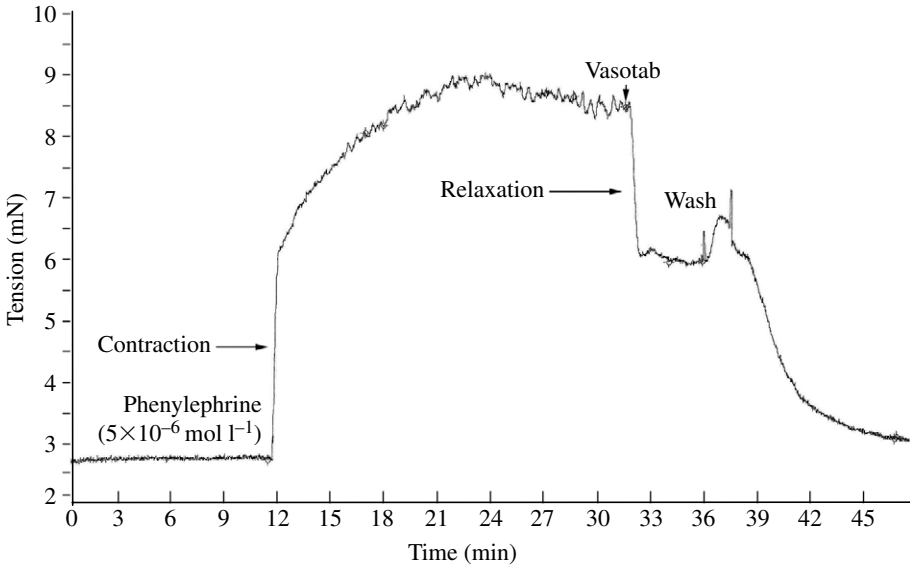

Fig. 4. Relaxation of contracted rat femoral artery induced by purified vasotab EV048, corresponding to the peak at 14.51 min (purification C, Fig. 2). The rat femoral artery was contracted with phenylephrine (5×10^{-6} mol l^{-1}).

The vasodilating responses of arterial rings induced by protein HPLC fractions (Fig. 2A) of *H. bimaculata* SGE obtained in the retention time range 5–28 min were compared (Fig. 3). Four active fractions were identified. The strongest vasodilation (47±2.5%) was induced by a fraction with a retention time of 13.77 min, EV048 (Fig. 4); 45±2.0% relaxation was measured in the peak with retention time 16.28 min. Less activity was obtained with fractions having retention times of 9.51 min (30±3.0%) and 22.47 min (15±1.0%; N=5).

Effect of SGE on isolated rat heart and sarcolemmal Na, K-ATPase

H. bimaculata SGE increased coronary flow and left ventricular contractility but had no significant effect on heart rate (Table 1). After application of 10 μl SGE (10 salivary glands) the coronary flow immediately increased from 10.6 to 14.2 ml min^{-1} (Fig. 5). The duration of the vasodilatatory effect was almost 11 min (data not shown). The most potent dose was 100 μl (Table 1).

H. bimaculata SGE caused a significant stimulation of the cardiac sarcolemmal Na,K-ATPase at all ATP concentrations. The relative increase in enzyme activity was higher at lower concentrations of ATP. At 0.08 mmol l^{-1} ATP, the stimulation represented 47%. With increasing concentrations of ATP, the effect decreased stepwise and at 4 mmol l^{-1} ATP the stimulation represented only 16% (Fig. 6A). These changes signal a significant alteration of K_m value, as shown in the Hanes plot (Fig. 6B). More precise evaluation of the data by non-linear regression revealed 3 μg of SGE proteins causes a significant 28% decrease in the K_m value of the Na, K-ATPase (P<0.05) although V_{max} was unchanged (Table 2).

Isolation and primary structure of the cDNA encoding vasotab

The HPLC fraction with retention time of 14.51 min (Fig. 2C), which was derived from the original 13.77 min peak (Fig. 2A), was subjected to N-terminal Edman degradation and yielded a sequence of 47 amino acid residues identical to

Table 1. *Effect of SGE from Hybomitra bimaculata on the isolated perfused rat heart*

	N	Dose SGE (μl)	CF (ml min^{-1})	LVP (mmHg)	HR (beats min^{-1})
Control	10	–	13.8±0.6	102.0±2.7	290.2±5.9
H. bimaculata	5	50	19.2±2.4	122.4±3.1	292.1±4.8
	5	100	20.7±3.3	145.6±4.7	274.2±5.2
	5	150	19.6±3.8	142.8±4.2	304.5±6.3

SGE, salivary gland extract; CF, coronary flow; LVP, left ventricular pressure; HR, heart rate.
Control, 100 μl PBS.

amino acids 21(D) to 67(Y) of Fig. 7. The full-length peptide corresponding to this partial sequence is termed vasotab.

The full-length cDNA for vasotab encodes a peptide of 76 amino acids (Fig. 7). This includes a 20-amino-acid putative signal peptide that is probably cleaved at VAA–DEC to generate the mature N terminus. The complete peptide has a predicted molecular mass of 8282.4 Da and a theoretical pI of 8.27. The 56-amino-acid mature peptide has a predicted molecular mass of 6146.7 Da and a theoretical pI of 7.78. The mass of the native protein determined by mass spectrometry was 6141 Da. The c. 6 Da difference between predicted and actual may be explained by the loss of 6 protons when the three disulfide bridges are formed. A N-linked glycosylation site is predicted at amino acid 26 (consensus sequence NRTF) of the mature peptide; there are no predicted O-linked sites. The sequence of the mature peptide has similarity with the large family (471 members and growing) of Kazal-type protease inhibitors (Fig. 8), including homology with rhodniin I and II, the Kazal-type inhibitors from *Rhodnius prolixus*, another haematophagous insect species (Friedrich et al., 1993; van de Locht et al., 1995), and with the protease inhibitor from the sea anemone *Anemonia sulcata* that appears to be specific for elastases (Tschesche et al., 1987).

The basic structure of a Kazal-type inhibitor is shown in the Fig. 9. All six cysteines are conserved in mature vasotab although the spacing between cysteine residues within the consensus pattern is unusual: C–x(7)–C–x(13)–F–x(3)–C–x(6)–C. The pattern is due to a sequence inserted between the third and fourth cyteines (PSGGRRS) that does not align with any other Kazal family member (Fig. 8). The second serine within this sequence has a high probability of phosphorylation but the rest of the 7-aminoacid sequence has no similarity to any other motifs in the PROSITE database. Homology modelling was performed with SWISS-MODEL available at ExPASy website (Schwede et al., 2003), and indicates that the insertion probably forms a loop and occurs within, or just before, the region of the peptide that may form the second β-sheet. The highly conserved tyrosine residue (between cysteines 3 and 4, Fig. 8) is a phenylalanine (a conservative

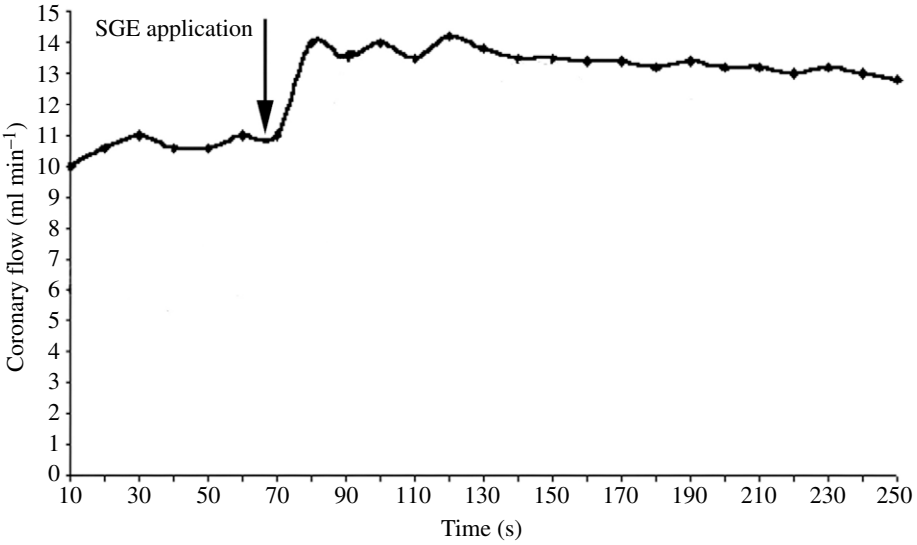

Fig. 5. Coronary flow record after application of *H. bimaculata* SGE (equivalent of 10 salivary glands).

Table 2. *Kinetic parameters of Na,K-ATPase in hearts from rats during activation with increasing concentrations of ATP*

[ATP] activation	V_{max} (μmol P_i mg^{-1} protein h)	K_m (mmol ATP l^{-1})
Control SL	2.71±0.12	0.487±0.058
SL+H.b.	3.05±0.20	0.353±0.048*

Six replicate experiments were performed for control sarcolemma alone (control SL), and sarcolemma with 3 μg of SGE-proteins from *Hybomitra bimaculata* (SL+H.b.).

Values are means ± s.e.m. *P<0.05 as compared to control group.

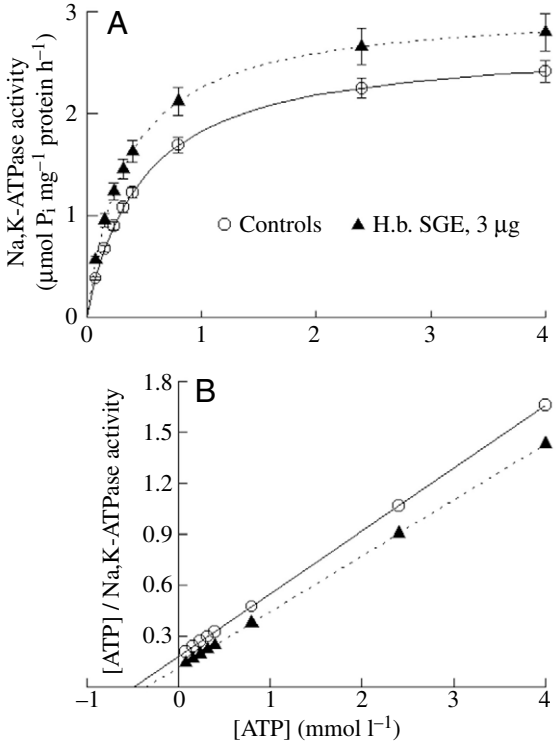

Fig. 6. *In vitro* effect of *H. bimaculata* (H.b.) SGE on cardiac Na,K-ATPase activity. SGE is expressed as total protein concentration. (A) Actual data of representative measurements. Estimation at each concentration was performed in triplicate. (B) Transformation of the data to a Hanes plot.

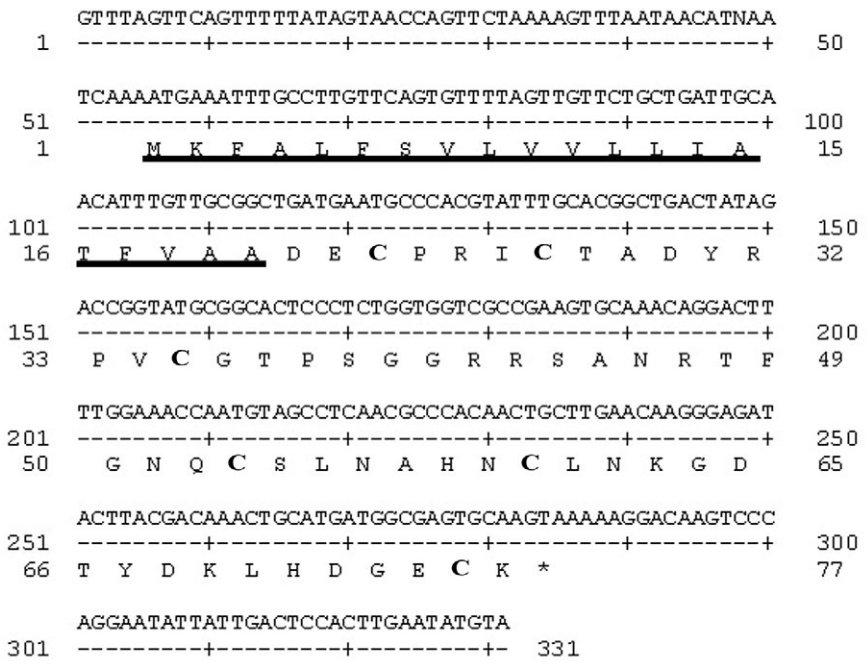

Fig. 7. Primary structure of peptide vasotab. The signal sequence is underlined, cysteine residues are shown in bold type, and the stop codon is indicated by an asterisk.

change) in mature vasotab and the putative active site residue P1 (indicated by the bold # in Fig. 8) is an alanine.

Baculovirus expression of mature vasotab

The majority (90%) of the expressed peptide was exported from the cell to the supernatant. Expression levels of mature vasotab (EV048) in the supernatant were approximately 0.3 μg ml^{-1} of Sf9 cells. Following metal affinity and cation exchange chromatography vasotab eluted as a single peak (data not shown), which was concentrated and visualised on a 4–12% Bis-Tris polyacrlamide gel (Fig. 10). The purified protein is approximately the size (7 kDa) expected for the mature peptide (including the glycine and 6x histidine tag added by PCR).

Changes in whole-cell I_{Ca}

Representative I_{Ca} traces recorded from control and protein treated myocytes of the rat are shown in Fig. 11A,B. The currents were induced by depolarizing the membrane from a holding potential of –45 mV up to –5 mV. The original records show that the amplitude of peak I_{Ca} was decreased almost to the same degree by both the crude (Fig. 11A) and the recombinant (Fig. 11B) proteins. These effects were highly reproducable. On average, 0.4 μg ml^{-1} of crude protein decreased I_{Ca} from –0.65±0.06 nA to –0.39±0.03 nA, N=5, P<0.005; 0.4 μg ml^{-1} of recombinant protein decreased I_{Ca} from –0.59±0.02 nA to –0.34±0.05 nA, N=5, P<0.001. These findings support the hypothesis that the protein exerts its vasodilating effect by blocking the L-type calcium channels, thus reducing the amount of trigger Ca^{2+}, decreasing the

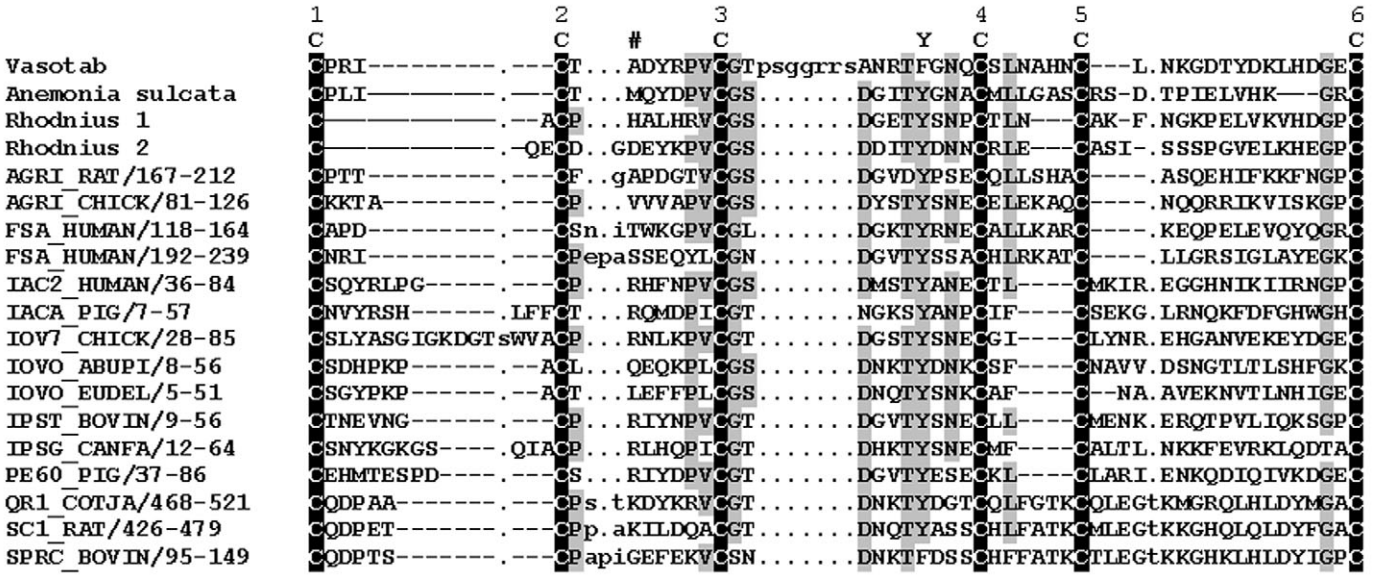

Fig. 8. Pfam alignment of vasotab with representative Kazal type proteins. The residues in lower case are outstandingly different from the overall consensus. Residues identical in more than half of the sequences depicted are highlighted in grey. Cysteines are highlighted in black. Identity of sequences shown: *Anemonia sulcata* inhibitor of elastase (IELA_ANESU/4–48); *Rhodnius prolixus* thrombin inhibitor domain 1 and 2 (THBI_RHOPR/6–48 and 57–101); AGRI, agrin; IAC, acrosin inhibitor; FSA, follistatin; PE60, PEC60; IPST, pancreatic secretory trypsin inhibitor; IPSG, double headed protease inhibitor; IOV, ovomucoid inhibitor; QR1, quail retinal 1; SC1, secreted calcium binding 1 matrix glycoprotein; SPRC, secreted protein acidic and rich in cysteine, also called basement membrane protein 40.

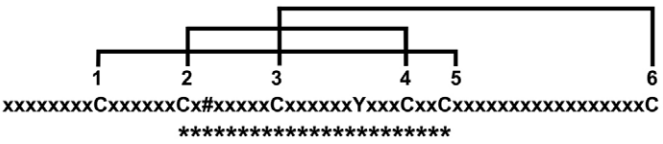

Fig. 9. Schematic representation of the primary structure of a Kazal-type protein. Disulphide bridging pattern of the conserved cysteine (C) residues is indicated by solid lines. Asterisks show the position of the Pfam consensus pattern: C–x(7–10)–C–x(6)–Y–x(3)–C–x(2–6)–C. All six cysteines are conserved in mature vasotab, although the spacing between cysteine residues within the consensus pattern is unusual: C–x(7)–C–x(13)–F–x(3)–C–x(6)–C. The active site residue of protease inhibitors is marked by a hash (#).

amount of activator Ca^{2+} released from the sarcoplasmic reticulum, and consequently weakening the contractile force.

Discussion

Vasodilatory and cardioactive effects of horse fly SGE

Artery relaxation induced by SGE has previously been reported in mosquitoes (Champagne and Ribeiro, 1994), black flies (Cupp et al., 1994), sand flies (Lerner and Shoemaker, 1991), ticks (Kemp et al., 1983) and triatomine bugs (Ribeiro et al., 1990, 1993). The mode of action of peptide vasodilators varies. Mosquito tachykinins induce endothelium-dependent vasorelaxation by binding endothelial cell tachykinin receptors eliciting release of nitric oxide (Champagne and Ribeiro, 1994). Other protein vasodilators are endothelium independent. Nitrophorins from triatomine bugs release nitric oxide, which induce direct relaxation of smooth muscle by increasing intracellular cGMP levels (Champagne, 1994; Weichsel et al., 1998). Sand fly maxadilan (7 kDa) is an agonist of the type I receptor for pituitary adenylate cyclase activating peptide (Moro and Lerner, 1997), which increases the intracellular level of cAMP within smooth muscle cells leading to relaxation (Grevelink et al., 1995). Our data suggest that horse flies may also use an endothelium-independent mechanism, since SGE from *H. bimaculata* induced higher levels of artery relaxation after removal of endothelium, which therefore represents a barrier rather than a part of the active process.

Salivary gland extracts from *H. bimaculata* exhibit potent cardioactive effects. SGE increased coronary blood flow and left ventricular contractility in an isolated perfused rat heart mode without effecting heart rate or rhythm. SGE also stimulated the cardiac sarcolemmal Na,K-ATPase especially at lower ATP concentrations, possibly by improving the substrate-binding properties of the enzyme. Na,K-ATPase is an enzyme involved in the active transport of Na^+ and K^+ ions across cell membranes causing potassium-dependent relaxation or so-called hyperpolarisation. The enzyme utilises the energy derived from hydrolysis of ATP. Increased activity of this enzyme can induce relaxation by hyperpolarizing vascular smooth muscle cells (Pomposiello et al., 1998), although in the heart activation of the Na,K-ATPase is likely to weaken cardiac contraction by lowering intracellular calcium levels through its effect on the Na^+/Ca^{2+} exchanger (James et al., 1999). We do not presently know whether the vasorelaxation observed in arteries is mediated *via* Na,K-ATPase and or whether the cardiac effects that were observed are mediated through one or more other bioactive molecules.

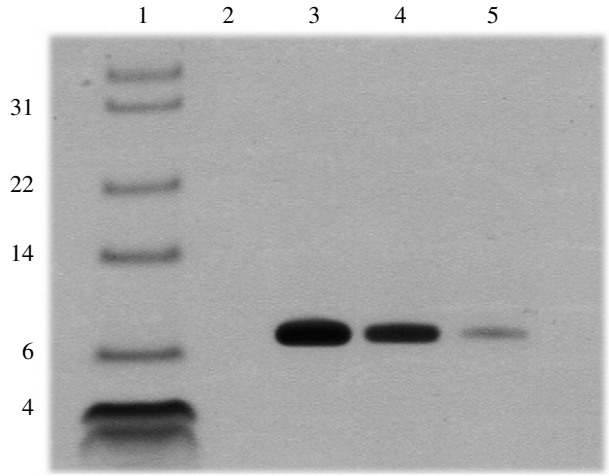

Fig. 10. Coomassie Blue stained NuPAGE^{TM} 4–12% Bis-Tris gel showing purified peptide vasotab. Lane 1, protein markers (molecular masses in kDa are indicated at left); lanes 2–5, serial fractions that elute at about 0.15 mol l^{-1} NaCl from a SP sepharose column.

Vasotab is a member of the Kazal type protease inhibitor family

N-terminal sequence analysis of the *H. bimaculata* HPLC fraction that induced the greatest amount of vasorelaxation (retention time 13.77 min), and subsequent analysis of the derived cDNA, indicated that the active peptide, termed vasotab, is closely related to Kazal-type protease inhibitors. The Kazal inhibitor family of proteins includes pancreatic

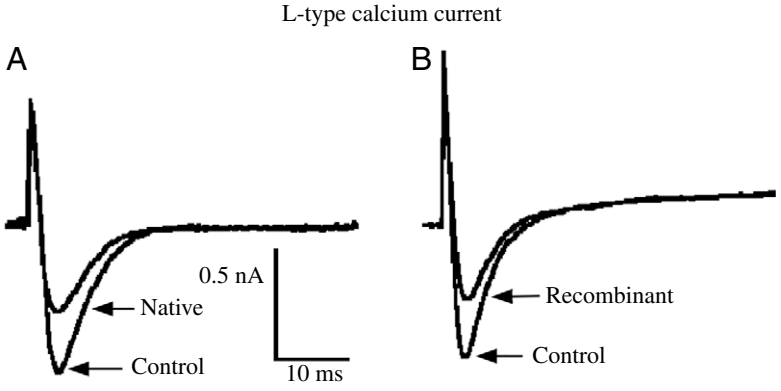

Fig. 11. Representative whole-cell voltage-clamp recordings of I_{Ca} at –5 mV depolarizing voltage steps in control myocytes and myocytes treated with 0.4 μg ml^{-1} native protein (A) and 0.4 μg ml^{-1} recombinant protein (B).

secretory trypsin inhibitor (Greene and Giordano, 1969), avian ovomucoid (Laskowski et al., 1987), acrosin inhibitor (Williamson et al., 1984) and elastase inhibitor (Tschesche et al., 1987). Kazal inhibitors contain between 1 and 9 Kazal-type inhibitor repeats. However, Kazal-like domains are also seen in the extracellular part of agrins and of PEC60 (a peptide with N-terminal glutamic acid, C-terminal cysteine, 60 residues), that are not known to be protease inhibitors. Agrins are multidomain proteins (including 8 Kazal-like domains) present at the synaptic basal lamina of the neuromuscular junction that cause the aggregation of acetylcholine receptors and acetylcholine esterase (Kleiman and Reichardt, 1996). PEC60 inhibits glucose-induced insulin secretion from perfused pancreas (Agerberth et al., 1989), may play a role in the immune system (Metsis et al., 1992) and, most notably, appears to activate the Na,K-ATPase (Kairane et al., 1994).

The structure of the Kazal repeat includes a large quantity of extended chain, a short α-helix and a 3-stranded anti-parallel β-sheet. The consensus contact residue set of the protease inhibitor family with the enzymes that they inhibit comprises 12 residues, of which 10 are variable (Lu et al., 2001). Altering the enzyme-contact residues, and especially that of the active site bond, affects the strength of inhibition and specificity of the inhibitor for particular serine proteases (Empie and Laskowski, 1982). Kazal protease inhibitors that inhibit trypsin-like proteinases often have basic residues (R, K or H) at their active (or P1) site whereas those that inhibit chymotrypsin-like proteases have large hydrophobic residues at the P1 position. The putative active site residue of vasotab is the small hydrophobic amino acid alanine. This residue is present at P1 in ovomucoid third domains of various bird species as well as agrins that are not known to be protease inhibitors. We do not yet know whether vasotab is a protease inhibitor; however, an algorithm for predicting the reactivity of Kazal family protein inhibitors (Lu et al., 2001) suggests that an active site sequence similar to that of vasotab would be a potent inhibitor of porcine pancreatic elastase.

The outstanding feature of the primary sequence of vasotab is the presently unique extra amino acid sequence (PSGGRRS) inserted between the third and fourth cysteine residues that may well play a key role in the function of the peptide. Homology modelling suggests that the additional amino acids exist at an exposed location, which may permit interaction with a target molecule.

Activity of crude and recombinant vasotab

To determine the mechanism of the muscle relaxing effect of the proteins, we examined their effects on the L-type calcium current (I_{Ca}) in single left ventricular myocytes isolated from rat hearts. Our data indicate that the mechanism by which the protein exerts its vasodilating effects is a strong calcium channel blocking action. The findings presented here undoubtedly prove that both the crude and the recombinant proteins have similar capability of blocking L-type calcium channels.

This possibility is not unprecedented since both Kunitz (bovine pancreatic trypsin inhbitor) and Kazal (chicken ovoinhibitor) serine proteinase inhibitors have been shown to exhibit specific interactions with calcium-activated potassium ion channels (Moss et al., 1996). The effect of vasotab on cardiomyocyte action potential persisted for more than 16 min after the active agent was washed away. Prolongation of the action potential without apparently inducing spontaneous oscillations suggests vasotab may have potential as an anti-arrhythmic agent.

Unlike SGE, the recombinant peptide did not show potent activity against isolated organs. Vasotab (40 μg) resulted in a transient increase in coronary flow in the perfused rat heart model, but we were unable to demonstrate an effect on rat femoral artery relaxation (data not shown). We speculate that the 6x histidine tag may have interfered with the peptide's vasodilatory activity, and plan further studies using untagged peptide and site-specific mutants to elucidate vasotab's mode of action.

List of abbreviations

ACN	acetonitrile
CF	coronary flow
EG	electrogram
HR	heart rate
LVP	left ventricular pressure
NR	non-redundant
PBS	phosphate buffer
PCR	polymerase chain reaction
RP-HPLC	reverse-phase high performance liquid chromatography
SGE	salivary gland extract
TFA	trifluoroacetic acid

References

Agerberth, B., Soederling-Barros, J., Joernvall, H., Chen, Z., Oestenson, C. G., Efendic, S. and Mutt, V. (1989). Isolation and characterization of a 60-residue intestinal peptide structurally related to the pancreatic secretory type of trypsin inhibitor: influence on insulin secretion. *Proc. Natl. Acad. Sci. USA* **86**, 8590-8594.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. (1997). Gapped BLAST and PSI-BLAST a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389.

Bateman, A., Birney, E., Durbin, R., Eddt, S. R., Howe, K. L. and Sonnhammer, E. L. (2000). The Pfam protein families database. *Nucleic Acids Res.* **28**, 263.

Bowman, A. S., Dollwith, J. W. and Sauer, J. R. (1996). Tick salivary prostaglandins: Presence, origin and significance. *Parasitol. Today* **12**, 388-396.

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

Champagne, D. E. and Ribeiro, J. M. C. (1994). Sialokinin I and II: Vasodilatory tachykinins from the yellow fever mosquito *Aedes aegypti*. *Proc. Natl. Acad. Sci. USA* **91**, 138-142.

Champagne, D. E. and Valenzuela, J. G. (1996). Pharmacology of haematophagous arthropod saliva. In *The Immunology of Host-Ectoparasitic Arthropod Relationships* (ed. S. K. Wikel), pp. 85-94. Wallingford (UK): Cab International.

Cupp, M. S., Ribeiro, J. M. C. and Cupp, E. W. (1994). Vasodilative activity in black fly salivary glands. *Am. J. Trop. Med. Hygiene* **50**, 241-246.

Empie, M. W., Laskowski, M. (1982). Thermodynamics and kinetics of single residue replacements in avian ovomucoid third domains: effect on inhibitor interactions with serine proteinases. *Biochem.* **21**, 2274-2284.

Friedrich, T., Kroeger, B., Bialojan, S., Lemaire, H. G., Hoeffken, H. W., Reuschenbach, P., Otte, M. and Dodt, J. (1993). A Kazal-type inhibitor with thrombin specificity from *Rhodnius prolixus*. *J. Biol. Chem.* **268**, 16216-16222.

Greene, L. J. and Giordano, J. S. J. (1969). The structure of the bovine pancreatic secretory trypsin inhibitor – Kazal's inhibitor. I. The isolation and amino acid sequences of the tryptic peptides from reduced aminoethylated inhibitor. *J. Biol. Chem.* **244**, 285-298.

Grevelink, S. A., Youssef, D. E., Loscalzo, J. and Lerner, E. A. (1993). Salivary-gland extracts from the deerfly contain a potent inhibitor of platelet aggregation. *Proc. Natl. Acad. Sci. USA* **90**, 9155-9158.

Grevelink, S. A., Osborne Loscalzo, J. and Lerner, E. A. (1995). Vasorelaxant and second messenger effects of maxadilan. *J. Pharmacol. Exp. Therap.* **272**, 33-37.

Hollander, A. L., Wright, R. E. (1980). Impact of tabanids on cattle: blood meal size and preferred feeding sites. *J. Econ. Entomol.***73**, 431-433.

James, P. F., Grupp, I. L., Grupp, G., Woo, A. L., Askew, G. R., Croyle, M. L., Walsh, R. A. and Lingrel, J. B. (1999). Identification of a specific role for the Na,K-ATPase alpha 2 isoform as a regulator of calcium in heart. *Mol. Cell* **3**, 555-563.

Jeanmougin, F., Thompson, J. D., Gouy, M., Higgins, D. G. and Gibson, T. J. (1998). Multiple sequence alignment with Clustal X. *Trends Biochem. Sci.* **23**, 403.

Kairane, C., Zilmer, M., Mutt, V. and Sillard, R. (1994). Activation of Na,K-ATPase by an endogenous peptide, PEC-60. *FEBS Lett.* **345**, 1-4.

Kazimírová, M., Šulanová, M., Kozánek, M., Takác, P., Labuda, M. and Nuttall, P. A. (2001). Identification of anticoagulant activities in salivary gland extracts of four horsefly species (Diptera, tabanidae). *Haemostasis* **31**, 294-305.

Kemp, D. H., Hales, J. R., Schleger, A. V. and Fawcett, A. A. (1983). Comparison of cutaneous hyperemia in cattle elicited by larvae of *Boophilus microplus* and by prostaglandins and other mediators. *Experientia* **39**, 725-727.

Kleiman, R. J. and Reichardt, L. F. (1996). Testing the agrin hypothesis. *Cell* **85**, 461-464.

Langendorff, O. (1895) Untersuchungen am uberlebenden Saugetiereherzen. *Pflugers Arch. Gesamte Physiol. Menschen Tiere* **61**, 291.

Laskowski, M., Kato, I., Ardelt, W., Cook, J., Denton, A., Empie, M. W., Kohr, W. J., Park, S. J., Parks, K., Schatzley, B. L., Schoenberger, O. L., Tashiro, M., Vichot, G., Whatley, H. E., Wieczorek, A. and Wieczorek, M. (1987). Ovomucoid third domains from 100 avian species: isolation, sequences, and hypervariability of enzyme-inhibitor contact residues. *Biochemistry* **26**, 202-221.

Law, J., Ribeiro, J. M. C. and Wells, M. (1992). Biochemical insights derived from diversity in insects. *Annu. Rev. Biochem.* **61**, 87-112.

Lerner, E. A., Ribeiro, J. M. C., Nelson, J. R. 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.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

Lu, S. M., Lu, W., Qasim, M. A., Anderson, S., Apostol, I., Ardelt, W., Bigler, T., Chiang, Y. W., Cook, J., James, M. N., et al. (2001). Predicting the reactivity of proteins from their sequence alone: Kazal family of protein inhibitors of serine proteinases. *Proc. Natl. Acad. Sci. USA* **98**, 1410-5.

Meszaros, J., Coutinho, J. J., Bryant, S. M., Ryder, K. O. and Hart, G. (1997). L-type calcium current in catecholamine-induced cardiac hypertrophy in the rat. *Exp. Physiol.* **82**, 71-83.

Metsis, M., Cintra, A., Solfrini, V., Ernfors, P., Bortolotti, F., Morrasutti, D. G., Oestenson, C.-G., Efendic, S., Agerberth, B., Mutt, V., Persson, H. and Fuxe, K. (1992). Molecular cloning of PEC-60 and expression of its mRNA and peptide in the gastrointestinal tract and immune system. *J. Biol. Chem.* **267**, 19829-19832.

Moro, O. and Lerner, E. A. (1997). Maxadilan, the vasodilator from sand flies, is a specific pituitary adenylate cyclase activating peptide type I receptor agonist. *J. Biol. Chem.* **272**, 966-970.

Moss, G. W., Marshall, J., Morabito, M., Howe, J. R. and Moczydlowski, E. (1996). An evolutionarily conserved binding site for serine proteinase inhibitors in large conductance calcium-activated potassium channels. *Biochemistry* **35**, 16024-16035.

Pomposiello, S. I., Alva, M., Wilde, D. W. and Carratero, O. A. (1998). Linoleic acid induces relaxation and hyperpolarization of the pig coronary artery. *Hypertension* **31**, 615-620.

Rajská, P., Pechánová, O., Takác, P., Kazimírová, M., Roller, L., Vidlicka, L., Ciampor, F., Labuda, M. and Nuttall, P. A. (2003). Vasodilatory activity in horsefly and deerfly salivary glands. *Med. Vet. Entomol.* **17**, 395-402.

Ribeiro, J. M. C. (1992). Characterization of a vasodilator from the salivary glands of the yellow fever mosquito *Aedes aegypti*. *J. Exp. Biol.* **165**, 61-71.

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

Ribeiro, J. M. C. and Nussenzveig, R. H. (1993). The salivary catechol oxidase/peroxidase activities of the mosquito *Anopheles albimanus*. *J. Exp. Biol.* **179**, 273-287.

Ribeiro, J. M. C. and Walker, F. A. (1994). High affinity histamine-binding and antihistaminic activity of the salivary nitric oxide-carrying heme protein (nitrophorin) of *Rhodnius prolixus*. *Biochem. J.* **180**, 2251-2257.

Ribeiro, J. M. C., Vachereau, A., Modi, G. B. and Tesh, R. B. (1989). A novel vasodilatory peptide from the salivary glands of the sand fly *Lutzomyia longipalpis*. *Science* **243**, 212-214.

Ribeiro, J. M. C., Marinotti, O. and Gonzales, R. (1990). A salivary vasodilator in the blood sucking bug *Rhodnius prolixus*. *Br. J. Pharmacol.* **101**, 932-936.

Ribeiro, J. M. C., Hazzard, J. M. H., Nussenzveig, R. H., Champagne, D. E. and Walker, F. A. (1993). Reversible binding of nitric oxide by a salivary heme protein from a bloodsucking insect. *Science* **260**, 539-541.

Schwede, T., Kopp, J., Guex, N. and Peitsch, M. C. (2003). SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Res.* **31**, 3381-3385.

Taussky, H. H. and Shorr, E. E. (1953). A microcolorimetric method for the determination of inorganic phosphorous. *J. Biol. Chem.* **202**, 675-685.

Tschesche, H., Kolkenbrock, H. and Bode, W. (1987). The covalent structure of the elastase inhibitor from *Anemonia sulcata* – a 'non-classical' Kazal type protein. *Biol. Chem. Hoppe-Seyler* **368**, 1297-1304.

van-de-Locht, A., Lamba, D., Bauer, M., Huber, R., Friedrich, T., Kroger, B., Hoffken, W. and Bode, W. (1995). Two heads are better than one: crystal structure of the insect derived double domain Kazal inhibitor rhodniin in complex with thrombin. *EMBO J.* **14**, 5149-5157.

Vrbjar, N., Soos, J. and Ziegelhoffer, A. (1984). Secondary structure of heart sarcolemmal proteins during interaction with metallic cofactors of (Na^+ + K^+)-ATPase. *Gen. Physiol. Biophys.* **3**, 317-325.

Weichsel, A., Anderson, J. F., Champagne, D. E., Walker, F. A. and Montfort, W. R. (1998). Crystal structure of a nitric oxide transport protein from a blood-sucking insect. *Nat. Struct. Biol.* **5**, 304-309.

Williamson, M. P., Marion, D. and Wuthrich, K. (1984). Secondary structure in the solution conformation of the proteinase inhibitor IIA from bull seminal plasma by nuclear magnetic resonance. *J. Mol. Biol.* **173**, 341-359.