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ISOLATION OF A NEUROPEPTIDE FROM LOCUST CORPUS CARDIACUM WHICH INFLUENCES ILEAL TRANSPORT

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Summary

1. *Schistocerca gregaria* ion-transport peptide (Scg-ITP) was isolated from aqueous extracts of the corpus cardiacum by a four-step procedure, utilizing reverse-phase high-performance liquid chromatography for separation and stimulation of a Cl^--dependent short-circuit current (I_{sc}) across locust ilea as the bioassay.

2. Scg-ITP has an unblocked N terminus and an apparent relative molecular mass of 7700. Thirty-one residues (of an estimated 65) were identified by sequence analysis.

3. Scg-ITP is structurally related to a crustacean family of neuropeptides which includes the crustacean hyperglycaemic hormones from the shore crab *Carcinus maenas* and the crayfish *Orconectes limosus* and moult-inhibiting hormone and vitellogenesis-inhibiting hormone from the lobster *Homarus americanus*.

4. Scg-ITP has no sequence homology with neuroparsins (Nps). Nps are the only other neuropeptides isolated to date that might regulate reabsorption in an insect hindgut (rectum).

Introduction

Ion and fluid transport across the hindgut (ileum and rectum) of the desert locust, *Schistocerca gregaria* Forskål, are influenced by proteinaceous stimulants from the central nervous system (reviewed by Phillips *et al.* 1986, 1988). However, our knowledge of the properties and chemical nature of these factors is limited. A partial purification of a factor (chloride transport stimulating hormone; CTSH) from the corpus cardiacum (CC) of the desert locust, which stimulated Cl^--dependent short-circuit current (I_{sc}) across the rectum was reported by Phillips *et al.* (1980), but no further attempts have been made since to isolate it. CTSH was found to be a proteinaceous compound with a relative molecular mass of approximately 8000. More recently, ion and fluid transport across the ileum of the desert locust has been shown to be influenced by proteinaceous factors from both the CC and ventral ganglia (VG; Lechleitner *et al.* 1989; Audsley and Phillips,

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1990). Factors from these sources acting on ileal I_{sc} are apparently different compounds because their physical properties differ (Audsley and Phillips, 1990). The factors in the CC are also the most potent and most stable stimulants. Some of the properties of ileal CC stimulants reported by Audsley and Phillips (1990) are also different from those of CTSH reported by Phillips *et al.* (1980); however, there was no compelling evidence that the stimulation of rectal and ileal I_{sc} was definitely due to different factors in the CC. This question can be resolved by isolating factor(s) from the CC and comparing the effects of specific fractions on both hindgut segments.

Neuroparsins (Nps) are two proteins (NpA and NpB) isolated from the CC storage lobes of *Locusta migratoria* (Girardie *et al.* 1989, 1990). NpB is a homodimer: a complete sequence (78 residues) of its monomer (8188 Da) has been determined (Girardie *et al.* 1989). NpA is identical to NpB, with an additional heterogeneous N terminal. The longest NpA chain is composed of 83 residues with a molecular mass of 8759 Da. NpB is believed to be formed from NpA by cleavage of the five N-terminal residues (Girardie *et al.* 1990). Recently, Hietter *et al.* (1991) published revised structures for Nps, indicating that they are actually monomeric.

Nps were shown to stimulate fluid reabsorption (J_v) across isolated everted rectal sacs of *L. migratoria* (Fournier and Girardie, 1988) but their effects on specific ion-transport processes have not been studied. To date, they are the only factors fully characterized which may control reabsorption in an insect hindgut (rectum).

In this paper we report the purification, amino acid analysis and partial sequence of a CC factor named *S. gregaria* ion-transport peptide (Scg-ITP or ITP), using reverse-phase high-performance liquid chromatography (RP-HPLC) for separation and voltage-clamped locust ilea as the bioassay. ITP and Nps are not structurally related; however, ITP has considerable sequence homology to crustacean hyperglycaemic hormone from the shore crab *Carcinus maenas* (Cam-CHH; Kegel *et al.* 1989) and the crayfish *Orconectes limosus* (Orl-CHH; Kegel *et al.* 1991) and moult-inhibiting hormone (Hoa-MIH; Chang *et al.* 1990) and vitellogenesis-inhibiting hormone (Hoa-VIH; Soyez *et al.* 1991) from the lobster *Homarus americanus*.

Materials and methods

The experimental animals were adult *Schistocerca gregaria*, 2–3 weeks past their final moult. They were reared at 28 °C and 55 % relative humidity under a 12 h:12 h light:dark cycle, and fed a diet of lettuce and a mixture of dried grass, bran and milk powder. Ilea from females were used because of their larger size.

Bioassay: electrogenic chloride transport

To test the activity of extracts and HPLC fractions, ilea were mounted as flat sheets between two modified Ussing chambers and voltage-clamped at 0 mV, as described by Hanrahan *et al.* (1984). Each chamber contained 2 ml of saline which was stirred by vigorously bubbling with a mixture of 95 % O_2/5 % CO_2 at 22±2 °C. Short-circuit current (I_{sc}), a direct continuous measurement of electronic Cl^- transport in this tissue (Irvine *et al.* 1988), was recorded continuously on a strip chart recorder (Soltec 1242, Soltec Corp.,

Sun Valley, CA). As this assay was sensitive to acetonitrile, samples of fractions to be assayed were dried in polypropylene microcentrifuge tubes (Robbins Scientific, CA) and rinsed with a 0.5% bovine serum albumin (BSA) solution by centrifugal evaporation (Speed-vac, Emerston Instruments Inc., Ontario). BSA was found to be necessary to prevent loss of activity due to non-specific binding to the surface of the tubes: if active peptide was dried alone it became insoluble. Fractions were resuspended in small volumes (10–100 μl) of physiological saline and samples were added to the haemocoel side of ilea once a steady-state level had been reached (1–2 h after dissection) to give the desired concentration. BSA, which does not affect hindgut I_{sc}, was also added to the bathing saline to reduce non-specific binding of active peptide to the walls of the Ussing chamber.

Salines

The physiological saline was based on the composition of locust haemolymph (Hanrahan *et al.* 1984) and contained (in mmol l^{-1}): 100 NaCl, 5 K_2SO_4, 10 $MgSO_4$, 10 $NaHCO_3$, 5 $CaCl_2$, 10 glucose, 100 sucrose, 2.9 alanine, 1.3 asparagine, 1.0 arginine, 5 glutamine, 11.4 glycine, 1.4 histidine, 1.4 lysine, 13.1 proline, 6.5 serine, 1.0 tyrosine and 1.8 valine, and was bubbled with 95% O_2/5% CO_2 and adjusted to pH 7.2.

Preparation of tissue and haemolymph extracts

Whole CC, storage lobes (NCC) and glandular lobes (GCC) of the CC were removed from adult male and female locusts, 2–6 weeks after their final moult, immediately frozen on dry ice and stored at −70 °C. Separate NCC and GCC were dissected from different animals and the connecting stalks were discarded to avoid contamination of each lobe with the products of the other. As the stalks from the NCC project part way into the GCC (Highnam, 1961), the anterior third of the GCC was removed to avoid potential contamination from NCC products. Extracts were prepared by mechanically homogenizing 50 CC in saline (for assay) or 1000 CC in HPLC water (for separation) using a Tissue Tearer homogenizer (Bartlesville, OK), and then centrifuging for 20 min at 12 000 ***g*** and at 4 °C. The supernatant was removed and after two further extractions of the pellet the supernatants were combined.

Haemolymph was collected from 50 locusts into 5 ml of ice-cold HPLC water agitated and centrifuged for 1 h at 12 000 ***g*** and at 4 °C. The supernatant was then applied to a preparative cartridge as described below.

Separation of CC extracts

Step 1: preparative stage

Supernatants were applied to a custom-made reverse-phase C_4 cartridge, 0.5 g, 30 nm, 10 μm packing (Hypersil, Phenomenex, CA) in a 3 ml polypropylene filtration column (Supelco, Ontario), equilibrated with 0.1% trifluroacetic acid (TFA). The cartridge was eluted stepwise with 2 ml each of 0.1% TFA, 30% CH_3CN in 0.1% TFA, 60% CH_3CN in 0.1% TFA, and 100% CH_3CN in 0.1% TFA. The active fraction (60% CH_3CN fraction) was concentrated by centrifugal evaporation (Speed-Vac) until volatile solvents were removed.

Step 2: C_8 RP-HPLC

The concentrated active fraction (1–2 ml) from the preparative stage (step 1) was injected *via* a Waters 2 ml loop injector onto a Nucleosil C_8 30 nm, 10 μm, reverse-phase column (250 mm×4.6 mm: Phenomenex, CA) fitted with a guard column (30 mm× 4.6 mm) of similar packing material. Chromatography was performed using two Beckman pumps, a Beckman 421A system controller (Beckman 114M Solvent Delivery Module) and a Waters variable-wavelength ultraviolet detector set at 225 nm. The column was eluted with a gradient of 35 % to 45 % CH_3CN in 0.1 % TFA for 10 min, then 45 % to 55 % for 5 min at a flow rate of 1 ml min^{-1}.

Nineteen fractions were collected manually, associated mainly with peaks. Samples of these fractions were dried in polypropylene Eppendorf tubes, previously rinsed with a 0.5 % bovine serum albumin (BSA) solution, by centrifugal evaporation (Speed-Vac). These fractions were resuspended in saline and assayed for activity on voltage-clamped ileal preparations. BSA alone had no effect on this bioassay.

Step 3: phenyl RP-HPLC

After concentration by centrifugal evaporation, the most active fraction (D) from step 2 was injected on to an Aquapore 30 nm, 7 μm RP-phenyl column (250 mm×4.6 mm) fitted with a guard column of the same type (15 mm×4.6 mm; Chromatographic Specialties, Ontario), using the same injector, pumps, controller and detector as in step 2. The column was eluted with a 28 % to 38 % CH_3CN in 0.1 % TFA gradient for 10 min at a flow rate of 1 ml min^{-1}.

Six fractions were manually collected and prepared for assay as described above. The active fraction was tested for purity by electrophoresis and by amino acid sequencing.

Step 4: phenyl RP-HPLC

The active fraction from step 3 was re-chromatographed on the RP-phenyl column isocratically at 29 % CH_3CN in 0.1 % TFA for 20 min and at a flow rate of 1 ml min^{-1}. The major peak and surrounding fractions were collected and bioassayed on ileal I_{sc}. The major peak was tested for purity by electrophoresis and by amino acid sequencing.

SDS–PAGE

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed on a PhastSystem utilizing PhastGels and PhastGel buffer strips (Pharmacia, Sweden). A high-density homogeneous polyacrylamide gel designed for the separation of peptides and small proteins (1000–10 000 relative molecular mass) was used.

Sample peptides equivalent to 10–20 CC in water were diluted (1:1) with SDS (non-reducing) or heated in the presence of SDS+β-mercaptoethanol (reducing conditions) before applying to the gel. The running conditions were; 400 V, 10 mA, 3.0 W for 25 min at 15 °C.

The relative molecular mass of the peptide was estimated by running calibration peptides on the same gel. The standard peptides used and their apparent relative molecular masses were insulin, 3.035×10^3; bovine trypsin inhibitor, 5.835×10^3; lysozyme, 14.4×10^3; α lactoglobulin, 18.4×10^3; carbonic anhydrase, 29.1×10^3.

The gels from the methods above were stained with silver as described by Heukeshoven and Dernick (1985).

Effect of proteolytic enzymes on purified CC

The purified fraction was resuspended in 0.1 mol l^{-1} Tris buffer and treated with trypsin at pH 7.6 or chymotrypsin at pH 7.8, at a concentration of 1 mg ml^{-1} for 2 h at 25 °C before testing extracts on short-circuited ilea. As controls, ileal preparations were exposed to proteases alone and to purified fractions which had been similarly incubated in the absence of proteases.

Amino acid analysis and sequencing

Amino acid analysis was conducted using an Applied Biosystems model 420A derivatizer–analyser system and sequence analysis using an Applied Biosystems 470A gas-phase sequencer with on-line PTH analyser and 900A system controller and data analyser. Both analyses were performed by Dr S. Kieland of the Regional Peptide Analysis Laboratory, University of Victoria, BC, Canada.

Results

C_4 separation

Activity from the preparative separation of whole CC, NCC, GCC and haemolymph was only associated with the 60 % CH_3CN fraction when assayed on ileal I_{sc}. At a concentration of 1 CC equivalent ml^{-1}, whole CC and NCC factors in the 60 % CH_3CN fraction produced a maximum stimulation of ileal I_{sc} (9.19±0.61 and 8.6±1.62 μequiv $cm^{-2}h^{-1}$, respectively; N=8) similar to that caused by 0.25 crude CC ml^{-1} (10.3±0.52 μequiv $cm^{-2}h^{-1}$; N=10). However, the GCC 60 % CH_3CN fraction produced very little response (2.3±0.54 μequiv $cm^{-2}h^{-1}$; N=4) even at high doses of 5 CC equivalents ml^{-1}. Haemolymph factors in the 60 % CH_3CN fraction from the equivalent of 12.5 locusts caused an increase in ileal I_{sc} of 5.53±0.62 μequiv $cm^{-2}h^{-1}$ (N=4). Control ileal I_{sc} (before fractions were tested) was generally close to zero and never greater than 1.0 μequiv $cm^{-2}h^{-1}$, as previously reported (Irvine *et al.* 1988; Audsley and Phillips, 1990).

C_8 separation

An absorption profile for HPLC separation of whole CC is shown in Fig. 1. The fractions collected are identified by letters and their effects on ileal I_{sc} are represented in Fig. 2.

Ileal I_{sc} stimulation in whole CC fractions is associated with two main peaks, D and F, which eluted from the column at CH_3CN concentrations of 38 % and 40 % respectively. A third fraction (G) had a small, but significant, effect on ileal I_{sc}. All other fractions gave no significant increase in ileal I_{sc}. Fraction D was the most potent source of stimulation, with a maximum response (9.97±0.84 μequiv $cm^{-2}h^{-1}$, N=8) observed at a concentration of 1 CC equivalent ml^{-1}. This is similar to the maximum stimulation of ileal I_{sc} achieved by crude CC extracts (0.25 CC ml^{-1}; Audsley and Phillips, 1990) and

5 mmol l^{-1} cyclic AMP (Irvine *et al.* 1988). At the same concentration, fraction F produced 46 % of this response (4.61±0.49 μequiv $cm^{-2} h^{-1}$, N=8), and fraction G only 13 % (1.30±0.44 μequiv $cm^{-2} h^{-1}$, N=8). Fraction D was also the most potent stimulant of rectal I_{sc}, but required higher doses than for ileal stimulation (Audsley, 1991).

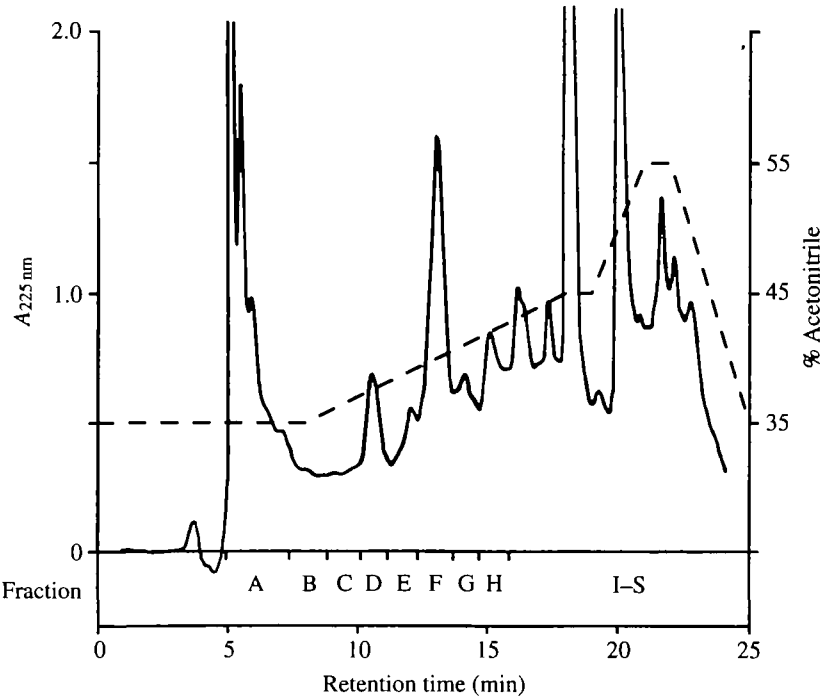

Fig. 1. Absorption profile of extracts from 300 whole corpora cardiaca (CC) from reverse-phase Nucleosil C_8 HPLC. Chromatographic conditions are described in Materials and methods. The dashed line represents the concentration of CH_3CN in 0.1 % TFA and the letters indicate the 19 fractions collected.

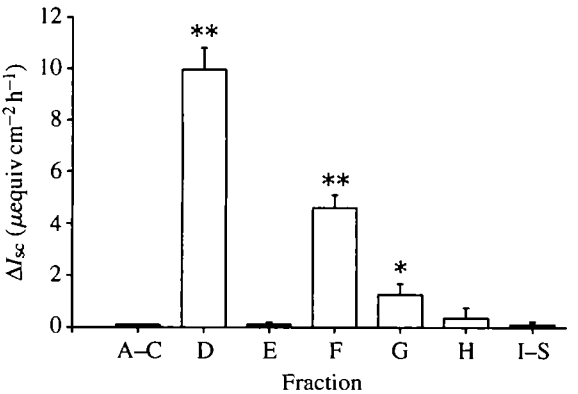

Fig. 2. The effect of the whole CC HPLC fractions (1 CC equivalent ml^{-1}) on ileal I_{sc}. ΔI_{sc} is the change from steady-state levels 1 h after addition of fractions (mean ± S.E., N=4–10). Mean increases were significantly different at P<0.05 (*) or P<0.01 (**).

The absorption profiles for HPLC separation of NCC and GCC are shown in Fig. 3A,B, and fractions stimulating ileal I_{sc} are identified by shading. NCC separation had two significant areas of activity (Fig. 3A), which correspond to the active fractions (D and F) of whole CC (Fig. 1). At a concentration of 1 CC equivalent ml^{-1}, fractions D and F from NCC separation increased ileal I_{sc} by 8.9±1.02 and 2.7±0.87 μequiv $cm^{-2}h^{-1}$, respectively (N=6). Some activity was also observed in a fraction from GCC separation (Fig. 3B) corresponding to fraction D of whole CC, but only at very high doses. An increase in I_{sc} of 1.62±0.83 μequiv $cm^{-2}h^{-1}$ was observed at a dose of 5 CC equivalents ml^{-1} (N=4).

Phenyl separation of fraction D

An absorption profile for RP-phenyl separation of fraction D from whole CC is shown in Fig. 4. The fractions collected are indicated by roman numerals. Using ileal I_{sc} as the

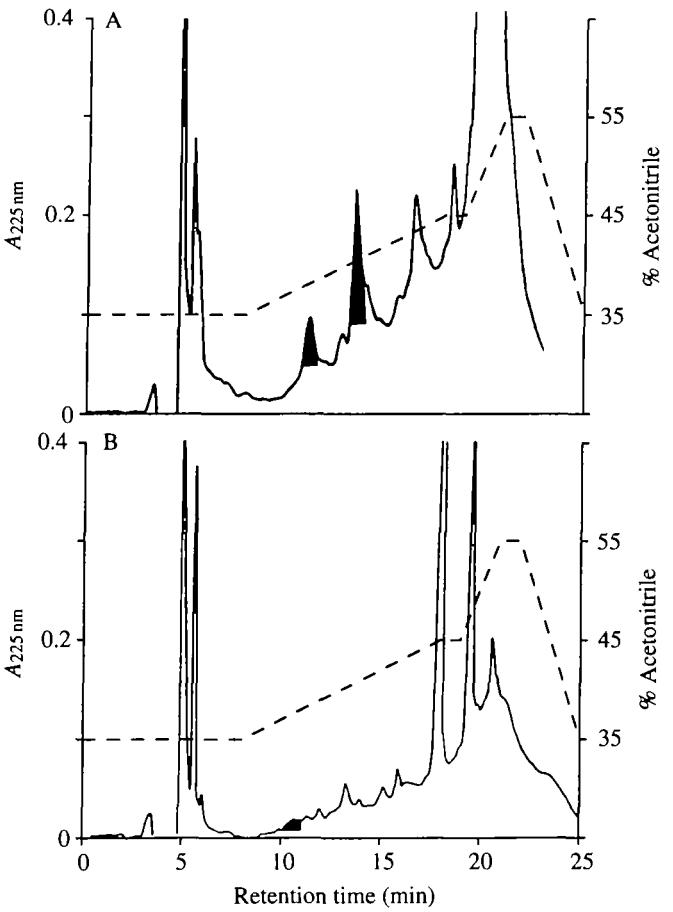

Fig. 3. Absorption profile of 50 NCC (A) and 50 GCC (B) on reverse-phase Nucleosil C_8 HPLC. Chromatographic conditions are described in Materials and methods. The dashed line represents the concentration of CH_3CN in 0.1 % TFA, and shaded areas indicate ileal I_{sc} activity.

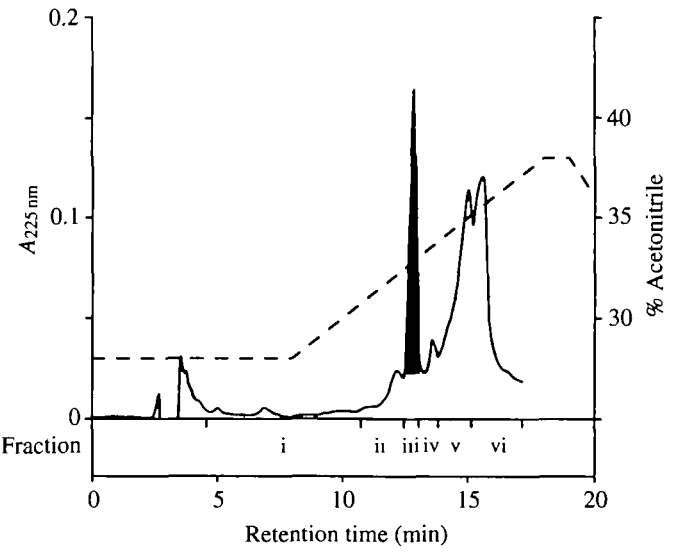

Fig. 4. Absorption profile of fraction D from phenyl RP-HPLC separation. Conditions are described in Materials and methods. The dashed line represents the concentration of acetonitrile in 0.1 % TFA. The roman numerals indicate the fractions collected, and the shaded area shows the active fraction.

bioassay, the activity from this separation was only associated with fraction iii (Diii; shaded). At a dose of 1 CC equivalent ml^{-1}, ileal I_{sc} was increased by 11.2± 1.09 μequiv $cm^{-2}h^{-1}$ (N=8), while all other fractions gave no response (N=4). This active fraction also stimulated rectal I_{sc}, but only to approximately 40 % of the maximum achieved with crude extracts, even at high doses (4 CC equivalents ml^{-1}; Audsley *et al.* 1992). Fraction Diii appeared to elute as a single peak and a single band on SDS–PAGE, but sequence analysis identified two peptides (Fig. 5). These were arbitrarily termed peptides 1 (P1) and 2 (P2). Both peptides had unblocked N termini, but the 20 residues identified had no homology with each other, and P1 was estimated to be present at 4–5 times the amount of P2.

Phenyl separation of fraction Diii (P1 and P2)

Using the same phenyl column as used in the previous chromatography step, P1 and P2 were separated by isocratic elution with 29 % acetonitrile in 0.1 % TFA. The absorption profile is shown in Fig. 6, where numbers (1–4) represent the fractions collected. The

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                    5         10        15        20
P1    S F F D I Q ? K G V Y D K S I F A R L D R I ?

P2    D A A D F G D P Y S F L D R L I E R G D
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Fig. 5. Amino acid sequences of P1 and P2 from fraction Diii.

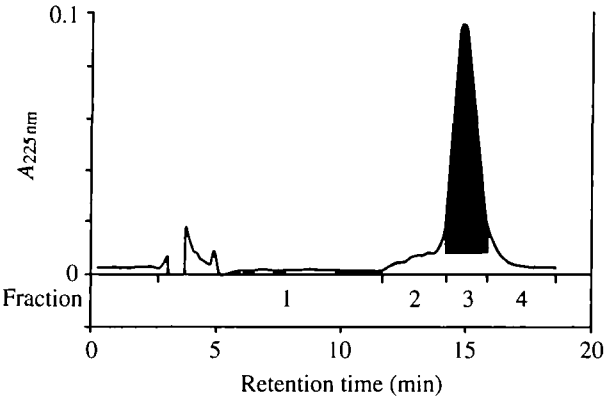

Fig. 6. Absorption profile of fraction Diii from phenyl RP-HPLC separation. Conditions are described in Materials and methods. Only fraction 3 (shaded) stimulated ileal I_{sc}.

active fraction eluted as a large single peak (3; shaded) and a small preceding shoulder (2). The peak and fractions around the peak were collected and assayed. Ileal I_{sc} activity was only associated with the major peak, which caused an increase in ileal I_{sc} of $9.96\pm0.66\,\mu equiv\,cm^{-2}h^{-1}$ (N=8) at a dose of 5.0 CC equivalents ml^{-1}. All other fractions (including P2) had no effect on ileal I_{sc} at the same dose (N=4).

SDS–PAGE

Fig. 7 shows electrophoretic separation of fraction Diii3 (lane B) by SDS–PAGE under non-reducing conditions, and calibrating peptides (lane A) used to estimate relative molecular mass. The active factor from HPLC runs as a single band, suggesting purity of this factor. The estimated relative molecular mass of Diii3 is approximately 5750 from a plot of the logarithm of relative molecular mass against relative mobility (data in Audsley, 1991).

Effect of proteolytic enzymes on fraction Diii3

The effects of pre-treatment with proteolytic enzymes on stimulatory activity in fraction Diii3 are shown in Table 1. Activity was reduced 65 % from control values by chymotrypsin and 78 % by trypsin, confirming the peptidic nature of Diii3. These results also suggest that the active peptide may contain the amino acids lysine or arginine (trypsin) and phenylalanine, tryptophan or tyrosine (chymotrypsin).

Amino acid analysis and sequence data

The amino acid analysis of Diii3 (Table 2) indicated a 65-residue peptide with a molecular mass of 7700 Da, which is greater than the value predicted by SDS–PAGE (M_r 5750). Cys and Met residues are labile under acidic hydrolysis conditions, so amino acid analysis did not accurately determine the number of these residues present.

Sequence analysis of fraction Diii3 identified one N terminal, confirming that purification of ITP was achieved. Sequence data for ITP are represented in Fig. 8 and are compared to the amino acid sequences of Cam-CHH, Orl-CHH, Hoa-MIH and Hoa-VIH

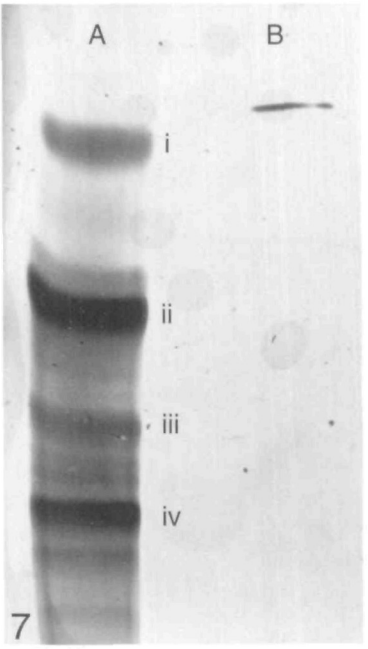

Fig. 7. SDS–PAGE of fraction Diii3 (lane B) and calibrating peptides (lane A; i, bovine trypsin inhibitor; ii, lysozyme; iii, α lactoglobulin; iv, carbonic anhydrase).

from crustaceans. A 34 amino acid sequence was obtained for ITP, including three unidentified amino acids in positions 7, 23 and 26. These sequence data confirmed that the most abundant peptide of two in fraction Diii (i.e. P1) was the active peptide. Considerable homology existed between the partial sequence of ITP and the first 34 residues of MIH (59%), Cam-CHH (44%) and Orl-CHH (50%). A 21% sequence identity was observed with VIH if a one-gap residue is inserted at position 11 of ITP.

The total amount of ITP used for amino acid analysis was estimated as 146 pmol from 300 CC equivalents. From this estimate, 1 CC equivalent was equal to 0.487 pmol (approximately) and 4.87 pmol of pure peptide was required for maximum stimulation of ileal I_{sc}.

Table 1. *Effect of protease pre-treatments of purified factor Diii3 (2.5 gland equivalents ml^{-1}) on stimulation of ileal* I_{sc}

Treatment	ΔI_{sc} (μequiv cm^{-2} h^{-1})
Control (no protease)	9.2±0.75
Chymotrypsin	3.2±1.0*
Trypsin	2.0±0.7*

Values are mean±S.E., N=4.

Protease alone at the concentration and over the period used in this experiment did not affect ileal I_{sc}.

*Highly significant difference from control value (P<0.005).

Table 2. *Amino acid analysis of Scg-ITP from fraction Diii3*

Amino acid	Residues per molecule
Asx	7.66 (8)
Glx	9.46 (9)
His	0.88 (1)
Lys	4.76 (5)
Arg	3.64 (4)
Ser	4.76 (5)
Thr	0.14 (0)
Tyr	2.38 (2)
Ala	2.00 (2)
Cys	1.3 (1)
Phe	5.78 (6)
Gly	2.3 (2)
Ile	4.08 (4)
Leu	7.82 (8)
Met	0.74 (1)
Pro	1.86 (2)
Val	1.70 (2)
Total	65

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                                5          10          15          20          25
Scg-ITP          S F F D I Q ? K G V   X D K S I F A R L D R I ? E D
Cam-CHH          pE I Y D T S C K G V   X D R A L F N D L E H V C D D
Orl-CHH          pE V F D Q A C K G I   X D R A I F K K L D R V C E D
Hoa-MIH          pE V F D Q A C K G V   X D R N L F K K L D R V C E D
Hoa-VIH  A S A W F T N D E C P G V M G N R D L Y E K V A W V C N D

                      30
Scg-ITP  ? X N L F R E P Q
Cam-CHH  C X N L Y R T S Y V A S A C R S N C Y S N L V F R Q C M D
Orl-CHH  C X N L Y R K P Y V A T T C R Q E C Y A E S V F R Q C L D
Hoa-MIH  C X N L Y R K P F V A T T C R E N C Y S N W V F R Q C L D
Hoa-VIH  C A N I F R N N D V G V M C K K D C F H T M W F L W C V Y

Cam-CHH  D L L M M D E F D Q Y A R K V Q M  V-NH2
Orl-CHH  D L L L I D V L D E Y I S G V Q T  V-NH2
HoA-MIH  D L L L S D V I D E Y V S N V Q  M-NH2
Hoa-VIH  A T E R H G E I D Q F R K W V S I L R
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Fig. 8. Comparison between the amino acid sequences of *S. gregaria* ion-transport peptide from fraction Diii3 (Scg-ITP), hyperglycaemic hormone from the shore crab *Carcinus maenas* (Cam-CHH) and the crayfish *Orconectes limosus* (Orl-CHH) and moult-inhibiting hormone (Hoa-MIH) and vitellogenesis-inhibiting hormone (Hoa-VIH) from the lobster *Homarus americanus*.

Discussion

Ion-transport peptide (Scg-ITP or ITP) was purified from *S. gregaria* CC by a four-step procedure, utilising RP-HPLC for separation and ileal I_{sc} as the main bioassay. ITP is the most potent stimulant of ileal I_{sc} in the CC, while the other factors (in fractions F and G) remain to be identified.

Purification of ITP caused a 20-fold loss in activity present in crude CC: 5 CC equivalents ml^{-1} of pure peptide was required to elicit a maximal ileal I_{sc} response, compared to 0.25 CC equivalent ml^{-1} of crude CC extract. Most of this loss (80%) occurred during the final separation step, but the active factor corresponded to the major of two peptides in fraction Diii from the penultimate purification step, when loss was much reduced. It was estimated that the amount of pure ITP recovered was equivalent to 0.487 pmol per CC. This was comparable to the amount of diuretic peptides detected in the CC of *Manduca sexta* (approximately 0.5 pmol; Kataoka *et al.* 1989) and *L. migratoria* (2.0 pmol; Morgan *et al.* 1987).

ITP is the first insect peptide purified which has been shown to act directly on the transport of a specific ion (i.e. Cl^-) in an insect excretory system, and the first peptide isolated which influences insect ileum. In an accompanying paper, Audsley *et al.* (1992) report that purified ITP also influences ileal transport of K^+, Na^+, H^+ and fluid.

HPLC separation of NCC and GCC extracts indicates that ITP is located in the NCC. The trace activity in the GCC may be due to contamination with neurosecretory material in stalks of the NCC which project into the GCC (see Highnam, 1961). Some part of these stalks may have been dissected with the GCC, although attempts were made to avoid this (see Materials and methods).

Spring and Phillips (1980) proposed that CTSH, which stimulates rectal I_{sc} and J_v, was released into the haemolymph on feeding. The same may also be true of CC factors acting on ileal transport, because a factor in locust haemolymph which stimulates ileal I_{sc} eluted from the C_4 preparative cartridge in the same fraction (60% CH_3CN) as active CC factors, including ITP.

Although Nps stimulate fluid reabsorption across rectal sacs of *L. migratoria in vitro*, it has not been established whether these factors act by altering the osmotic permeability of the rectal epithelia or by stimulating the transport of a specific ion or solute, which drives the secondary movement of water. Possible actions of Nps have not been reported for any ion-transport process or on ileal fluid absorption. Fournier *et al.* (1987) demonstrated that crude extracts of CC only stimulated the reabsorption of water in the presence of Cl^-. It therefore seems probable that Nps may act first by stimulating the transport of this ion, and might be expected to have a similar physiological function to ITP and CTSH.

There is no sequence homology between ITP (first 34 residues) and NpA or NpB. Clearly ITP and Nps are different compounds. ITP and Nps influence reabsorption across the locust hindgut, but they act on different tissues (ileum and rectum respectively) and therefore may not be expected to have similar structures. Conceivably Nps could be related to CTSH, which acts on the rectum, but CTSH must first be characterized to determine whether this is the case.

The partial purification of CTSH from the CC of *S. gregaria* reported by Phillips *et al.* (1980) estimated a relative molecular mass of approximately 8000 by gel filtration

chromatography. Although CTSH is approximately the same size as ITP, it is unlikely that they are the same compound because of the much reduced stimulatory effect of ITP on rectal I_{sc} and their different extraction properties (Phillips *et al.* 1980; Audsley and Phillips, 1990; Audsley *et al.* 1992). Moreover, ITP does not stimulate rectal fluid transport (J_v) or potassium permeability (I_K), whereas crude CC containing CTSH stimulates both rectal J_v and I_K in a similar manner to cyclic AMP (Audsley, 1991). The ileum and rectum of the desert locust therefore appear to be influenced by different factors (ITP and CTSH respectively); however, this prediction must be confirmed by characterizing CTSH and testing its actions on the ileum.

ITP has structural similarities with a novel neuropeptide family from crustaceans and is the first non-crustacean member to be reported. ITP (estimated 65 residues; 7700 Da) is of similar size to Cam-CHH (72 residues; 8524 Da), Orl-CHH (72 residues; 8400 Da), Hoa-MIH (71 residues; 8483 Da) and Hoa-VIH (77 residues; 9135 Da) and the partial sequence of ITP has considerable homology with the first 34 residues of all these peptides (Fig. 7; Chang *et al.* 1990; Kegel *et al.* 1989, 1991; Soyez *et al.* 1991). It is probable that the three unknown residues in the partial sequence of ITP are Cys, because they align with the crustacean Cys, and in all four crustacean peptides the six Cys residues present occupy identical positions. This will be determined by further sequence analysis after reduction and carboxymethylation of ITP. Both ITP and VIH have unblocked N termini, whereas MIH, Orl-CHH and Cam-CHH have a pGlu N terminus.

The adipokinetic hormone/red pigment-concentrating hormone peptide family is a large group of peptides isolated from the neurohaemal organs of insects and crustaceans. They are structurally related but functionally diverse (reviewed by Gäde, 1990). These reports of ITP, MIH, VIH and CHH may be the first evidence of another such family of arthropod peptides.

In conclusion, a unique peptide from the CC of *Schistocerca gregaria* which stimulates Cl^--dependent I_{sc} across the ileum *in vitro* has been purified and partially sequenced.

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