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ANALYSES OF cDNA AND RECOMBINANT PROTEIN FOR A POTENT VASOACTIVE PROTEIN IN SALIVA OF A BLOOD-FEEDING BLACK FLY, *SIMULIUM VITTATUM*

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Summary

A cDNA was cloned from the salivary glands of a blood-feeding black fly *Simulium vittatum*. The encoded protein has been given the name *Simulium vittatum* erythema protein or SVEP, because of its ability to increase blood perfusion in skin capillaries, resulting in the well-characterized erythema of black fly bites. The full-length cDNA contains 548 base pairs which encode 152 amino acid residues of the nascent protein. Post-translational processing produces a mature, secreted protein of 133 residues with a molecular mass of 15.4 kDa. Recombinant SVEP (rSVEP) was produced in a baculovirus expression system and purified by a one-step reversed-phase HPLC procedure. Analyses of physical properties and biological potency demonstrated fidelity of rSVEP to the native protein. Recombinant SVEP relaxed rabbit aorta preparations when preconstricted with 2 μmol l^{-1} phenylephrine or 25 mmol l^{-1} K^+ but not with 60 mmol l^{-1} K^+. Further, the rSVEP-induced relaxation response of phenylephrine-constricted aorta was inhibited by glibenclamide (10 μmol l^{-1}), suggesting that at least part of its action to relax smooth muscle may result from the opening of ATP-dependent K^+ channels. SVEP is a novel salivary-gland-derived vasoactive protein that may be essential for blood feeding by black flies and could potentially enhance transmission of filarial parasites.

Key words: erythema, vasoactive protein, *Simulium vittatum*, black fly, salivary gland, cDNA.

Introduction

Blood-feeding insects cause a wide range of problems for humans and other vertebrates, ranging from annoyance and skin irritation to transmission of a variety of pathogenic organisms that can result in acute disease, debilitation or death. Of the many different insects studied, all secrete one or more potent salivary factors into their vertebrate hosts to facilitate blood feeding (Ribeiro, 1987). These factors may increase the rate of blood detection and engorgement (Ribeiro *et al.* 1984, 1985, 1986), keep blood fluid during feeding (Hudson, 1964) or modulate the host immune response (Cross *et al.* 1993*a*,*b*, 1994; Titus and Ribeiro, 1988; Qureshi *et al.* 1996). Although an insect's saliva may contain factors with one or more of these properties, recent investigations have focused heavily on salivary anti-hemostatic properties. Vertebrate hemostasis is critical to survival and thus has interconnecting arms that serve (1) to decrease, by vasoconstriction, the volume of blood loss from an injured vessel, (2) to seal a lesion site rapidly by platelet adherence and (3) to reinforce and stabilize the platelet seal by deposition of a fibrin meshwork as a result of the coagulation cascade. Vasoconstriction especially poses important consequences to a blood-seeking insect because it decreases the likelihood that the insect can feed to repletion before host detection.

Insects vary in the nature of their mouthparts and the method by which they blood-feed. Vessel-feeding insects have relatively long, tube-like mouths that can penetrate skin to the depth of arterioles and venules and feed directly from vessels (Gordon and Crewe, 1948). The second general type has mouthparts that are shorter and unable to cannulate vessels and thus relies on mouthparts to cut the smaller capillaries to form a pool of blood for feeding (Sutcliffe and McIver, 1984). For many of these insects, including black flies, sand flies and other gnats, it may be essential also to increase blood flow to the skin capillary bed.

The structures and modes of action of vasoactive salivary factors often differ between species, perhaps reflecting convergent evolution to blood feeding (Ribeiro, 1987). The differences encountered among closely related species, however, may also show fine tuning due to host selection, as suggested for black flies (Cupp and Cupp, 1997). Thus, these novel vasoactive factors, which have been shaped by evolutionary processes, may also have potential as therapeutic agents for human and animal hemostatic disorders (Ribeiro,

1995). Extracting salivary components (usually proteins) from insect glands in adequate quantities to address important biological questions and to elucidate modes of action requires extremely large numbers of organisms and a heavy investment in time for dissection. Thus, molecular cloning and recombinant protein production offer a reasonable solution for obtaining large quantities of pure, highly active proteins for study. Here, we describe the cloning and recombinant protein production of a novel *Simulium vittatum* erythema protein (SVEP) and demonstrate its structural identity to the native protein and its potency. In addition, we present initial evidence for its mode of action to increase blood flow to the capillary bed of skin which results in the skin erythema that is characteristic of black fly bites.

Materials and methods

Molecular analysis of SVEP

SVEP activity was purified from 900 pairs of salivary glands of *Simulium vittatum*, as described previously (Cupp *et al.* 1994); 170 pmol of the protein was used for physical analysis, including molecular mass determination by matrix-assisted laser-desorption mass spectroscopy (MALD/MS), tryptic digestion to produce peptides, and subsequent amino acid analysis of two of the peptides by automated Edman degradation (Harvard Microchemistry Facility).

Amplification by polymerase chain reaction and cloning of an SVEP cDNA fragment

Messenger RNA (mRNA) was isolated from salivary gland extract (SGE) derived from 50 pairs of *S. vittatum* salivary glands using the Micro-Fast Track mRNA isolation kit version 1.2 (Invitrogen Corporation, San Diego, CA). Single-stranded cDNA was synthesized using Superscript II (Life Technologies, Inc., Gaithersburg, MD), according to manufacturer's instructions, and divided into two equal parts. Two polymerase chain reactions (PCRs) were set up using two pairs of degenerate primers (200 ng each) designed from amino acid sequences of the SVEP peptides. PCR was carried out for 35 cycles in a thermocycler (Perkin Elmer, Foster City, CA). Amplified cDNA was resolved in a 1 % agarose gel, and DNA was purified from excised bands using the Sephaglas Band Prep Kit (Pharmacia Biotech, Uppsala, Sweden). DNA (7 ng) was inserted into the pCR vector (Invitrogen Corporation, San Diego, CA) using a T4 DNA ligase reaction at 12 °C overnight. OneShot cells (Invitrogen Corporation, San Diego, CA) were transformed with the product of the ligation reaction, according to manufacturer's instructions, and seeded on LB/agar plates (Sambrook *et al.* 1989) containing ampicillin (50 $\mu g\,ml^{-1}$) for overnight growth at 37 °C. White colonies (containing insert) were picked and grown in LB medium (Sambrook *et al.* 1989) containing ampicillin (30 $\mu g\,ml^{-1}$) at 37 °C in a shaking incubator. Plasmid DNA was isolated from the cellular growth of two colonies; both were sequenced in the forward and reverse directions, using primers for Sp6 and T7 promoters, by the Molecular Instrument Facility, University of Georgia (Athens, GA). A digoxigenin-labeled SVEP probe (DIG-SVEP) was synthesized by PCR using the cloned SVEP fragment as template and the Genius system (Boehringer Mannheim, Indianapolis, IN) using a ratio of digoxigenin-11-dUTP to dTTP of 1:5. DIG-SVEP DNA was purified by agarose gel electrophoresis.

Simulium vittatum *salivary gland cDNA library construction*

The linear phase for synthesis of salivary gland secretions was determined previously (Cupp *et al.* 1993), and this information was used to time the removal of salivary glands to optimize collection of corresponding mRNA. Total RNA was isolated from *S. vittatum* salivary glands using a micro RNA isolation kit (Stratagene, La Jolla, CA) and the manufacturer's instructions. Poly(A) Quik reagents (Stratagene, La Jolla, CA) were used to isolate mRNA and 5 µg of the purified material was used to construct the library in the ZAP Express vector following the manufacturer's protocol (Stratagene, La Jolla, CA). The completed library was packaged in Gigapack II Gold packaging extract (Stratagene, La Jolla, CA). The library was amplified once and the titer determined by standard procedures (Sambrook *et al.* 1989).

Cloning and sequencing of a full-length SVEP

XL1-Blue cells were transfected with 50 000 library plaque-forming units (p.f.u.) for screening using nylon membranes for plaque lifts and the DIG-SVEP probe. Two plaque picks from the first screening were confirmed positive in a secondary screen. Phage were extracted and amplified by growth in XL1-Blue MRF cells on NZY plates. SVEP-positive clones were tested by PCR amplification with SVEP-specific forward (SVEPF1) and reverse (SVEPR1) internal primers, which were based on a sequence in the cloned SVEP fragment. Two positive clones were further tested by an additional plaque assay and shown to be pure by hybridization of all colonies with the DIG/SVEP probe.

Phagemids containing SVEP cloned inserts were obtained by automatic excision using ExAssist helper phage and the protocol of Stratagene. XLOLR cells were infected with excised phagemids and colonies established on LB-kanamycin plates (Sambrook *et al.* 1989); colony picks were amplified by liquid culture. Phagemid DNA was extracted (QIAprep Spin Miniprep Kit, Qiagen Inc., Santa Clarita, CA) and analyzed by automated cycle sequencing (model 373A, Applied Biosystems, Arizona Research Laboratories, Tucson, AZ) in the forward direction using primers T3 and SVEPF1 and in the reverse directions using primers T7 and SVEPR1.

Preparation of BacPAK8/SVEP plasmid and transformation of baculovirus

SVEP plasmid DNA and the transfer vector pBacPAK8 (CLONTECH, Palo Alto, CA) were each digested with *Pst*I and *Xho*I restriction enzymes and purified by gel electrophoresis in Tris-acetate-EDTA buffer. Digested bands were excised from the gel, and DNA was extracted with

Sephaglas Band Prep Kit (Pharmacia Biotech, Uppsala, Sweden). A 1:2 (vector:insert) ligation reaction was set up to run overnight at 15 °C. OneShot cells were transformed with the BacPAK8 plasmid containing the SVEP insert (BacPAK8/SVEP) as described for the SVEP PCR fragment. Four colonies were selected for amplification at 37 °C. Plasmid DNA was isolated for sequencing using the Bac 2 primer (CLONTECH).

A recombinant baculovirus containing the SVEP insert was prepared by co-transfection of Sf9 cells with BacPAK8/SVEP plasmid and BSU36I-digested BacPAK6 viral DNA using lipofectin (Life Technologies, Grand Island, NY) as transfection reagent and SF900 serum-free medium (Life Technologies, Grand Island, NY). SVEP-virus clones were isolated and purified by plaque assay using standard methods contained in the BacPAK protocol of CLONETECH. Clear, positive plaques were picked, and virus was eluted in TNM-FH medium. Sf9 cells were infected with eluted virus and incubated for 4 days at 27 °C to generate passage 1 virus.

Cells were collected in phosphate-buffered saline (10 mmol l^{-1}, pH 7.4) and DNA was extracted using the Stratagene DNA micro extraction kit and protocol II in the instruction manual. Extracted DNA was used as template for PCR with an SVEP forward and reverse primer pair and the Bac1/Bac2 primer pair (CLONTECH). A secondary plaque assay was conducted to ensure clone purity.

Characterization of rSVEP

Sf9 cells were infected with virus (multiplicity of infection = 2) and incubated at 27 °C; media were collected at 12, 24, 48, 72 and 96 h. Virus was concentrated and removed from the medium by centrifugation in Centriplus 100 concentrators (Amicon, Beverly, MA) at 3000 ***g*** for 2 h at room temperature. Total protein in the fraction smaller than 100 kDa was estimated using the modified Lowry assay (Peterson, 1977). Test compounds were sterilized by passage through syringe filters (0.2 μm pore size; Gelman Sciences, Ann Arbor, MI) and injected intradermally into the shaved skin of New Zealand White rabbits (NZW), as previously described (Cupp *et al.* 1994). The appearance of visible redness (erythema) within 30 min, centered at the site of injection, was designated as a positive result (+).

Large molecular mass components (≥10 kDa) in the virus-free cell culture supernatant were concentrated by centrifugation at 3000 ***g*** for 4 h in Centriplus 10 microconcentrators. RP/HPLC using a C18 macrosphere column and elution with an acetonitrile/water (0.1 % trifluoroacetic acid) gradient was used for isolation of rSVEP from other medium components. RP/HPLC-purified rSVEP or *S. vittatum* SGE were diluted 1:1 with 2× electrophoresis sample buffer (Laemmli, 1970), heated to 95 °C for 5 min, centrifuged for 5 min at 12 000 ***g*** before resolving by SDS/15 % PAGE (Laemmli, 1970) and visualizing by silver staining (Oakley *et al.* 1980). Stained gels were scanned for densitometry analysis of band intensity (Personal Densitometer S.I., ImageQuaNT for Windows NT, Molecular Dynamics, Sunnyvale, CA).

Rabbit aorta relaxation test

Rabbit aorta rings, bathed in Krebs–Ringer's solution and bubbled with 95 % O_2/5 % CO_2, were contracted with 2 μmol l^{-1} phenylephrine and 25 mmol l^{-1} or 60 mmol l^{-1} K^+ before the addition of rSVEP at 1, 3, 10 or 30 μg ml^{-1} to the bathing solution. Increases in K^+ concentration were matched with equivalent decreases in Na^+ concentration to preserve solution tonicity. Glibenclamide (5 mmol l^{-1} solution in ethanol) was tested by adding to preconstricted aortic rings to yield a final concentration of 10 μmol l^{-1} prior to the addition of rSVEP.

Results

MALD/MS analysis of *S. vittatum* erythema protein, isolated from salivary gland extracts by RP/HPLC, had a molecular mass of 15 373±38 Da (mean ± S.D.), in agreement with the value of 15 351±1.3 Da previously reported from electrospray ionization mass spectroscopy (ESI/MS) (Cupp *et al.* 1994). SVEP was digested with trypsin, and the N-terminal amino acid sequences of two peptides (designated CT29 and CT51 in the HPLC-separated digest) were obtained (Fig. 1). Because the orientation of the two peptides in the native protein was unknown, two pairs of degenerate DNA primers were synthesized on the basis of the amino acid sequences designated in Fig. 1 by the forward or reverse arrows. Two cDNAs of approximately 300 and 400 base pairs were amplified by PCR in the reaction with the primer pair CT51 forward/CT 29 reverse and template copied from *S. vittatum* salivary gland mRNA. No product was produced in the analogous reaction using the opposite set of primers. Cloning and sequencing of the two PCR products revealed seven base differences (designated in italics), with only two base differences resulting in a change of amino acid (designated in bold, Fig. 2).

S. vittatum salivary gland mRNA was isolated during the first 48 h after adult emergence, during the period when salivary gland apyrase activity was known to be increasing at

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                  →→→→→→→→
SVEP-CT29:        GKNWGVMKDGEKHYFTVENCQ[E]
                  ←←←←←←←←

                     →→→→→→→
SVEP-CT51:           KPGQEFPYYVYMIPK
                   ←←←←←←←←←←←←←
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Fig. 1. Sequence of two peptides from a trypsin digest of SVEP used for constructing degenerate nucleotide primers. HPLC-purified SVEP was digested with trypsin and the resulting peptides separated by RP/HPLC. The N termini of two of the more abundant peptides were sequenced by automated Edman degradation (Harvard Microchemistry Facility, Cambridge, MA). Right-pointing arrows indicate the residues used to design degenerate nucleotide primers in the forward direction, and left-pointing arrows indicate amino acid sequences used for degenerate nucleotide reverse primer construction.

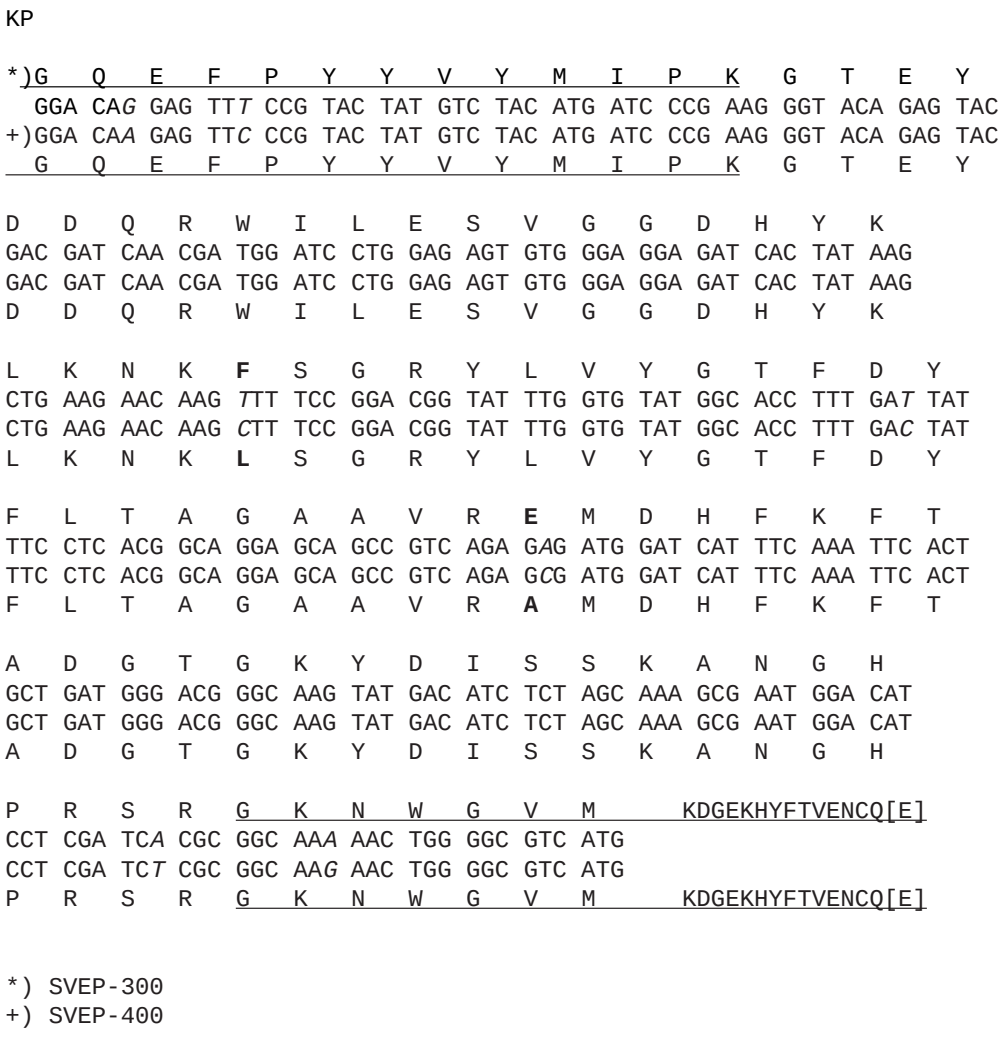

Fig. 2. Nucleotide sequences and amino acid translation of SVEP clones obtained by PCR. Two nucleotide products, SVEP-300 (*) and SVEP-400 (+), generated by PCR using the primer pair SVEP-CT51 forward and SVEP-CT29 reverse, were cloned and sequenced. Base composition varied at seven sites, but only two differences coded for amino acid changes. Base differences are in italics and amino acid changes are noted with bold letters. Underlined amino acids indicate ones known from protein sequencing.

a linear rate (Cupp *et al.* 1993). It was assumed that levels of other proteins secreted in saliva would also be increasing in a similar time frame. mRNAs were reverse-transcribed, and a DNA library was constructed in the lambda ZAP Express vector. The library was estimated to contain 2.8×10^6 clones and was amplified once to produce a titer of 2.3×10^9 p.f.u. ml^{-1}. The 300-base-pair DNA fragment of SVEP was used as template to synthesize a digoxigenin-labeled probe by PCR for screening the cDNA library to isolate a full-length clone. An initial screen of 50 000 p.f.u. resulted in the identification of 35 strongly positive clones. Two clones with the highest signal were further subcloned to ensure purity.

DNA isolated from preparations of the first clone (designated 5A) was sequenced from the T3 and T7 promoters contained in the ZAP Express vector and from two internal primers designed from the SVEP PCR clone. The cDNA insert contained 548 base pairs, including 48 base pairs preceding the putative methionine start site (Fig. 3). Inspection of the 3′ end revealed two stop codons in succession, which overlapped the signal for the poly(A) tail (AATAAA). There was a relatively short non-coding sequence of 19 bases between the end of the stop codon and the addition of the poly(A) tail.

Back-translation of the cDNA sequence predicted a polypeptide of 133 amino acid residues. Hydropathy plots, using GCG version 7 (Genetics Computer Group, Inc., Madison, WI) indicated a hydrophobic leader sequence of 19 residues which would result in a processed, secreted protein of 114 residues. The calculated molecular mass of such a protein was 15 349 Da, which was 1–2 Da less than the molecular mass of SVEP purified from salivary glands and determined by ESI/MS. A BLAST search of the GenBank nucleotide and protein data bases revealed no significant relationships to other

Table 1. *Analysis of SVEP protein concentration by SDS/ polyacrylamide gel electrophoresis and densitometry of cell culture supernatants from SVEP/baculovirus-infected cells*

Time (h)	rSVEP band intensity	Percentage of total
12	–	–
24	–	–
48	59	100
72	123	67
96	125	42

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CTGAAGTGTAAAGTACTTAAATCATTCGGTGGGAATTATCCAGCAAGTATGAGCATC
                                                  M  S  I

ACACAAAGCTTCTTTGTTTTAACCCTTGCCATATTTGGTGCTGCATCAGACAACCCA
T  Q  S  F  F  V  L  T  L  A  I  F  G  A  A  S *D  N  P

ATTGCTGATAGAAAATGTATCGTCATCAGTGACGGGGACCTGGTTATGCACGAGCGA
I  A  D  R  K  C  I  V  I  S  D  G  D  L  V  M  H  E  R

AAACCCGGTCAAGAGTTCCCATACTATGTCTACATGATCCCGAAGGGTACAGAGTAC
K  P  G  Q  E  F  P  Y  Y  V  Y  M  I  P  K  G  T  E  Y

GACGATCAACGATGGATCCTGGAGAGTGTGGGAGGAGATCACTATAAGCTGAAGAAC
D  D  Q  R  W  I  L  E  S  V  G  G  D  H  Y  K  L  K  N

AAGTTTTCCGGACGGTATTTGGTGTATGGCACCTTTGATTATTTCCTCACGGCAGGA
K  F  S  G  R  Y  L  V  Y  G  T  F  D  Y  F  L  T  A  G

GCAGCCGTCAGAGAGATGGATCATTTCAAATTCACTGCTGATGGGACGGGCAAGTAT
A  A  V  R  E  M  D  H  F  K  F  T  A  D  G  T  G  K  Y

GACATCTCTAGCAAAGCGAATGGTCATCCTCGATCTCGCGGCAAAAATTGGGGAGTC
D  I  S  S  K  A  N  G  H  P  R  S  R  G  K  N  W  G  V

ATGAAAGATGGTGAGAAGCACTATTTCACTGTTGAAAATTGTCAGGAATAATAAATA
M  K  D  G  E  K  H  Y  F  T  V  E  N  C  Q  E  †  †

AGAAATGTTGAAGTTGAAAAAAAAAAAAAAAAAAA
```

Fig. 3. Nucleotide sequence and amino acid translation of SVEP clone from *Simulium vittatum* salivary gland cDNA library. An SVEP clone was obtained by library screening using the digoxigenin-labeled SVEP nucleotide fragment synthesized by PCR. In addition to the full-length coding sequence, 48 base pairs preceding the methionine start site were obtained. The end of the putative hydrophobic leader sequence (*) and the polyadenylation signal (underline) are marked. These sequence data are available from GenBank under accession number U94515. The daggers indicate stop codons.

database holdings (Altschul *et al.* 1990). The nucleotide base and amino acid sequences for SVEP are deposited in GenBank accession U94515.

Cloned SVEP DNA was ligated into a baculovirus plasmid vector (BacPAK8) and used to generated a recombinant virus by co-transfection of Sf9 cells. DNAs extracted from cells infected with wild-type virus, and co-transfected with the SVEP/BacPAK8 construct or virus-negative control DNA, were tested for PCR amplification using baculovirus and SVEP-specific primers. Only DNA from co-transfected cells had amplifiable DNA in both tests. Recombinant SVEP/baculovirus was purified by one round of plaque purification before further analysis.

Protein secretion into the cell culture medium by Sf9 cells in the period 12–96 h post-infection was followed by PAGE and silver staining of virus-free cell culture medium to determine the time course of rSVEP production. Densitometric analysis of the stained gel revealed no protein bands until 48 h post-infection, when the putative rSVEP band (approximately 16 kDa) stained as the only detectable protein. By 72 h post-infection, the intensity of the band had doubled, and it represented 67 % of the total detectable protein (Table 1). There was no further change in intensity of the putative rSVEP band at 96 h, but increases in the intensity of other protein bands (also seen in the wild-type virus control lane) resulted in a decrease in proportion to 42 % (Table 1). Vasodilative activity in serial dilutions of media from wild-type virus-infected, SVEP virus-infected or *S. vittatum* SGE showed no activity, and endpoints of 1 ng and 0.017 pairs of glands, respectively, in the rabbit skin erythema test (Table 2).

SVEP protein was semi-purified and concentrated by centrifugation of virus-free cell culture supernatant in Centriplus 10 microconcentrators. RP/HPLC of this concentrated sample yielded a single peak of vasodilative activity that co-migrated with a similar approximately 16 kDa band contained in SvSGE (Fig. 4). SVEP represented

Table 2. *Presence of visible erythema in New Zealand White rabbit skin following intradermal injection of salivary gland extracts (SGE) of female* Simulium vittatum *or semi-purified recombinant* S. vittatum *erythema protein (rSVEP)*

Test compound	Quantity/reactivity							
SGE (pairs of glands)	1	0.5	0.05	0.3	0.025	0.020	0.017	0.014
	+	+	+	+	+	+	+	–
rSVEP (ng)	1650	165	82.5	8.25	4.13	2.04	1.03	0.51
	+	+	+	+	+	+	+	–

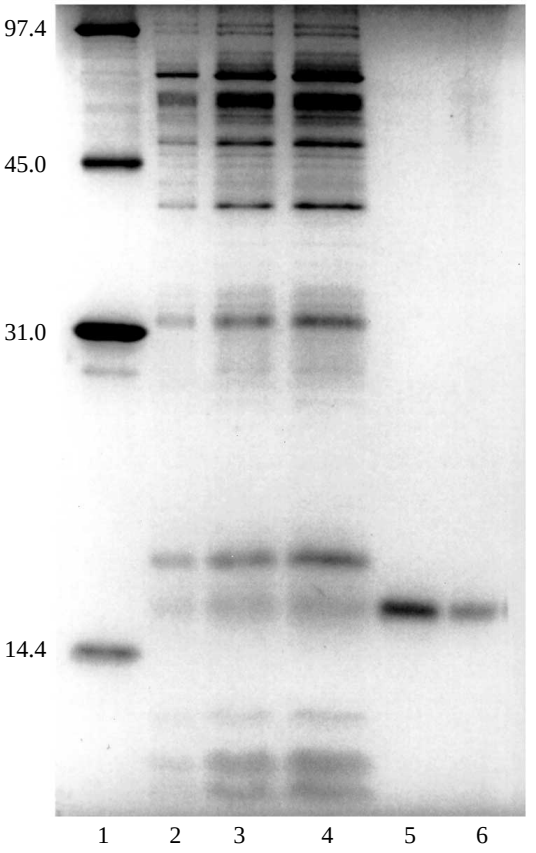

Fig. 4. SDS–PAGE of *Simulium vittatum* salivary gland extract (SvSGE) and HPLC-purified rSVEP. Lane 1 contains the molecular mass standards phosphorylase b (97.4 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa) and lysozyme (14.4 kDa). Lanes 2, 3 and 4 contain extracts of *S. vittatum* salivary glands (SGE) equivalent to one, two and three pairs of glands. HPLC-purified rSVEP is in lane 5 (6 μg) and lane 6 (3 μg). The electrophoretic mobility of rSVEP is consistent with a protein contained in *S. vittatum* SGE of approximately 16 kDa, based on the molecular mass standards. Densitometric comparison of the SVEP protein bands in SGE with those of rSVEP indicated that SGE contains 0.14±0.01 μg of SVEP per pair of glands (mean ± S.D., *N*=3).

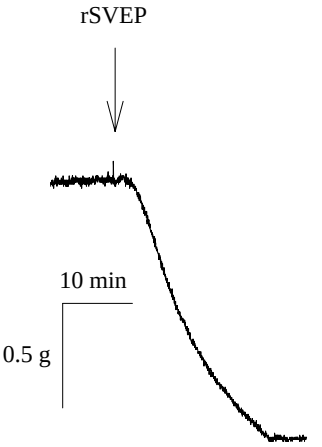

Fig. 5. Relaxation of contracted rabbit aortic ring in response to rSVEP. RP/HPLC-purified rSVEP was dried to remove solvents and resuspended in Tyrode's solution. The rabbit aortic ring was bathed in Krebs–Ringer's solution with 5 % CO_2, 95 % O_2 and contracted with 2 μmol l^{-1} phenylephrine. Addition of rSVEP (30 μg ml^{-1}=1.7 μmol l^{-1}) is indicated at the arrow. Note some delay and slow relaxation.

7.0±0.4 % of the total protein in SvSGE, which was equivalent to 0.14±0.01 μg of SVEP in a pair of glands (means ± S.D., *N*=3). MALD/MS analysis of purified rSVEP gave a mass of 15 377±38 Da, which matched the molecular mass of SVEP isolated from SvSGE.

RP/HPLC-purified rSVEP was dried and resuspended in Tyrode's solution and tested for vasodilative activity in the rabbit aorta relaxation test. Aortic rings, preconstricted with 2 μmol l^{-1} phenylephrine, showed a slow relaxation following a brief delay (Fig. 5). Furthermore, this relaxation occurred in a dose-related manner after preconstriction of the aorta with either 2 μmol l^{-1} phenylephrine or 25 mmol l^{-1} K^+ (Fig. 6A). In contrast, when increasing amounts of rSVEP were added to the aorta bathing solution following preconstriction with 60 mmol l^{-1} K^+, the effect was to increase constriction (Fig. 6A). Glibenclamide (10 μmol l^{-1}), a compound that inhibits opening of ATP-dependent K^+ channels, decreased the relaxation response to 10 μg ml^{-1} rSVEP by 64±7 % (Fig. 6B). This degree of inhibition is similar to that observed for cromakalim and pinacidil in the presence of glibenclamide (McPherson and Angus, 1990).

Discussion

Blood-feeding insects with short mouthparts that penetrate skin to shallow depths may require pharmacological, vasoactive compounds in their saliva to increase blood perfusion in superficial regions of the skin to assist in the location of the blood supply. The presence of potent erythema-inducing activity in salivary glands of all black flies tested supports the hypothesis that such factors are crucial for survival and were strongly selected for during the evolution of the New World species of Simuliidae. Further, the association between increased concentrations of erythema activity with anthropophilic blood-feeding behavior strongly correlates this activity with vector competency, at least in the species of *Simulium* examined to date.

In order to investigate further the means by which black fly saliva is able to increase perfusion of the papillary dermis, we have cloned a salivary gland cDNA for the vasoactive protein from *S. vittatum* and used it to produce a recombinant protein that appears to be identical in structure and function with the native salivary protein. Evidence for this conclusion is provided by the following observations: (1) identical migration characteristics of an approximately 16 kDa protein in silver-stained gels following SDS–PAGE of *S. vittatum* SGE and rSVEP; (2) the molecular mass of HPLC-purified rSVEP is the same as that of native SVEP isolated from salivary glands (see ESI/MS and MALD/MS data in Results); (3) the potency for

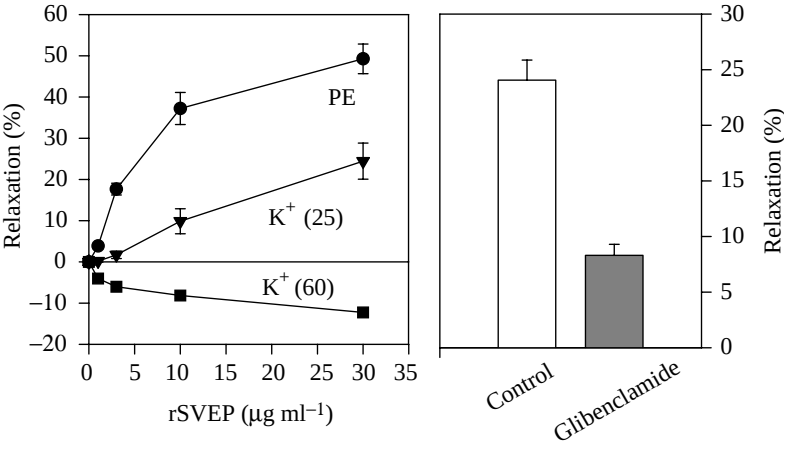

Fig. 6. rSVEP-dose-related relaxation of rabbit aortic rings and its inhibition by glibenclamide. (A) Dose–response curve. Aortic rings were contracted with 2 μmol l^{-1} phenylephrine (PE) and 25 or 60 mmol l^{-1} K^+ before addition of rSVEP at 1, 3, 10 or 30 μg ml^{-1}. SVEP induced dose-related relaxation in the presence of PE or 25 mmol l^{-1} K^+, but caused increased contraction in the presence of 60 mmol l^{-1} K^+. Increased K^+ concentrations were matched by an equivalent reduction in Na^+ concentration. Values are means ± S.E.M., *N*=4. (B) Inhibition of rSVEP-induced relaxation of rabbit aorta by glibenclamide. Glibenclamide (5 mmol l^{-1} solution in ethanol, final concentration 10 μmol l^{-1}) or ethanol (control) was added to aortic rings 17 min after contraction with 2 μmol l^{-1} PE. Recombinant SVEP (10 μg ml^{-1}) was added at 27 min and inhibition was measured at 47 min. Values are means + S.E.M., *N*=4–5.

erythema induction in the rabbit skin test was 1 and 2 ng for rSVEP and salivary gland SVEP, respectively. PCR-generated fragments that differed by two amino acid residues indicated that at least two isotypes of SVEP may be present in *S. vittatum* saliva. The baculovirus expression system used to produce rSVEP with native properties also provided high yields (30 mg l^{-1}), which should facilitate further investigation of its importance to black fly parasite transmission, its mechanism of action in skin and its potential therapeutic uses.

SVEP is potent in its action on the peripheral circulation with an end point for induction of visible erythema in the shaved skin of a rabbit of approximately 65 fmol. This potency equals that of maxadilan, the erythema protein of sand flies, and thus SVEP is among the most powerful vasodilator compounds yet described (Grevelink *et al.* 1995). Although these two insect-derived erythema proteins show little primary structural identity (discussed below), their overall molecular nature, which reflects secondary and tertiary properties, may show more similarity. This notion is indicated by the striking observation that each protein contains 14.29 % basic amino acid residues which could strongly influence its reactivity. Processing and amidation of maxadilan results in the loss of two C-terminal residues, increasing the basic nature of the mature protein to 14.75 %. There is no indication that SVEP is processed to a shorter protein.

SVEP and maxadilan are secreted by flies that have a similar manner of feeding, i.e. from shallow pools of blood that collect after capillaries have been lacerated by their short rasping mouth parts. Thus, on the basis of the site of saliva release and the erythema that is induced, it is likely that these proteins target blood flow to the capillary bed that supplies the papillary dermis. Endogenous blood flow to these vessels is under the control of smooth muscle cells that form a precapillary sphincter at the point where they branch from arterioles (Wilson, 1972). Muscle relaxation allows blood flow into the capillary loop, imparting a red blush at the skin surface known as erythema.

Muscle control can be mediated by neuropeptides, which are often co-localized in neurons with non-peptide transmitters (Morris, 1995). They are released after stimulation with high-frequency impulses and often have postsynaptic effects that develop more slowly and are of longer duration than their non-peptide counterparts (Morris, 1995), as would be expected from a receptor-mediated event. The slow relaxation of rabbit aorta in response to rSVEP, following a short delay, indicates a similar receptor-mediated effect of this insect protein. Further studies indicate that rSVEP also relaxes other non-vascular smooth muscles such as the guinea pig tenia coli and trachea (J. Ribeiro, personal observation). SVEP did not induce relaxation of aorta preconstricted by 60 mmol l^{-1} K^+, indicating that, under standard concentrations of extracellular K^+, rSVEP results in increased flow of K^+ out of the cell to cause hyperpolarization and smooth muscle relaxation. With high extracellular [K^+] abolishing the diffusion potential, membrane depolarization, along with opened K^+ channels, resulted in increased muscle contraction. The inhibition of rSVEP action by glibenclamide further indicates that opening of ATP-dependent K^+ channels may explain the vasoactive effect of SVEP. Further evidence will be needed to prove that SVEP works in a similar manner on vascular smooth muscle to induce erythema and that its mode of action relies on opening ATP-dependent K^+ channels.

Several channel inhibitors were tested but none could block the effect of maxadilan and thus reveal how it leads to muscle relaxation (Grevelink *et al.* 1995). Other studies, however, have shed light on the biochemical processes involved in its mechanism of action, including the elevation of intracellular cyclic AMP concentrations (Grevelink *et al.* 1995). Recently, maxadilan was shown to bind specifically to type 1 receptors for the neurotransmitter pituitary adenyl-cyclase-activating peptide (PACAP) (Moro and Lerner, 1997). Binding studies indicate that most peptidic neurotransmitters, including PACAP, have multiple cellular receptors, with varying ligand affinities. In humans, only one of these three receptors (designated PACAP type 1 receptor) showed unique specificity for PACAP, while the other two (PACAP/VIP R-1 and R-2)

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rSVEP    ...KLKNKFSGRYLVYGTFDYFLTAGAAVREMDHFKFTADGTGKYDISSKANGHPRSRGKNWGVMKDGEKHYFTVENCQE
Maxadilan  CDATCQFRKAIDDCQKQAHHSNVLQTSVQTTATFTSMDTSQLPGNSVFKECMKQKKKEFKA
PACAP 38                                  HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK
PACAP 27                                  HSDGIFTDSYSRYRKQMAVKKYLAAVL
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Fig. 7. Sequence alignment of SVEP, maxadilan, PACAP 38 and PACAP 27. The full sequence is shown for maxadilan and the PACAP peptides, but only the latter part of the sequence for SVEP is listed for simplicity. The sequences are aligned to place the dipeptides phenylalanine plus tyrosine in register (see Moro and Lerner, 1997). Identities and conservative amino acid differences that occur owing to this alignment are identified in bold type.

also bound vasoactive intestinal peptide (VIP) (Meyer *et al.* 1996). Expression of each receptor shows tissue specificity, with overlapping expression among the three types (Wei and Mojsov, 1996). The specific, high-affinity binding of maxadilan to PACAP type 1 receptor reveals a greater complexity to the PACAP receptor family than previously understood. This observation is highlighted by the lack of strong sequence identity between maxadilan and the other members of the PACAP/VIP family (Moro and Lerner, 1997). Alignment of SVEP with maxadilan and PACAP 38 and 27 so that a common pair of residues, phenylalanine (F) and tyrosine (T), is in register reveals few other similarities (Fig. 7). Six residues are in similar positions for maxadilan and the PACAP peptides, while five identical residues and three conservative differences occur between maxadilan and SVEP.

There are currently many gaps to fill before the structurally significant epitopes of these two proteins and their cellular targets are revealed. Resolving the remaining questions will provide a better understanding of how these flies manipulate host blood supply and may further elucidate endogenous control mechanisms of peripheral circulation. Recombinant proteins, now available for each of these factors, should hasten the discovery of the missing components.

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