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LOCUST ION TRANSPORT PEPTIDE (ITP): PRIMARY STRUCTURE, cDNA AND EXPRESSION IN A BACULOVIRUS SYSTEM

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Summary

Ion transport peptide (ITP) purified from locust nervous corpus cardiacum (CC) has previously been shown to stimulate salt and water reabsorption and inhibit acid secretion in the ileum of *Schistocerca gregaria*. We used the partial amino acid sequence of purified ITP to derive degenerate primers. These were used to amplify a cDNA from brain RNA using reverse transcription and the polymerase chain reaction (RtPCR). This sequence was extended using anchored PCR to yield a partial, 517 bp cDNA clone. This cDNA encodes a putative ITP prohormone which could be cleaved at two dibasic amino acid sites to yield a 72 residue active amidated peptide. The deduced amino acid sequence from the cDNA agrees completely with the amino acid sequence and molecular mass (8564 Da) derived from analysis of purified ITP. Relative to a family of crustacean hyperglycaemic hormones (CHH), all six cysteine residues and many other amino acid residues are conserved in ITP, establishing that ITP is a homologue. However, CHH, crab eyestalk and CC extracts from distantly related insects have no action, whereas CC extracts from closely related insects are active on the locust ITP assay, showing that the bioassay is selective. Insect Sf9 cells transfected with a baculovirus containing our partial cDNA secreted a potent stimulant of locust ileal transport, confirming that the peptide encoded by our ITP clone has biological activity. The mRNA for ITP is restricted to the brain and CC. Interestingly, a related mRNA is observed in other tissues which are not active on the ITP bioassay.

Key words: *Schistocerca gregaria*, locust, ion transport peptide (ITP), cDNA sequence, amino acid sequence, baculovirus expression, tissue mRNA, receptor selectivity.

Introduction

Osmotic and ionic homeostasis and body water content in insects depend on the excretory system and its hormonal control (reviewed by Phillips *et al.* 1986, 1988; Phillips and Audsley, 1995). The production of the primary urine by Malpighian tubules and its hormonal control have been well studied (Coast *et al.* 1994; Patel *et al.* 1995; Wheeler and Coast, 1990; Pannabecker, 1995). However, regulation of haemolymph composition and body water content ultimately depends on controlled and selective reabsorption of essential solutes and most of the Malpighian tubule fluid in the anterior (ileum) and posterior (rectum) hindgut. The locust (*Schistocerca gregaria*) ileum is functionally analogous to the proximal tubules of vertebrate kidneys (Phillips *et al.* 1994; Audsley *et al.* 1992*a*) in that bulk iso-osmotic reabsorption of fluid occurs as a consequence of active transport of Cl^- and Na^+, and passive movement of K^+ by electrical coupling. H^+ and NH_4^+ secretion associated with bicarbonate absorption in locust ileum contribute to pH regulation and nitrogen excretion by cell transport mechanisms which are also analogous to those in vertebrate proximal kidney tubule epithelia. The locust ileum is the major site of Na^+ reabsorption, NH_4^+ secretion and their control. The applied electrical current (short-circuit current, I_{sc}) required to clamp trans-ileal voltage at 0 mV is a direct measure of active Cl^- transport after stimulation of both locust ileum and rectum. Measurement of I_{sc} across flat sheet preparations of locust ileum and rectum has been used as a bioassay to survey the locust neuroendocrine system for stimulants of salt (Cl^-) and hence fluid reabsorption, which is dependent on Cl^- transport (i.e. antidiuretic activity; Phillips *et al.* 1982; Lechleitner and Phillips, 1989; Audsley and Phillips, 1990; Proux *et al.* 1984, 1985).

Previous workers in our laboratory have identified neuropeptide stimulants with different physico-chemical properties in locust corpus cardiacum (nervous lobe), and

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hence brain, that act on the ileum (ion transport peptide, ITP: Audsley and Phillips, 1990; Audsley *et al.* 1992*a*,*b*) and rectum (chloride transport stimulating hormone, CTSH; Phillips *et al.* 1980). Neuroparsins (Girardie *et al.* 1989) from the nervous corpora cardiaca are reported to stimulate fluid reabsorption by rectal sacs of *Locusta migratoria* (Fournier and Girardie, 1988), but Jeffs and Phillips (1996; also Jeffs, 1993) observed no effect of this neuropeptide on either I_{sc} or fluid transport across *Schistocerca gregaria* ileal and rectal preparations. Saline extracts of locust ventral ganglia also stimulate I_{sc} and fluid transport across both hindgut segments (Audsley and Phillips, 1990). However, the ventral ganglia factor has quite different extraction properties and a distinct I_{sc} time course compared with nervous corpora cardiaca stimulants. Moreover, Bilgen (1994) in our laboratory has recently partially purified the acid-labile ventral ganglia factor, which has an approximate molecular mass (37 000 Da) four times that of either ITP (Audsley *et al.* 1992*b*) or CTSH (Phillips *et al.* 1980). The haemolymph of locusts has both ITP-like and CTSH-like activity on hindgut I_{sc} (Phillips *et al.* 1982; Audsley *et al.* 1992*a*). ITP and CTSH both apparently use cyclic AMP as a second messenger, with the exception of ITP action on ileal acid secretion (Chamberlin and Phillips, 1988; Audsley and Phillips, 1990; Audsley *et al.* 1992*b*).

Using reverse-phase HPLC, Audsley *et al.* (1992*a*,*b*, 1994) purified ITP (which has a reported molecular mass of 8652±3 Da measured by mass spectroscopy) and obtained a partial amino acid sequence (33 amino acid residues; Audsley *et al.* 1994). At picomole levels, ITP fully stimulates (compared with cyclic AMP or crude nervous corpora cardiaca extracts) Cl^-, Na^+, K^+ and fluid reabsorption and almost completely inhibits acid secretion by locust ileum. Thus, a single neuropeptide (ITP) mimics all of the actions of crude nervous corpora cardiaca extracts on the ileum (Audsley *et al.* 1992*b*). After several exhaustive and unsuccessful attempts to complete the amino acid sequence of ITP by enzymatic cleavage and HPLC purification of the products, we used the polymerase chain reaction (PCR) to recover a cDNA which we cloned and sequenced.

In this paper, we report the complete amino acid sequence of an ITP propeptide deduced from its cDNA nucleotide sequence; ITP appears to be cleaved from this propeptide at dibasic amino acid sites. Our predicted amino acid sequence for ITP was recently published in abstract form (Meredith *et al.* 1995). To confirm that we have obtained a cDNA for ITP, we used a baculovirus expression system to obtain a culture medium containing a factor(s) that fully stimulates ileal I_{sc}.

ITP has considerable sequence similarity to a family of crustacean hormones of similar length (about 72 amino acid residues), including hyperglycaemic (CHH), moult-inhibiting (MIH) and vitellogenesis-inhibiting (VIH) factors from several species (reviewed by Audsley *et al.* 1992*a*, 1994). ITP is the first non-crustacean member of this protein family to be described. We report that CHH and crude extracts of crab eyestalk expected to contain related neuropeptides have no stimulatory effect on locust ileal I_{sc}, confirming the high specificity of our bioassay (i.e. ITP receptor selectivity). Only corpora cardiaca extracts from more closely related insects (e.g. other locusts, crickets, cockroaches) stimulate locust ileal I_{sc}, whereas those from more distantly related orders (Diptera, Lepidoptera, Hymenoptera) do not, suggesting considerable sequence divergence of ITP-like neuropeptides amongst insects. Finally, we have used PCR to study the tissue distribution of ITP mRNA in locusts. As predicted, ITP mRNA was detected only in the brain and corpus cardiacum, consistent with the bioassay results for ITP.

Materials and methods

Adult *Schistocerca gregaria* Forskål 2–4 weeks past their final moult and reared as described previously (Audsley *et al.* 1992*a*,*b*) were used.

ITP-like activity was tested in physiological saline extracts of whole heads, brains and corpora cardiaca of several insects obtained from laboratory colonies at the University of British Columbia, Simon Fraser University and York University. These included adults of the following: the cockroach *Periplaneta americana*; the grasshopper *Melanoplus sanguinipes*; the cricket *Acheta domesticus*; a hemipteran, *Rhodnius prolixus*; a dipteran, *Neobellaria* (=*Sarcophaga*) *bullata*; a lepidopteran, *Galleria mellonella*; and a hymenopteran, *Vespula pennsylvanica* (collected locally). In addition, a larval lepidopteran, the cutworm *Peridroma saucia* (last instar), was tested. Eyestalks of the green shore crab (*Hemigrapsus oregonensis*) were tested immediately after their collection from the local seashore.

Locust ileal I_{sc} *bioassay*

The bioassay for ITP was identical to that described by Hanrahan *et al.* (1984) and used by Audsley *et al.* (1992*a*,*b*) to purify ITP from corpora cardiaca of *Schistocerca gregaria*. Ilea were mounted as flat sheets between modified Ussing chambers containing 2 ml of physiological saline resembling locust haemolymph at 22±2 °C. Saline was oxygenated and mixed by bubbling with 95 % O_2/5 % CO_2. The applied transepithelial current required to maintain the measured transepithelial potential (PD) at 0 mV (i.e. the short-circuit current, I_{sc}) was continuously recorded. The increase in I_{sc} (ΔI_{sc}) caused by stimulants is a direct measure of active transport of Cl^- from the lumen side. Open-circuit PD was measured and transepithelial resistance calculated at intervals in most experiments by briefly stopping the I_{sc} as previously described. The I_{sc} was allowed to fall close to zero (usually slightly below) over the first hour after dissection of ilea. Crude extracts of *Schistocerca gregaria* corpora cardiaca and purified ITP on the haemocoel side increase the value of I_{sc} from near 0 μequiv h^{-1} cm^{-2} to a maximum of 13±3 μequiv h^{-1} cm^{-2} in a dose-dependent manner (Audsley and Phillips, 1990; Audsley *et al.* 1992*a*,*b*): maximum stimulation is achieved with 0.25 locust corpora cardiaca ml^{-1} and 2.4 pmol ml^{-1} of pure ITP. As little as 0.01 locust corpora cardiaca ml^{-1} gives a measurable ΔI_{sc} and half-maximal ΔI_{sc} is achieved within 15 min at higher dosages.

To detect ITP-like activity, 1–50 μl samples of solution containing the test sample were added to the 2 ml of saline bathing the haemocoel side of ilea, with appropriate solvent controls if physiological saline was not used. Various animal tissues were homogenized in locust saline at 4 °C, centrifuged, and the supernatant tested on ileal I_{sc} as described previously for locust corpora cardiaca by Audsley and Phillips (1990). *Carcinus* hyperglycaemic hormone obtained from Dr R. Keller (University of Bonn) was dissolved with vortexing in saline containing 0.001 % bovine serum albumin to reduce surface binding of neuropeptides (see Audsley *et al.* 1992*a*,*b*). Viability of ileal preparations was confirmed at the end of each experiment by maximum stimulation of I_{sc} with locust corpora cardiaca extracts.

Isolation of total and poly(A+) RNA

Tissues were dissected from adult *Schistocerca gregaria* into liquid nitrogen and stored at −80 °C until use. Total RNA was extracted using the acid guanidinium thiocyanate–phenol–chloroform procedure of Chomczynski and Sacchi (1987) or using total RNA extraction reagent (Trizol, Gibco BRL). Poly(A+) RNA was selected from total RNA using oligo dT cellulose (Celano *et al.* 1993) giving about 0.85 μg of total RNA and 0.03 μg of poly(A+)-enriched RNA per brain. For library construction, tissue-specific mRNAs were isolated from 1 mg of total RNA using the PolyATract mRNA isolation system (Promega).

Oligonucleotide synthesis

All gene-specific oligonucleotides were synthesized using an automated DNA synthesizer (Applied Biosystems 380B). They were deprotected under standard conditions and purified using *n*-butanol (Sawadogo and Van Dyke, 1991). In some cases, the sequences synthesized included a restriction site and flanking region at the 5′ end.

cDNA synthesis and amplification by polymerase chain reaction (PCR)

Total RNA (1 μg) from brain was reverse-transcribed at 37 °C for 1 h using 50 pmol of random hexamer (Pharmacia) and 200 units (for all units of enzyme activity, see manufacturers' specifications) of Moloney murine leukaemia virus reverse transcriptase (Superscript, Gibco BRL) in a 20 μl reaction mixture containing 50 mmol l^{-1} Tris/HCl (pH 8.3), 75 mmol l^{-1} KCl, 10 mmol l^{-1} dithiothreitol, 3 mmol l^{-1} $MgCl_2$, 0.5 mmol l^{-1} each dNTP and 20 units of human placental ribonuclease inhibitor (Gibco BRL).

cDNA-encoding amino acids at positions 2–30 of ITP (Audsley *et al.* 1992*a*) were amplified using 20 pmol of two degenerate oligonucleotide primers (1 and 2) based

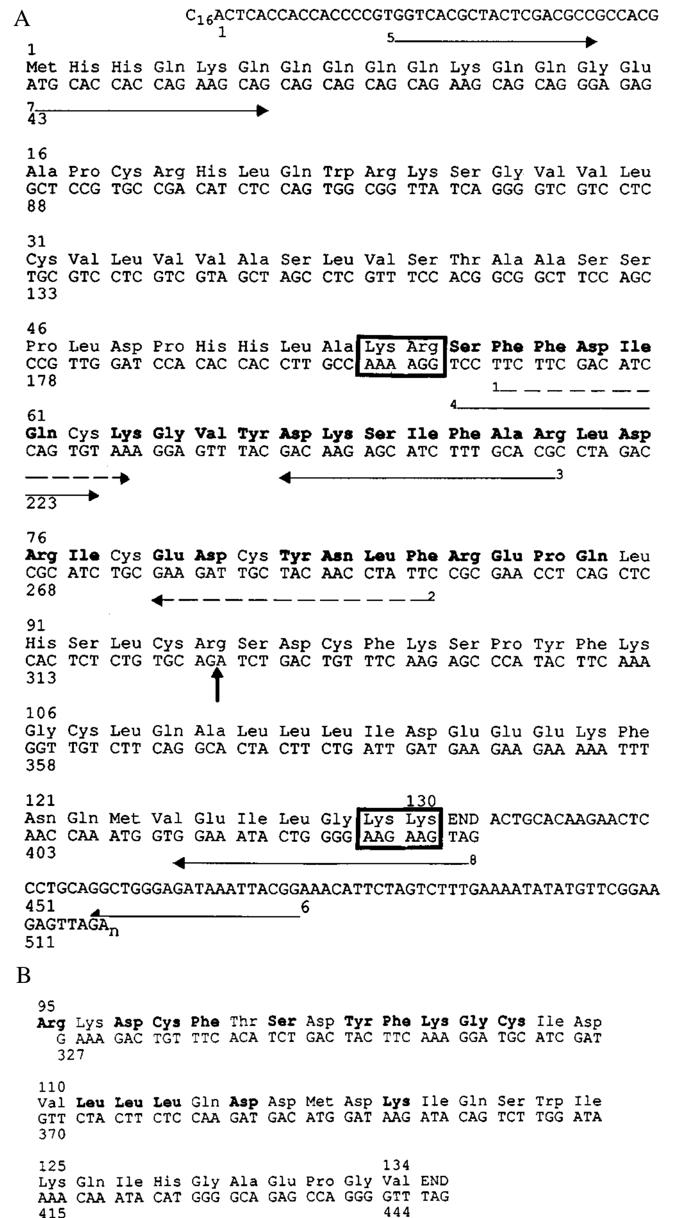

Fig. 1. (A). Nucleotide and deduced amino acid sequence of a partial ion transport peptide (ITP) cDNA. Nucleotides are numbered below the sequence and amino acid residues beginning with the open reading frame are numbered above the sequence. Positions of primers described in the text are indicated with numbered arrows (degenerate primers with dashed arrows) and the vertical arrow indicates the position of the ITP-L insert (see B,C). Dibasic cleavage sites are boxed. A known partial amino acid sequence of ITP is printed in bold type. (B) Nucleotide and deduced amino acid sequence of ITP-L insert. The position of the insert is indicated in A and C. Amino acid residues conserved in the C-terminal region of the two ITP forms are indicated in bold type. (C) Comparison of ITP (upper bar) and ITP-L (lower bar) cDNA open reading frames. Numbers above bars correspond to those in A and B. Shaded boxes represent dibasic cleavage sites and black boxes represent termination codons. The dashed lines indicate co-linear sequences and the hatched box represents the sequence found in ITP-L but absent from ITP.

on the partial amino acid sequence reported and assuming cysteine residues at positions 7 and 26 (Fig. 1: primer 1, sense strand amino acid residues 57–63; 5′-TTT/CTTT/CGAT/CATICAA/GTGT/CAA-3′; and primer 2, antisense strand amino acid residues 79–85; 5′-A/GAAIAGA/GTTA/GTAA/GCAA/GTCT/CTC-3′). PCR was conducted using 2.5 units of Taq DNA polymerase (Boehringer Mannheim) in a 100 μl reaction volume containing 33.3 mmol l^{-1} Tris/HCl (pH 8.3), 50 mmol l^{-1} KCl, 6 % dimethylsulphoxide, 0.2 mmol l^{-1} each dNTP and 3–10 mmol l^{-1} $MgCl_2$. A Perkin-Elmer/Cetus DNA thermal cycler was programmed as follows: 94 °C for 2 min, followed by 35 cycles consisting of denaturation at 94 °C for 1 min, annealing at 37 °C for 1 min, extension at 72 °C for 1.5 min, followed by a final extension at 72 °C for 3 min.

Analysis of PCR products

PCR products were separated on a 2 % agarose gel, purified by excision and electroelution and cloned into the *Sma*I site of pBluescript KS^+ (Stratagene) using the method of Liu and Schwarz (1993). DH5α cells were transformed and transformants selected using standard techniques (ampicillin resistance and α complementation of β-galactosidase, Sambrook *et al.* 1989) and by hybridization screening. Individual bacterial colonies were grown on nitrocellulose filters (Hybond, Amersham) and hybridized to primer 2 end-labelled with [γ-^{32}P]ATP (Amersham) and 8 units of T4 polynucleotide kinase (Promega) at 42 °C overnight in a solution containing 20 % formamide, 0.2 % SDS, 5.8× SSPE (180 mmol l^{-1} NaCl, 10 mmol l^{-1} NaH_2PO_4, 1 mmol l^{-1} EDTA), 0.2 mg ml^{-1} salmon sperm and 0.5× Denhardt's solution (Sambrook *et al.* 1989). They were washed twice in 1× SSPE at 37 °C and exposed to Kodak X-OMAT AR film overnight. Plasmids from positive colonies were sequenced.

5′ extension and amplification of ITP cDNA

The 5′ RACE System (Gibco BRL) was used according to the manufacturer's instructions. Total brain RNA (1 μg) was reverse-transcribed using 25 pmol of primer 2 and PCR was performed with 20 pmol of anchor primer supplied by the manufacturer and primer 3 (antisense strand encoding amino acid residues 67–73: Fig. 1; 5′-CCACGAATTCC-GTGCAAAGATGCTCTTGTC-3′) as described previously. PCR products were purified, cloned and sequenced as before.

3′ extension and amplification of ITP cDNA

The 3′ RACE system (Gibco BRL) was used according to the manufacturer's instructions with the following changes. 1 μg of poly(A+)-enriched (10×) brain RNA was reverse-transcribed at 45 °C using the anchor primer supplied. The resulting cDNA was amplified using 10 pmol each of the supplied universal primer and primer 4 (sense strand amino acid residues 56–62 of Fig. 1; 5′-CCACGGATCCTCCTTC-TTCGACATCCAGTGT-3′). PCR conditions were as described previously with the exception that annealing temperatures were reduced from 61 to 55 °C over eight cycles followed by 27 cycles of annealing at 55 °C. PCR products were purified, cloned and sequenced as before.

Library construction

Tissue-specific cDNA libraries were constructed using the lambda ZAP Express vector (Stratagene). A brief summary of the procedures follows. Tissue-specific poly(A+) RNA (10 μg) was denatured at 65 °C for 5 min and reverse-transcribed in a 100 μl reaction volume with avian myoblastosis virus reverse transcriptase (USB) at 42 °C for 1.5 h. The reaction was primed with 2.5 μg of oligo $dT_{12–18}$ (Pharmacia). Second strand cDNA was synthesized at 16 °C for 3 h using 100 units of DNA polymerase I (Gibco BRL) and 10 units of RNAase H (Pharmacia) in a 400 μl reaction volume. The double-stranded cDNA was extracted, made blunt-ended using the Klenow fill-in reaction (Sambrook *et al.* 1989) and ligated to *Eco*RI-*Not*I adaptors (Promega) using 1 unit of T_4 ligase (Boehringer Mannheim) at 8 °C overnight. Unligated adaptors and small DNA (less than 400 bp) were removed using a SizeSep 400 spin column (Pharmacia). The size-selected cDNA was extracted and phosphorylated with [γ-^{32}P]ATP and 8 units of T_4 polynucleotide kinase (Promega) and ligated to *Eco*RI-digested lambda ZAP Express vector. Packaging the cloned cDNA and subsequent manipulations of the libraries were performed according to instructions from Stratagene.

Northern analysis

Total RNA (5 μg) from brain was denatured in buffer containing 1 mol l^{-1} *N*-(morpholino)-propane-sulphonic acid, 3 % formaldehyde, 50 % formamide and 0.02 mg ml^{-1} ethidium bromide and separated on an agarose (1.1 %) formaldehyde (17.7 %) denaturing gel. The RNA was transferred overnight to a nitrocellulose membrane and cross-linked using ultraviolet radiation. The membrane was hybridized to a 461 bp probe amplified from total RNA by PCR using primers 5 (Fig. 1; 5′-TGGTCACGCTACTCGACGCC-3′) and 6 (Fig. 1; 5′-TCCGTAATTTATCTCCCAGCC-3′) under conditions already described. The probe was randomly primed using [α-^{32}P]ATP (Amersham) and 3 units of Klenow fragment (Gibco BRL) to a specific activity of 2.8×10^9 cts min^{-1} μg^{-1}. Filters were hybridized overnight at 45 °C in a solution containing 50 % formamide, 0.5× Denhardts, 5× SSPE, 0.1 % SDS and 0.2 mg ml^{-1} salmon sperm DNA. They were washed at increasing temperatures up to 60 °C in 1× SSPE + 0.1 % SDS and exposed to Kodak X-OMAT AR film overnight.

Baculovirus expression

Total brain RNA (1 μg) was reverse-transcribed and amplified as previously described using primers 7 (sense strand amino acid residues 1–6 of Fig. 1; 5′-CCACCCCGGGATGCACCACCAGAAGCAGCAG-3′) and 8 (antisense strand amino acid residues 124–130, two stop codons of Fig. 1; 5′-CCACGAATTCCTACTACTTCTTC-CCCAGTATTTCC-3′). The resulting 416 bp PCR product

was cloned into the baculovirus transfer vector pVL1393 (Webb and Summers, 1990) and recombinant plasmids were selected as described previously and sequenced. Fresh cultures of *Spodoptera frugiperda* (Sf9) cells were co-transfected with recombinant plasmid and linearized viral DNA (Baculogold, Pharmagen). Individual plaques were purified and propagated using standard techniques (Summers and Smith, 1987). After 13 days, the culture medium (supernatant) and cultured infected cells were separated by centrifugation (735 ***g***, 5 min). Cells were resuspended in one-third of their volume of saline and lysed by sonication for 180 s on ice. Cell debris was removed by centrifugation (16 000 ***g***, 10 min) and cell lysate and culture medium were then tested on short-circuited ilea.

Sequencing

Both strands of double-stranded DNA were sequenced using the dideoxy chain-termination method (Sanger *et al.* 1977) and sequenase (USB). Both universal primers for pBluescript as well as gene-specific primers were used for sequencing.

Results

cDNA and predicted ITP sequence

Degenerate primers (dashed arrows) deduced from a partial amino acid sequence of ITP (bold type; indicated in Fig. 1A), were used to amplify a cDNA from brain mRNA. After confirming its sequence, 5′ and 3′ RACE were used to obtain sequences extending further 5′ and 3′, respectively, to yield a 517 bp cDNA whose sequence is shown in Fig. 1A. The cDNA encodes a complete open reading frame of 130 amino acid residues, beginning with a methionine codon and ending with an in-frame termination codon, showing that we have the complete peptide sequence.

Comparison of the deduced amino acid sequence of the cDNA with the partial amino acid sequence of purified ITP obtained by Audsley *et al.* (1992*a*) reveals several interesting features (Fig. 1A,C). First, the ITP peptide sequence determined by Audsley *et al.* (1992*a*) begins 56 residues downstream from the putative initiation codon of the cDNA open reading frame, suggesting that the cDNA encodes a propeptide. This suggestion is supported by the observation that a dibasic cleavage site at residues 54–55 (Lys-Arg) (boxed Fig. 1A, shaded box Fig. 1C) lies immediately upstream from the partial ITP sequence. There is another putative dibasic cleavage site (Lys-Lys) at residues 129–130. Second, the deduced amino acid sequence from the cDNA agrees with all of the partial sequence established by Audsley *et al.* (1992*a*; residues 56–89 Fig. 1A) and confirms the presence of cysteines at positions 62, 78 and 81.

Ileal library clone

We probed an ileal mRNA library using standard conditions with the two PCR clones obtained from 5′ and 3′ extension of ITP cDNA as described above. A clone positive to both probes containing a 1.9 kb insert was isolated and sequenced. The sequence of this clone is identical to that of the brain cDNA except for an additional 121 base pairs (Fig. 1B) starting at nucleotide position 327 (vertical arrow Fig. 1A). We have termed this clone ITP long form (ITP-L). Fig. 1C compares features of ITP and ITP-L. ITP-L has an open reading frame of 134 residues. The positions of the six cysteines are conserved, as is one dibasic cleavage site (residues 54, 55). Of the 40 C-terminal amino acids of ITP-L, 15 are conserved (Fig. 1B bold type) relative to ITP.

Analysis of ITP transcripts

Primers derived from the untranslated regions (primers 5 and 6 in Fig. 1A) were used in RtPCR with brain RNA. Two fragments were obtained, the expected fragment of 461 bp and a larger fragment of about 580 bp (data not shown). The smaller fragment was purified and used to probe brain RNA on a northern blot, which revealed the presence of two mRNAs of 1.5 and 1.9 kb (Fig. 2). Together, these data suggest that there are two ITP transcripts present in the brain. The northern analysis also suggests that our shorter cDNA lacks about 1.0–1.4 kb. Analysis of clones from cDNA libraries indicates that the missing sequence is at the 3′ end of the transcript (J. Meredith and M. Ring, unpublished data).

To determine the tissue distribution of ITP and ITP-L mRNAs, cDNA produced from 1 μg of total RNA from flight muscle, Malpighian tubules, ilea, ventral ganglia, corpora cardiaca and brains was amplified using primers 5 and 8 (Fig. 1). A 430 bp product (432 bp is the expected size of ITP) predominated in brain, was clearly detected in the corpora cardiaca and was not detected in the other tissues (Fig. 3). A larger 550 bp product (553 bp is the expected size of ITP-L) was present in every tissue examined.

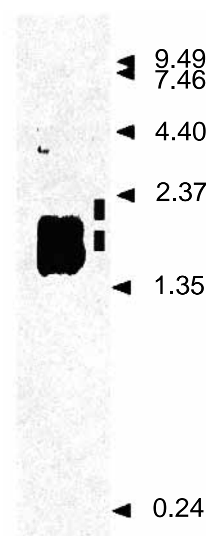

Fig. 2. Northern analysis of total brain RNA hybridized with 461 bp probe (see Materials and methods). Positions of ribosomal bands are indicated by a bar, whereas positions of RNA standards are shown on the right-hand edge (sizes in kb).

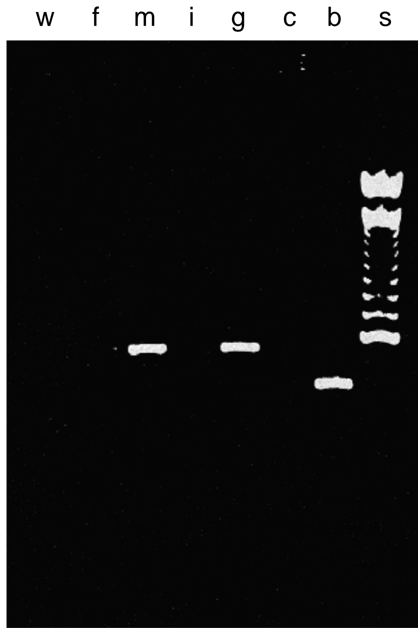

Fig. 3. Tissue distribution of ITP mRNA. RtPCR using primers 5 and 8 was performed with 1 μg of total RNA extracted from various tissues. w, water control; f, flight muscle; m, Malpighian tubules; i, ilea; g, ventral ganglia; c, corpora cardiaca; b, brain; s, 100 bp DNA ladder. A 430 bp product is detected in RNA from brain and corpora cardiaca and a 550 bp product is detected in all tissues examined.

Ileal I_{sc} *bioassays*

Saline extracts of whole heads from a variety of insect species were first tested to detect any ITP-like activity in brain or corpora cardiaca. Heads from insects not closely related to locusts had no effect on ileal I_{sc}. These include the wax moth *Galeria mellonella* at dosages up to 3.5 heads ml^{-1} (N=6), the flesh fly *Neobellaria bullata* at up to 1.5 heads ml^{-1} (N=6), the wasp *Vespula pennsylvanica* at 1.5 heads ml^{-1} (N=3), the blood-sucking bug *Rhodnius prolixus* at doses of 3.0 heads ml^{-1} (N=3) and the variegated cutworm *Peridroma saucia* at 3.0 heads ml^{-1} (N=3). Likewise, eyestalks of the crab *Hemigrapsus oregonensis* (5 eyestalks ml^{-1}; N=4), which should contain several crustacean neuropeptide hormones related to locust ITP, had no effect on locust ileal I_{sc}.

Heads from insects more closely related to *Schistocerca gregaria* all stimulated locust ileal I_{sc} maximally (i.e. as determined by thousands of assays with locust corpora cardiaca and cyclic AMP). We therefore tested corpora cardiaca (CC) dissected from these species at three or more concentrations to obtain crude dose–response relationships (Fig. 4). Approximately 10-fold higher dosages (CC equivalents ml^{-1}) of cricket (*Acheta domesticus*) and cockroach (*Periplaneta americana*) corpora cardiaca were required for full stimulation of locust ileal I_{sc} compared with corpora cardiaca from *Schistocerca gregaria* or the grasshopper *Melanoplus sanguinipes*. These differences may be partially explained by the different sizes and neuropeptide contents of corpora cardiaca from these species. However, subsequent extensive studies of *Acheta* corpora cardiaca, conducted by L. McLean in our laboratory, suggested some structural difference for the ITP-like factor from this species. The initial rate of rise in locust ileal I_{sc} caused by cricket corpora cardiaca extracts was less than 25 % (i.e. 1 h for half-maximal stimulation) of that caused by locust corpora cardiaca (i.e. 10–15 min for half-maximal stimulation), even at excessive dosages (data not shown). *Carcinus* hyperglycaemic hormone (CHH) has substantial amino acid sequence identity (39 %) and even higher similarity (67 %) to locust ITP (see Discussion), but increasing the levels of CHH from 2.5×10^{-7} to 10^{-6} mol l^{-1} had no effect on ileal I_{sc} (Fig. 5), electropotential difference or transepithelial resistance (data not shown). This experiment was repeated with similar results using a second batch of CHH from Dr Keller and confirms the high selectivity of the ileal I_{sc} bioassay, and hence ITP receptors, for locust ITP.

Culture medium (50 μl from a total volume of 3 ml) from Sf9 cells infected with baculovirus containing locust ITP cDNA caused a maximal stimulation of locust ileal I_{sc}, i.e. 10.5±0.4 μequiv h^{-1} cm^{-2} (N=5), while control culture media from Sf9 cells infected with wild-type baculovirus gave no significant response, 0.14±0.26 μequiv h^{-1} cm^{-2} (N=4). Homogenates of virus-infected Sf9 cells tested at dosages equivalent to that of culture media gave no response, and at 2–6 times higher dosages gave submaximal stimulations of locust ilea (about 40 %), while control cell homogenates were without effect. These data indicate that introduction of a locust ITP cDNA into Sf9 cells causes them to synthesize and secrete a factor that stimulates locust ilea as tested in a highly specific bioassay. Structural analysis of the secreted ITP-like stimulant is being undertaken in a separate detailed study to determine what post-translational modifications and cleavage events

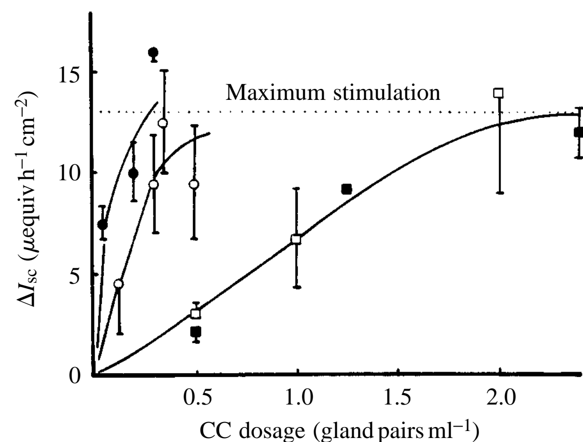

Fig. 4. Dose–response curves for saline extracts of corpora cardiaca (CC) from desert locusts (filled circles, N=11–12), grasshopper (*Melanoplus sanguinipes*; open circles, N=5–7), house cricket (*Acheta domesticus*, filled squares, N=6) and American cockroach (*Periplaneta americanus*, open squares, N=6) on ileal I_{sc}, i.e. active Cl^{-} absorption (ΔI_{sc}). The mean maximum stimulation is 13±3 μequiv h^{-1} cm^{-2} from over 1000 previous assays. Ileal I_{sc} was at or below 1 μequiv h^{-1} cm^{-2} prior to stimulation. Values are means ± S.E.M.

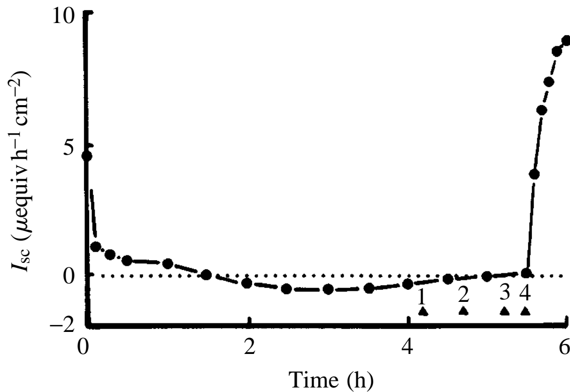

Fig. 5. A typical bioassay for the effect of pure *Carcinus* hyperglycaemic hormone (CHH) on locust ileal I_{sc}. CHH was added to the saline bathing the haemocoel side to achieve final concentrations of (1) 2.5×10^{-7} mol l^{-1}, (2) 7.5×10^{-7} mol l^{-1} and (3) 10^{-6} mol l^{-1}, without any effect. Crude locust corpora cardiaca extracts (0.5 gland-pair ml^{-1}) added at 4 rapidly and fully stimulated ileal I_{sc}. Four replicate bioassays gave similar results.

occur during expression and are required for the observed activity. A less active prohormone containing ITP may dominate inside the cells or ITP may be rapidly secreted after synthesis, thereby keeping Sf9 intracellular levels low.

Discussion

We have obtained a cDNA that encodes a 130 residue peptide that we believe is likely to be a propeptide of ITP (see Fig. 1A). Audsley *et al.* (1992*a*) purified active ITP and found it to have a molecular mass of 8652 Da (Audsley *et al.* 1994), much smaller than the molecular mass of the propeptide encoded by the complete cDNA. The presence of dibasic cleavage sites at residues 54–55 and 129–130 is consistent with the hypothesis that the active peptide is cleaved from the prohormone. In strong support of this hypothesis, the amino acid sequence of the active ITP purified by Audsley *et al.* (1992*a*) begins at residue 56.

Crab hyperglycaemic hormone is processed from a 140 bp precursor by cleavage on the C-terminal side of a Lys-Arg sequence to produce the N terminus of active CHH (Weidemann *et al.* 1989). The C-terminal tripeptide Gly-Lys-Lys of ITP propeptide may be the site of cleavage and amidation. Endoproteolytic cleavage at two or three basic residues, their removal by carboxypeptidase, followed by removal of the Gly residue to generate an amidated carboxyl group, is a common maturation pathway producing physiologically active peptides (Sossin *et al.* 1989). Mature CHH is amidated and produced from a propeptide with the C-terminal sequence Gly-Arg-Lys-Lys (Weidemann *et al.* 1989) and indeed most insect neuropeptides that have been described are amidated. If ITP is cleaved from the propeptide and amidated in a similar fashion, and all the cysteines are reduced, then the predicted molecular mass of ITP deduced from the cDNA is 8564 Da, in reasonable agreement with the molecular

A

	1		12		22	
S	FFDIQ	CKGVY	DKSIF	ARLDR	ICEDC	YNLFR
Q	IYDTS	CKGVY	DRALF	NDLEH	VCDDC	YNLYR
pE	VFDQA	CKGVY	DRNLF	KKLDR	VCEDC	YNLYR
R	IFDTS	CKGFY	DRGLF	AQLDR	VCEDC	YNLYR
ASAW	FTNDE	CPGVMG	NRDLY	EKVAW	VCNDC	ANIFR
R	VINDE	CPNLIG	NRDLY	KKVEW	ICEDC	SNIFR

32		42		52		62
EPQLH	SLCRS	DCFKS	PYFKG	CLQAL	LLIDE	EEKFN
TSYVA	SACRS	NCYSN	LVFRQ	CMDDL	LMMDE	FDQYA
KPFVA	TTCRE	NCYSN	WVFRQ	CLDDL	LLSDV	IDEYV
KPHVA	AECRR	DCYTT	EVFES	CLKDL	MMHDF	INEYK
NNDVG	VMCKK	DCFHT	MWFLW	CVYAT	ERHGE	IDQFR
KTGMA	SLCRR	NCFFN	EDFVW	CVHAT	ERSEE	LRDLE

	72
QMVEI	L - NH_2*
RKVQM	V - NH_2
SNVQM	V - NH_2*
EMALM	VS - NH_2
KWVSI	LR
EWVGI	LGAGRD

B

Sequence	Percentage identity	Percentage similarity
ITP	100	100
Crab CHH	39	67
Lobster CHHA	44	67
Woodlouse CHH	42	71
Lobster VIH	29	53
Crab MIH	30	54

Fig. 6. (A) Comparison of the deduced amino acid sequence for locust ITP (1) with some crustacean hormones (2–6). Sequence identity with ITP is indicated in bold type and similarity in italic type. Amino acid residues are numbered from the beginning of the partial amino acid sequence of Audsley *et al.* (1992*a*), (2) *Carcinus maenas* hyperglycaemic hormone (Kegel *et al.* 1989), (3) *Homarus americanus* hyperglycaemic hormone A (Tensen *et al.* 1991*a*), (4) *Armadillidium vulgare* hyperglycaemic hormone (Martin *et al.* 1993), (5) *H. americanus* vitellogenesis-inhibiting hormone (Soyez *et al.* 1991), and (6) *C. maenas* moult-inhibiting hormone (Klein *et al.* 1993). *Terminal amidation assumed (see Discussion). (B) A comparison of ITP with the crustacean hormones in A on the basis of conserved amino acids (percentage identity) plus neutral amino acid substitutions (percentage similarity).

mass obtained by Audsley *et al.* (1994) for the purified ITP. The difference of 88 Da might indicate that ITP is phosphorylated or sulphated. It is also possible that the purified ITP analysed by mass spectroscopy was a potassium salt. If there are two K^+ per ITP, this would account for most of the discrepancy (D. A. Schooley, personal communication). Further work will be required to demonstrate how active ITP is derived from the prohormone.

The complete agreement between the deduced amino acid sequence from the ITP cDNA sequence and that published by Audsley *et al.* (1992*a*) supports the conclusion that we have cloned ITP. Audsley (1991) anticipated cysteine at position 78 (Fig. 1A) given the difficulties encountered in detecting this amino acid in his study. Audsley (1991) obtained additional amino acid sequence data for ITP that have not been published because accuracy declines towards the end of long sequence

analyses; however, our cDNA sequence agrees with Audsley's unpublished sequence for residues 90–100, 102 and 104 (Fig. 1A), strengthening the conclusion that our cDNA is authentic. Further support comes from the finding that expressing the cDNA in Sf9 cells *via* a baculovirus produces a culture medium containing biological activity. We have not established whether the active factor secreted by Sf9 cells has exactly the same molecular structure as ITP from locust corpora cardiaca, i.e. that the processing and post-translational modifications are identical in Sf9 and corpora cardiaca cells.

The evidence presented here shows that there are two ITP-like mRNAs, one encoding a 130 residue propeptide in brain and corporus cardiacum, and the other (ITP-L) encoding a 134 residue peptide expressed in many other tissues. On the basis of preliminary analyses of cDNA clones from brain and ileal libraries, we believe that these alternatively spliced mRNAs are derived from one primary transcript, rather than being the products of two separate genes (J. Meredith, unpublished results). Furthermore, we have reason to believe that the smaller PCR product encodes the active ITP detected in the locust ileal assay. ITP is believed to be synthesized in the neurosecretory cells of the pars intercerebralis of the brain and transported to the corpora cardiaca for storage and release. Indeed, CHH-immunoreactive material has previously been reported in the brain and corpus cardiacum of the stick insect *Carausius morosus* (Jaros and Gäde, 1982). Only the smaller PCR product is abundant in brain and corpora cardiaca, the two tissues active in the ileal assay (Audsley and Phillips, 1990). By contrast, the larger PCR product (ITP-L) is ubiquitous and abundant in flight muscle, Malpighian tubules and ileal tissues, extracts of which have no activity on ileal I_{sc} assays (Audsley and Phillips, 1990; J. Meredith, unpublished data). This suggests that the ITP-L peptide has a different function from that of ITP. The much larger neuropeptide stimulant (37 000 Da) of locust ileal I_{sc} partially purified by Bilgen (1994) in our laboratory from ventral ganglia is apparently unrelated to the ITP because ITP mRNA was not detected in this tissue (Fig. 3).

On the basis of their partial sequence for ITP, Audsley *et al.* (1992*b*, 1994) first reported the considerable sequence similarity to a family of crustacean hormones, including hyperglycaemic (e.g. CHH), moult-inhibiting (MIH) and vitellogenesis-inhibiting (VIH) hormones from several species. ITP is the first member of this protein family found outside crustaceans. We are now in a position to compare the complete amino acid sequences (Fig. 6). These peptides are of similar length (72–78 residues), with the six cysteine residues conserved in all cases. The majority exhibit terminal amidation and several other highly conserved sequences (residues 7–12, 16, 19–31, 39–43, 49 and 52–61). There is greatest divergence in the last 10 residues, which the Chou–Fasman and Robson–Garnier methods (as used by the protein analysis toolbox in Macvector software) predict is an alpha-helix for ITP. As a working hypothesis, we assume that there are three disulphide bridges in locust ITP at the same locations as have been determined for CHH (Kegel *et al.* 1989), namely residues 7–43, 23–39 and 26–52. Together, these data establish that ITP is homologous to these crustacean hormones.

The 55 amino acid residues preceding the ITP sequence do not exhibit sequence homology to the well-defined crustacean hyperglycaemic hormone precursor related peptides (Weidemann *et al.* 1989; Tensen *et al.* 1991*b*), although there is a putative dibasic cleavage site at positions 24 and 25 (Fig. 1A).

The failure of *Carcinus* hyperglycaemic hormone and crab eyestalk extracts to stimulate locust ileal I_{sc} indicates that the ITP receptor in the ileum is quite specific. Species specificity is also observed amongst crustaceans. Hyperglycaemic hormones from crayfish and crab (*Orconectes* and *Carcinus*) are ineffective in woodlice (*Porcellio*) and that from woodlice is ineffective in crab (*Uca*) (Keller *et al.* 1985). We predict that the failure of heads (i.e. brain + CC) from dipteran, lepidopteran and hymenopteran species to stimulate locust ileal I_{sc} indicates divergence of ITP amino acid sequence among insect orders rather than the absence of this protein family. The partial cDNA for locust ITP that we report here should greatly facilitate the detection of ITP-like peptides in other insects. In the case of cockroaches and crickets, the locust ileal I_{sc} bioassay can be used to confirm expression of their ITP-like cDNA.

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