Latrophilin is required for toxicity of black widow spider venom in *Caenorhabditis elegans*

Christopher J. MEE, Simon R. TOMLINSON[1], Pavel V. PERESTENKO[2], David DE POMERAI, Ian R. DUCE, Peter N. R. USHERWOOD and David R. BELL[3]

School of Biology, University of Nottingham, University Park, Nottingham NG7 2RD, U.K.

Black widow spider venom (BWSV) kills *Caenorhabditis elegans* after injection owing to the presence of heat- and detergent-sensitive components, which are high-molecular-mass latrotoxins. A *C. elegans* homologue of latrophilin/CIRL (calcium-independent receptor for latrotoxin), B0457.1, was identified and shown to have five conserved domains. RNAi (RNA interference) of this gene rendered *C. elegans* resistant to BWSV, whereas RNAi for CYP37A1 or a neurexin I homologue, and a deletion mutant of the related B0286.2 gene, had no effect on BWSV toxicity. The latrophilin RNAi mutants exhibit changes in defaecation cycle and alterations in drug sensitivity. These results demonstrate that latrophilin mediates the toxicity of BWSV and provide evidence for a physiological function of this receptor.

Key words: black widow spider, *Caenorhabditis elegans*, latrophilin/CIRL (calcium-independent receptor for latrotoxin), latrophilin, latrotoxin, neurotransmission.

INTRODUCTION

The venom of the black widow spider (*Latrodectus mactans tredecimguttatus*; BWSV) elicits massive neurotransmitter release from vertebrate, insect and crustacean nerve terminals [1–3], owing to its content of the high-molecular-mass proteins latrotoxins (LTXs) [4]. α-LTX is well known for its potent ability to trigger neurotransmitter release from vertebrate nerve terminals, and it has been an invaluable tool for characterizing the mechanisms involved in presynaptic neurotransmitter release (see e.g. [5–7]). The mechanism of action of BWSV appears to be similar in vertebrates, invertebrates and crustaceans [8]; however, α-LTX is inactive at invertebrate nerve terminals [8], and other LTXs, such as latroinsectotoxins (LITs), specifically release transmitter at insect neuromuscular junctions [4]. The fact that the LTXs have a similar structure and elicit similar physiological responses in vertebrates, invertebrates and crustaceans suggests common mechanisms controlling neurotransmitter release across phyla, and emphasizes their utility as tools for studying these mechanisms.

α-LTX binds with high affinity to two proteins, the neurexins [9] and latrophilin/CIRL (calcium-independent receptor for α-LTX) [10–13]; however, the function of these receptors and their role in mediating the toxicity of LTXs is unclear. Both proteins are capable of enhancing the function of α-LTX in cultured cells, including truncated proteins lacking putative signalling domains [14–17]. This had reinforced the hypothesis that the receptors are merely a 'tether' for the toxin, which then forms ion channels [18,19]. However, these results do not exclude the additional hypothesis that the receptor may be activated by the binding of LTXs, and may transduce signals in the specialized microcosm of the neural synapse. The delineation of a function for BWSV receptors would be an important step forward in defining this action of BWSV toxins.

We have investigated the action of BWSV using the model organism, *Caenorhabditis elegans*. Although it has a simple nervous system, we demonstrate that a specific high-molecular-mass protein from BWSV is a potent and lethal toxin for *C. elegans*. We identified a homologue of latrophilin in *C. elegans* that is essential for the toxicity of BWSV, and we have shown that RNAi (RNA interference) with this receptor leads to a profound phenotype in *C. elegans*.

MATERIALS AND METHODS

Animals

All strains of *C. elegans* were maintained as hermaphrodites at 15 °C on NGM (nematode growth medium) plates fed with either the standard OP50 *Escherichia coli* strain or P90C, a lacZ strain [20]. Young adults were visualized with an Olympus CK003 microscope, and BWSV was micro-injected into the body cavity using a Leitz micromanipulator and a General Valve Corp. Picospritzer II to control fluid injection, in a buffer of 50 mM Tris (pH 8.0)/50 mM NaCl. Worms were allowed to recover on NGM plates for up to 2 days at 15 °C.

Behavioural assays

Defaecation was scored by direct observation of the muscle contractions that constitute the defaecation motor programme (pBoc, aBoc and exp) in single animals on P90C-coated NGM plates over a span of ten consecutive cycles at 20 °C. The wild-type defaecation motor programme consists of sequentially occurring posterior body muscle contractions (pBoc), anterior body wall contractions (aBoc) and expulsion (exp) contractions every 45–50 s [21].

Abbreviations used: BWSV, black widow spider venom; dsRNA, double-stranded RNA; EST, expressed sequence tag; GPS, G-protein-coupled receptor protease cleavage site; LIT, latroinsectotoxin; LTX, latrotoxin; NGM, nematode growth medium; RNAi, RNA interference.

[1] Present address: Computational Genome Analysis Laboratory, ICRF, 44 Lincoln's Inn Fields, London WC2 A3PX, U.K.
[2] Present address: School of Medicine, Department of Anatomy, University of Bristol, University Walk, Bristol BS8 1TD, U.K.
[3] To whom correspondence should be addressed (e-mail david.bell@nottingham.ac.uk).

© 2004 Biochemical Society

A 100 μM stock solution of levamisole, a 1 mM stock solution of aldicarb in 100 % ethanol or a 5 mg/ml stock solution of imipramine dissolved in water was dissolved into molten NGM to give the required range of experimental concentrations. The strain of worm to be exposed was placed on the levamisole or aldicarb plates as a population of 25 and left to equilibrate for 15 min. This was taken as the zero time. After a 15 min exposure, the number of *C. elegans* paralysed was recorded. Paralysis was assessed by the observed lack of locomotion, a rod-like appearance and a lack of movement observed after prodding the worm with a worm pick. This procedure was repeated at 15 min intervals for a total of 3 h. For imipramine plates, 25 worms were placed on the plates and left to equilibrate for 15 min. This was taken as the zero time. Pharyngeal pumping was counted using light microscopy for each concentration for 5 min in total.

RNAi

For micro-injection, cDNAs were cloned into pGEM-T (Promega) or pBluescript II, and RNAs were transcribed *in vitro* using T3/T7/SP6 polymerases according to standard methods. Sense and anti-sense strands were mixed, heated to 88 °C and then slowly allowed to cool. The dsRNA (double-stranded RNA) was then micro-injected into the gonadal syncitium of adult N2 worms. After 12 h, animals were transferred on to fresh plates. Alternatively, an RNA feeding method was used. The oligonucleotides GGATGCCCT**GAATTC**GGACGTTATAAAACC and TTCAG-ATCGTGTCC**GAATTC**TCACATCAAAACAGC (bold sites represent an *Eco*RI recognition sequence) were used to PCR-amplify bases 495–1631 of the latrophilin cDNA; subsequently, they were cloned into pGEM-T and *Eco*RI-digested into pL4440 to give pL4440.LPH. Plasmids were transformed into the *E. coli* strain HT115 [22], and RNAi feeding was performed as described in [23] using dhc-1 as a control [24].

Cloning

Putative homologues of latrophilin were identified by searching the *C. elegans* genomic DNA database (http://www.sanger.ac.uk/Projects/C_elegans/; http://www.dna.affrc.go.jp/htdocs/SWsrch/index.html). Full-length clones of latrophilin (CE02945) and CYP37A1 (CE18566) were cloned by PCR of cDNA, and subcloned into pGEM-T. ESTs (expressed sequence tags) of putative neurexin I, yk346b3 and yk23f8 (CE25791) were obtained from Professor Y. Kohara (National Institute of Genetics, Shizuoka, Japan) as phage clones. These were subjected to phage excision to yield clones in pBluescript II. Clones were sequenced on both strands using an ABI sequencer, and analysed using GCG (http://www.accelrys.com/products/gcg_wisconsin_package/index.html).

Chromatography of BWSV

BWSV (obtained from Latoxan, Valence, France) was resuspended in 50 mM Tris/150 mM NaCl (pH 8.0) and subjected to size-exclusion chromatography on Superdex 200 (HR10/30; Amersham Biosciences); 1 ml fractions were assayed by diluting to 1:1000 in 50 mM Tris/50 mM NaCl (pH 8) and micro-injected into *C. elegans*. Positive fractions were analysed with a 40–300 mM NaCl gradient in 50 mM Tris (pH 8.0) on Uno-Q (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.) and then with a 40–400 mM NaCl gradient in 50 mM sodium succinate (pH 6.2) on Uno-S. Proteins were run on a 10 % (w/v) Bis-Tris denaturing polyacrylamide gel in Mops buffer according to the manufacturer's instructions (Novex, Invitrogen, Paisley, U.K.), followed by silver staining. Proteins were diafiltered into 150 mM Tris (pH 8.16) and subjected to tryptic digestion followed by MS/MS analysis.

RESULTS

Wild-type N2 *C. elegans* were refractory to incubation in BWSV. However, micro-injection of the venom resulted in death of the worms within a few hours, characterized by a paralysed, rod-like appearance of the worms. The BWSV was toxic over a wide range of concentrations, i.e. inducing 100 % lethality in ten injected worms over the concentration range 1.2 mg/ml to 1.2 ng/ml, whereas worms injected with vehicle showed 0 % mortality (0/10). At lower concentrations, the toxic response was highly variable, perhaps owing to non-specific adsorption of BWSV on glass or plastic containers. To determine if a protein component of BWSV was responsible for the lethality, the venom was treated with 0.1 % SDS or heated at 68 °C for 10 min. BWSV at 1.2 μg/ml killed 10/10 worms, but BWSV heated at 100 °C for 5 min, or buffer control, killed 0/10 animals. Injection of 0.1 % (w/v) SDS killed 0/10 worms, whereas BWSV at 1.2 μg/ml killed 10/10 worms; BWSV preincubated in 0.1 % SDS killed 0/10 worms. Thus both of these treatments totally ablated the toxicity of the venom, suggesting that a proteinaceous component of the venom was responsible for toxicity.

It is known that high-molecular-mass LTXs mediate the toxicity of BWSV in mammals and insects. It was necessary to characterize further the active principle of the venom for this system to be useful as a model for mammalian and insect toxicities. Therefore purification of toxins from the venom was undertaken and fractions were tested by micro-injection into *C. elegans*. Micro-injection of highly purified 2 μM α-LTX (a gift from Y. A. Ushkaryov, Imperial College, London, U.K.) [7] demonstrated that α-LTX had no measurable toxicity to *C. elegans* (0/10 animals killed). The fractionation scheme used an initial size-exclusion column [7], followed by a procedure based on the method described by Krasnoperov et al. [4]. Fractions containing components that were toxic to *C. elegans* eluted early from the size-exclusion column, with a peak of activity suggesting a molecular mass of approx. 250 kDa in comparison with protein molecular-mass standards (Figure 1A), although this fraction contained proteins of 110–130 kDa on denaturing PAGE (Figure 1C), suggesting oligomerization [7]. These fractions were pooled, and then fractionated on Uno-Q, yielding multiple peaks with activity against *C. elegans* (Figure 1B). One fraction of the toxin, which eluted at approx. 180 mM NaCl from the Uno-Q column, was subsequently separated on Uno-S to yield a protein of 110 kDa, which appeared homogenous on PAGE (Figure 1C). The elution of proteins from the anion- and cation-exchange columns showed a remarkable similarity to the work of Krasnoperov et al. [4] in terms of elution order and mass of the eluted proteins; the purified protein with toxicity to *C. elegans* showed similar chromatographic properties as described for ε-LIT. Insufficient protein was harvested for N-terminal sequence determination, but the protein was subjected to tryptic digestion followed by MS/MS sequencing to give three peptide sequences, namely EA(L/I)(L/I)GHR, (AT)FQEV(L/I)DA(L/I)(L/I)EK and (FT)-TDYVNN(L/I)AEDVR. We were unable to match these sequences with other known toxin sequences; however, the known LTX sequences are highly divergent, with approx. 30 % amino acid identity. The ε-LIT fraction had an LD_{50} of approx. 1–2 μg/kg on *C. elegans*. Several other distinct proteins were highly purified and showed high toxicity to *C. elegans*; however, the small

© 2004 Biochemical Society

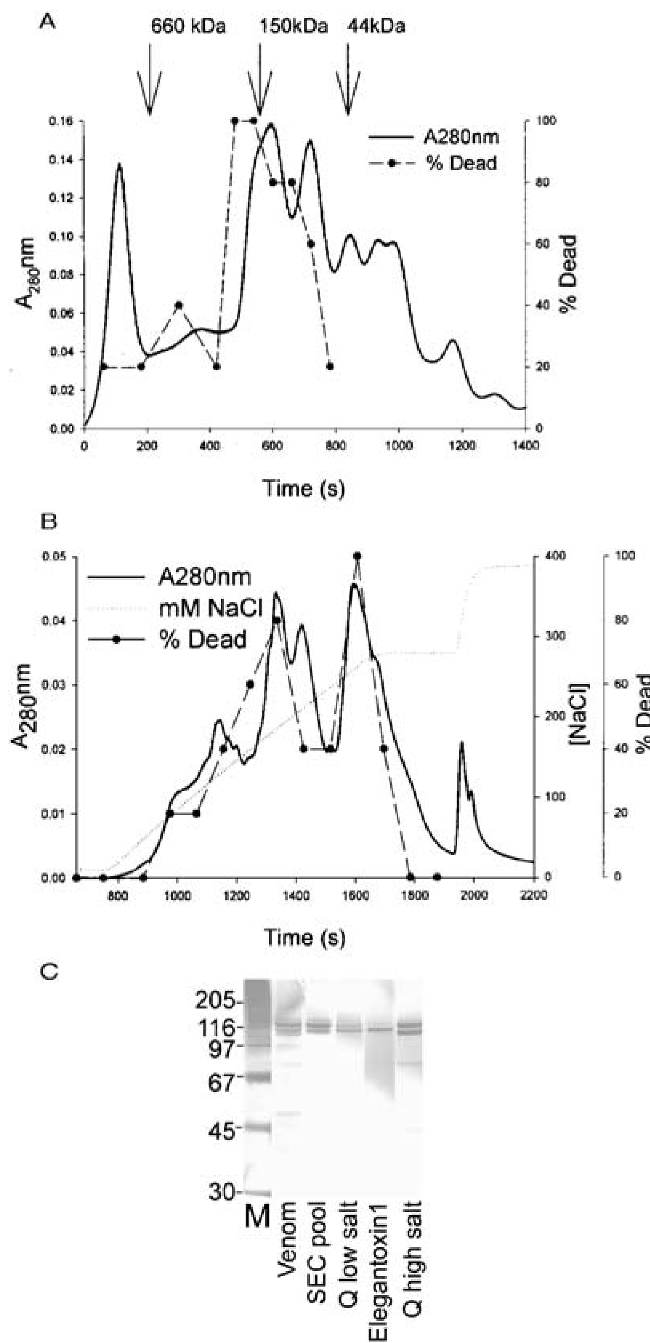

Figure 1 Purification of LTXs that kill *C. elegans*

(**A**) Size-exclusion chromatography of BWSV. BWSV was dissolved in 50 mM Tris (pH 8.0) and 150 mM NaCl, and loaded on to a Superdex 200 column at 0.5 ml/min; the relevant part of the trace is shown. The absorbance at 280 nm (A_{280}) of the eluate is shown by a solid black line. Fractions (1 ml) were collected and assayed by diluting to 1:1000 in an injection buffer and micro-injecting into *C. elegans*; the percentage dead is shown ($n = 5$) by a broken line with closed circles. The positions of elution of molecular-mass standards (bovine thyroglobulin, bovine γ-globulin and chicken ovalbumin) are indicated by arrows. (**B**) Peak fractions from (**A**) were pooled, diluted to 30 mM NaCl and applied on to a Uno-Q1 column in 50 mM Tris (pH 8), followed by a salt gradient elution (· · · · · ·). The absorbance of the eluate at 280 nm is shown by a thick black line (A_{280}), and the activity of the fractions on *C. elegans* is shown by a broken line with circles. (**C**) Denaturing PAGE of venom fractions. The indicated samples [venom, size-exclusion pool (SEC pool), high- and low-salt fractions from Q-Sepharose (Q low salt and Q high salt) and purified elegantoxin1] were subjected to PAGE as described in the Materials and methods section, alongside molecular-mass standards (M), followed by silver staining. The molecular-mass standards are given in kDa.

amounts of these proteins prevented their further characterization. Notably, several fractions containing high-abundance LITs, such as α-LIT, which had been further purified on phenyl-Sepharose HIC (hydrophobic interaction chromatography) [4], were only marginally toxic to *C. elegans*. These results demonstrate that BWSV contains specific high-molecular-mass proteins that are toxic to *C. elegans*.

Both latrophilin and neurexin Iα are known to bind the mammalian-specific α-LTX; therefore homologues of these genes were candidates for mediating the effect of LTXs in *C. elegans*. A database search of the *C. elegans* genome revealed the presence of two genes with similarity to latrophilin, namely B0457.1 and B0286.2, the former being more closely related to latrophilin. The cDNA for the B0457.1 gene was PCR-amplified, using primers designed from analysis of the genomic sequence. Figure 2(A) shows the structure of this latrophilin cDNA, which encodes a peptide of 1014 amino acids with a pI of 7.2. Although the global amino acid sequence identity is only 29 %, several features of the sequence are worth noting. As shown in Figure 2(B), the *C. elegans* latrophilin contains motifs in common with its rat homologue, as identified by Pfam6.6 [26]. These are the galactose-binding lectin domains at residues 51–133 (Pfam PF02140), a hormone receptor motif at residues 181–240 containing four cysteine residues conserved in the rat latrophilin (Pfam PF02793), a GPS (G-protein-coupled receptor protease cleavage site) motif at residues 493–541 with four cysteine residues conserved in the rat latrophilin (Pfam PF01825) and a seven-transmembrane domain of the secretin family at residues 548–799 (Pfam PF00002). Additionally, a sequence containing four conserved cysteine residues is also present in *C. elegans* latrophilin at amino acids 511–566, which shows strong conservation with the rodent latrophilin homologues (815–870 of rat latrophilin), and various other proteins, including lactomedin 1α and β and orphan G-protein-coupled receptors O94910 and ETL (Q9HBW9). It is of interest to note that the region of rat latrophilin required for binding to α-LTX is amino acids 467–891 [27], and that this region has relatively high (39 %) identity to the *C. elegans* homologue.

The *C. elegans* latrophilin was 'knocked out' by RNAi using micro-injection of dsRNA, and F1 worms were tested for toxicity of BWSV. The offspring of worms injected with vehicle showed 100 % survival after injection with vehicle (10/10 worms survived), but injection of BWSV at 1200, 120, 12, 1.2, 0.12 or 0.012 μg/ml produced 100 % mortality (10/10 worms for each data point). However, the offspring of worms injected with dsRNA for latrophilin were completely resistant to the effects of BWSV over the same concentration range of venom (10/10 worms surviving at each point). To determine if this resistance of BWSV was related to the process of micro-injection of dsRNA leading to RNAi, micro-injection of dsRNA of the unrelated *C. elegans* gene CYP37A1 was undertaken; the F1 offspring showed 100 % lethality when injected with BWSV at 1.2 μg/ml ($n = 10$ worms). Additionally, subcloning a fragment of the latrophilin gene into pL4440 for RNAi by bacterial feeding also yielded substantially similar results (results not shown). The RNAi effect was possibly caused by interference with a closely related gene. To test this, the *C. elegans* strain VC158 has a 1083 bp deletion in the closely related B0286.2 gene, and these animals were injected with BWSV. However, these worms showed 100 % lethality to BWSV injection at 1.2 μg/ml ($n = 10$ worms). Thus the ability of RNAi of the latrophilin gene to confer BWSV insensitivity is not due to the process of RNAi or interference with related genes. Neurexin Iα is also known to bind the mammalian α-LTX. Therefore we investigated whether a neurexin I homologue in *C. elegans*, Q18291, has a role in BWSV toxicity. An EST for Q18291 was used to generate neurexin I 'knockout'

© 2004 Biochemical Society

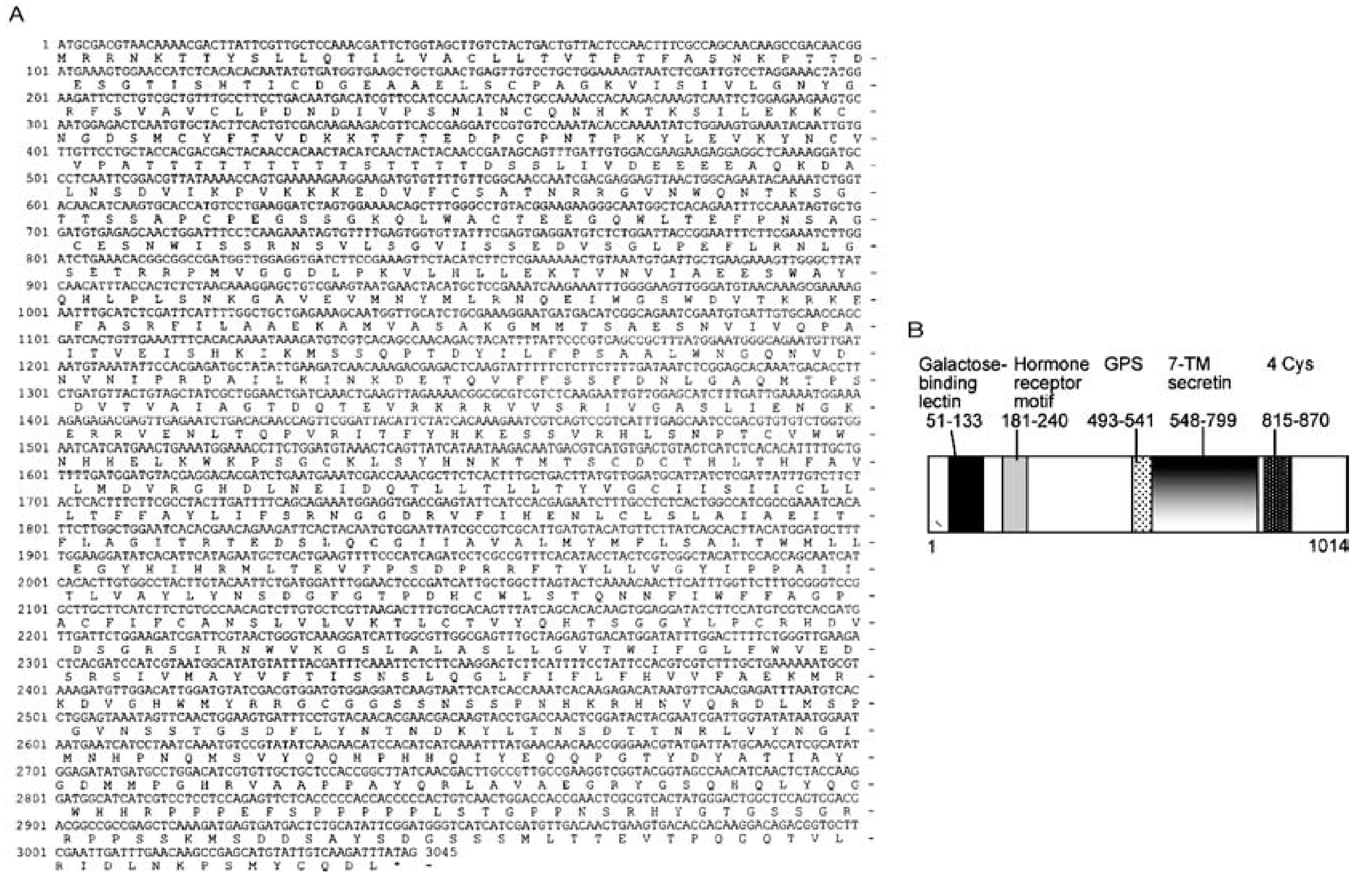

Figure 2 A latrophilin homologue in *C. elegans*

(**A**) cDNA and deduced amino acid sequence of B0457.1 transcript; (**B**) structural domains of latrophilin. Domains were identified by manual alignment with the rat latrophilin sequence, Pfam6 and database searching. A galactose-binding lectin domain is shown by a black box and a 'hormone receptor motif' as a solid grey box. The GPS motif is shown by black dots on a white background, and the seven-transmembrane domain (7-TM secretin) is shown by a box with black to white fade. A further domain with four conserved cysteine residues is shown by a black box with white dots. Positions of the domains are indicated by amino acid numbers.

worms by RNAi. These worms showed 100 % lethality to BWSV injection at 1.2 μg/ml ($n = 10$ worms). Thus the ability of the RNAi technique to confer resistance to BWSV was specific and unique to the latrophilin gene.

Close examination of the latrophilin RNAi worms showed that they had a distended gut (Figures 3A and 3B). Moreover, there was an extended period of gut peristalsis when compared with controls, as measured by the interval between expulsion contractions (Figure 3C). These effects on the gut and extended gut peristalsis interval were also seen in *C. elegans* where RNAi was induced by feeding with bacteria containing dsRNA for latrophilin, demonstrating that the effect is not due to the administration of dsRNA by micro-injection. The effect on the defaecation cycle is reminiscent of several other neural mutants, such as aex-3 [28]. To test whether there was altered neural function, the sensitivity of wild-type *C. elegans* and latrophilin RNAi *C. elegans* to drugs was investigated. Levamisole is an acetylcholine receptor agonist in *C. elegans* (see e.g. [29]). Latrophilin RNAi worms were less sensitive to levamisole when compared with wild-type worms (Figure 4A). They were also less sensitive to an imipramine-induced decrease in pharyngeal pumping compared with wild-type (Figure 4B). However, latrophilin RNAi worms did not have significantly altered sensitivity to the acetylcholinesterase inhibitor aldicarb (Figure 4C).

DISCUSSION

Although *C. elegans* has a simple nervous system, it has been used informatively as a model system to study the fundamental mechanisms of neurotransmission (see e.g. [30,31]). We now show that the mechanism of action of BWSV is conserved from mammals to *C. elegans*, opening up the possibility of using *C. elegans* genetic analysis to understand the function of its receptor.

We are unaware of any previously published reports of the effects of BWSV on *C. elegans*. BWSV was acutely toxic to *C. elegans* after micro-injection, over a 10^5-fold concentration range, demonstrating a potent action of the venom. Separate components of the venom are active against mammals, insects and crustaceans, but each toxin is known to be a high-molecular-mass (approx. 120 kDa) protein. If the mechanism of action of venom components was conserved, it would be predicted that the venom component which killed the *C. elegans* would also be a high-molecular-mass protein. Therefore the venom was treated with heat or with dilute SDS; both treatments ablated the toxicity of BWSV, suggesting that the active component was proteinaceous. Protein purification of the active components was undertaken to characterize and identify these proteins. The active components of the venom eluted from a size-exclusion

© 2004 Biochemical Society

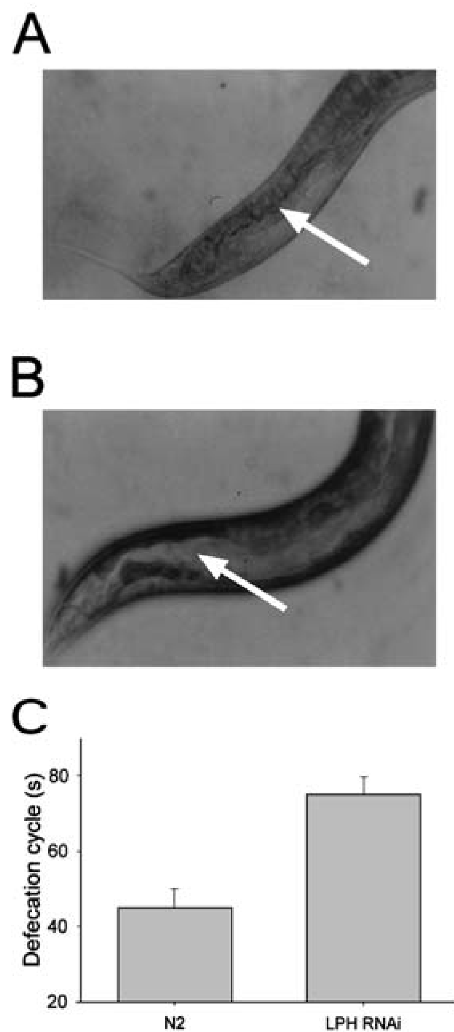

Figure 3 Constipation in latrophilin RNAi worms

(**A**) Wild-type worm, with the gut shown by a white arrow. (**B**) Latrophilin RNAi worm, with the distended gut shown by a white arrow. (**C**) Wild-type (N2) and latrophilin RNAi (LPH RNAi) worms were observed under DIC (differential interference contrast) optics and the time between expulsions was counted for ten consecutive cycles for each of the ten animals. Results are presented as means $\pm$ S.D.

column at approx. 260 kDa, but consisted principally of proteins of approx. 110–130 kDa. This is consistent with the formation of dimers, as reported for other LTXs [7]. Further fractionation of the size-exclusion fraction on ion-exchange chromatography revealed the presence of multiple proteins of high molecular mass (approx. 120 kDa) that are toxic to *C. elegans*. One such protein was purified to apparent homogeneity by PAGE and shows similar chromatographic properties and mass after PAGE to the ε-LIT reported by Krasnoperov et al. [4], although its toxicity to *C. elegans* ($LD_{50} \approx 1$–$2\ \mu$g/kg) is much higher when compared with its toxicity to insects ($LD_{50} \approx 1000\ \mu$g/kg) [4]. This *C. elegans*-specific toxin has an apparent molecular mass of 110 kDa, the same as δ-LIT; however, this protein is not δ-LIT, as δ-LIT and α-LTX elute at a much higher salt concentration from the anion-exchange column (300 mM versus approx. 180 mM for the *C. elegans* toxin and ε-LIT [4]). The toxicity of this LTX to *C. elegans* does not appear to be a feature common to all LITs, since a fraction containing α-LIT had only marginal toxicity to *C. elegans*, although

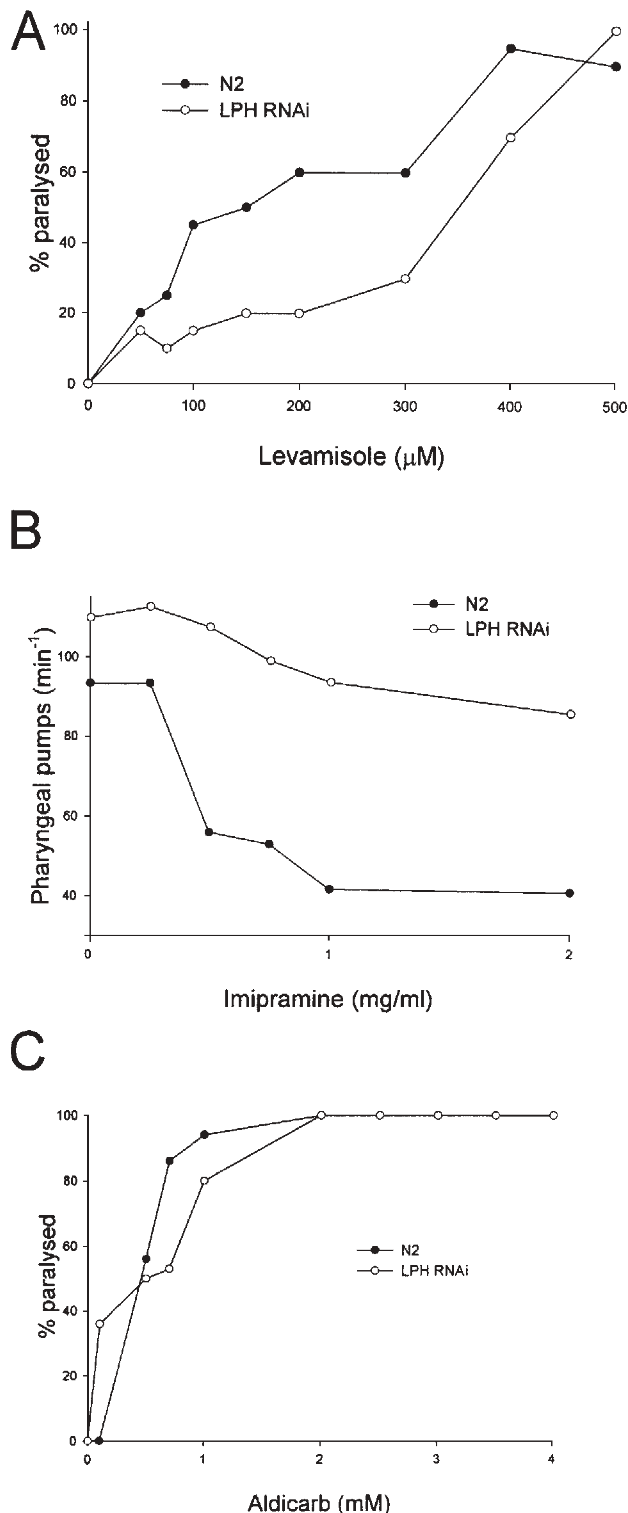

Figure 4 Sensitivity of latrophilin RNAi worms to neuroactive agents

(**A**) Wild-type worms (N2) or latrophilin-RNAi worms (LPH RNAi) were exposed to levamisole and the number of paralysed worms was counted, as described in the Materials and methods section. Each data point reflects an analysis of 25 worms. (**B**) Wild-type or latrophilin RNAi worms were exposed to imipramine and the rate of pharyngeal pumping was measured, as described in the Materials and methods section. Each data point represents 25 worms per group. (**C**) Wild-type or latrophilin RNAi worms were exposed to aldicarb and the number of paralysed worms was counted, as described in the Materials and methods section. Each data point represents 25 worms per group.

© 2004 Biochemical Society

it is highly potent against insects ($LD_{50} \approx 15\ \mu g/kg$). Further confirmation of the specificity of LTXs was the demonstration that highly purified 2 μM α-LTX was non-toxic after its injection into *C. elegans* (C. J. Mee, D. R. Bell and Y. A. Ushkaryov, unpublished work). There are reports that α-LTX activates a latrophilin homologue from *Haemonchus contortus* [32] and that it activates *Drosophila melanogaster* nerve junctions [33]. However, α-LTX must be of high purity, and δ-LIT is a common contaminant of α-LTX [7]. Our results are consistent with the widely accepted view that LTXs show pronounced species specificity; moreover, they show that specific high-molecular-mass LTXs are responsible for the toxicity of BWSV to *C. elegans*.

A latrophilin homologue, B0457.1, was identified from genomic DNA sequence, and cloned from *C. elegans*. Whereas the overall sequence identity is low, at approx. 30 %, the sequence identity to rat latrophilin in a minimal domain defined for binding α-LTX [27] is significantly higher, at approx. 39 % identity; this shows that sequence conservation is higher in domains associated with LTX binding. Several domains that have been identified previously in latrophilin remain conserved, including a galactose-binding lectin domain, a hormone receptor motif at residues 181–240 containing four cysteine residues conserved in rat latrophilin, a GPS motif with four cysteine residues conserved in rat latrophilin and a seven-transmembrane domain of the secretin family. We were also able to identify a novel sequence motif immediately C-terminal to the seven-transmembrane domain, which contains four conserved cysteine residues. This domain, although only 55 amino acids in length, shows marked conservation (approx. 50 % identity) with a variety of higher mammalian proteins of the G-protein-coupled receptor superfamily. The function of this domain is not clear, but it is probable that the two pairs of conserved cysteine residues form a higher-order structure.

Next, we investigated whether the knockout of the latrophilin gene affected the toxicity of BWSV, using RNAi to knock out the gene. The results show that knockout of the latrophilin gene ablates the toxicity of BWSV over a 10^5-fold range of concentrations; the results also show that this effect is specific to latrophilin and is not shared by cytochrome P450 (CYP37A1), the related G-protein-coupled receptor B0286.2 or a neurexin Iα homologue. These results demonstrate a remarkable specificity of BWSV action, insofar as RNAi of the latrophilin gene completely removes toxicity of BWSV, thus demonstrating that latrophilin is absolutely required for BWSV toxicity. This is in marked contrast with mammals, where both neurexin Iα and latrophilin [34] mediate the effects of BWSV in cells. In neurexin Iα knockout mice, there is a decrease in BWSV effects, as measured by glutamate release from synaptosomes [16]; however, it is unclear how this effect would be reflected in whole animal studies on the effect of BWSV. Our results show that latrophilin plays the principal role in mediating the effects of BWSV in *C. elegans*, and suggests that latrophilin may have the primary role in mediating the effects of BWSV in mammals.

C. elegans with RNAi of latrophilin showed a pronounced phenotype, with a constipated phenotype, associated with lengthened exp intervals (Figure 3). Mutants of several genes that affect synaptic function are known to cause similar phenotypes [28,35], but equally, so does the flr-1 gene, which is expressed in intestinal cells [36]. As a further test of possible involvement of the nervous system, the sensitivity of *C. elegans* to a variety of pharmacological agents was assayed. When compared with wild-type worms, latrophilin RNAi worms were more resistant to (cholinergic agonist) levamisole-induced paralysis and (reuptake inhibitor) imipramine-induced slowing of pharyngeal pumping (Figures 4A and 4B), but there was no difference in sensitivity to the acetylcholinesterase inhibitor, aldicarb. These results show the different sensitivities to two classes of pharmacological agents in latrophilin RNAi worms, and are consistent with a generalized altered function in neuromuscular control. Further studies will be needed to resolve the endogenous function of latrophilin.

In summary, we have shown that BWSV is acutely toxic to *C. elegans* and there are multiple high-molecular-mass (approx. 120 kDa) LTXs in BWSV, which mediate the toxicity of BWSV to *C. elegans*. A latrophilin homologue, B0457.1, can be knocked out in *C. elegans*, and renders the worms resistant to BWSV; this effect is specific to latrophilin and is not seen with a neurexin Iα homologue or a knockout of the G-protein-coupled receptor B0286.2. Moreover, RNAi of the latrophilin gene results in a markedly constipated phenotype, associated with increased time for expulsion cycles, and in altered sensitivity to several pharmacological agents. This system offers a powerful opportunity to understand the endogenous role of the latrophilin gene.

We thank Virginie Vallet, Anne-Sophie Collin and Coralie Boulignat (University of Bourgogne, Dijon, France) for experiments on latrophilin during their Erasmus projects, Declan Brady for technical support, Dr Valerii Krasnoperov (New York University, New York, NY, U.S.A.) for help and advice on LTX purification, Dr Y. A. Ushkaryov for providing purified α-LTX and Professor Yuji Kohara for providing *C. elegans* cDNAs. This work was supported by Biotechnology and Biological Sciences Research Council (BBSRC) grant nos. 42/S06591 (S. R. T. and D. R. B.) and 42/d11591 (D. R. B.), Wellcome Trust grant no. 054778 (D. R. B.), a Royal Society Fellowship (P. V. P.) and a University of Nottingham studentship (C. J. M.). D. R. B. wishes to thank the EMBO *C. elegans* workshop for training and advice. Some of the strains used in this work were provided by the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN, U.S.A.), which is funded by the NIH National Centre for Research Resources. The B0286.2 deletion strain was prepared by the *C. elegans* Knockout Consortium.

REFERENCES

1 Elrick, D. B. and Charlton, M. P. (1999) α-Latrocrustatoxin increases neurotransmitter release by activating a calcium influx pathway at crayfish neuromuscular junction. J. Neurophysiol. **82**, 3550–3562
2 Longenecker, H. E. J., Hurlbut, W. P., Mauro, A. and Clark, A. W. (1970) Action of black widow spider venom on the frog neuromuscular junction. Nature (London) **225**, 701–703
3 Cull-Candy, S. G., Neal, H. and Usherwood, P. N. R. (1973) Action of black widow spider venom on an aminergic synapse. Nature (London) **241**, 353–354
4 Krasnoperov, V. G., Shamotienko, O. G. and Grishin, E. V. (1992) Isolation and properties of insect and crustacean-specific neurotoxins from the venom of the black widow spider (*Latrodectus mactans tredecimguttatus*). J. Nat. Toxins **1**, 17–23
5 Sudhof, T. C. (2001) α-Latrotoxin and its receptors: neurexins and cirl/latrophilins. Annu. Rev. Neurosci. **24**, 933–962
6 Grishin, E. V. (1998) Black widow spider toxins: the present and the future. Toxicon **36**, 1693–1701
7 Ashton, A. C., Rahman, M. A., Volynski, K. E., Manser, C., Orlova, E. V., Matsushita, H., Davletov, B. A., van Heel, M., Grishin, E. V. and Ushkaryov, Y. A. (2000) Tetramerisation of α-latrotoxin by divalent cations is responsible for toxin-induced non-vesicular release and contributes to the Ca^{2+}-dependent vesicular exocytosis from synaptosomes. Biochimie **82**, 453–468
8 Frontali, N., Ceccarelli, B., Gorio, A., Mauro, A., Siekevitz, P., Tzeng, M. C. and Hurlbut, W. P. (1976) Purification from black widow spider venom of a protein factor causing the depletion of synaptic vesicles at neuromuscular junctions. J. Cell Biol. **68**, 462–479
9 Ushkaryov, Y. A., Petrenko, A. G., Geppert, M. and Sudhof, T. C. (1992) Neurexins – synaptic cell-surface proteins related to the α-latrotoxin receptor and laminin. Science **257**, 50–56
10 Krasnoperov, V. G., Bittner, M. A., Beavis, R., Kuang, Y. N., Salnikow, K. V., Chepurny, O. G., Little, A. R., Plotnikov, A. N., Wu, D. Q., Holz, R. W. et al. (1997) α-Latrotoxin stimulates exocytosis by the interaction with a neuronal G-protein-coupled receptor. Neuron **18**, 925–937
11 Krasnoperov, V. G., Beavis, R., Chepurny, O. G., Little, A. R., Plotnikov, A. N. and Petrenko, A. G. (1996) The calcium-independent receptor of α-latrotoxin is not a neurexin. Biochem. Biophys. Res. Commun. **227**, 868–875
12 Davletov, B. A., Shamotienko, O. G., Lelianova, V. G., Grishin, E. V. and Ushkaryov, Y. A. (1996) Isolation and biochemical characterization of a Ca^{2+}-independent α-latrotoxin-binding protein. J. Biol. Chem. **271**, 23239–23245

© 2004 Biochemical Society

13 Lelianova, V. G., Davletov, B. A., Sterling, A., Rahman, M. A., Grishin, E. V., Totty, N. F. and Ushkaryov, Y. A. (1997) α-Latrotoxin receptor, latrophilin, is a novel member of the secretin family of G protein-coupled receptors. J. Biol. Chem. **272**, 21504–21508
14 Ichtchenko, K., Khvotchev, M., Kiyatkin, N., Simpson, L., Sugita, S. and Sudhof, T. C. (1998) α-Latrotoxin action probed with recombinant toxin: receptors recruit α-latrotoxin but do not transduce an exocytotic signal. EMBO J. **17**, 6188–6199
15 Davletov, B. A., Meunier, F. A., Ashton, A. C., Matsushita, H., Hirst, W. D., Lelianova, V. G., Wilkin, G. P., Dolly, J. O. and Ushkaryov, Y. A. (1998) Vesicle exocytosis stimulated by α-latrotoxin is mediated by latrophilin and requires both external and stored Ca^{2+}. EMBO J. **17**, 3909–3920
16 Geppert, M., Khvotchev, M., Krasnoperov, V., Goda, Y., Missler, M., Hammer, R. E., Ichtchenko, K., Petrenko, A. G. and Sudhof, T. C. (1998) Neurexin Iα is a major α-latrotoxin receptor that cooperates in α-latrotoxin action. J. Biol. Chem. **273**, 1705–1710
17 Volynski, K. E., Meunier, F. A., Lelianova, V. G., Dudina, E. E., Volkova, T. M., Rahman, M. A., Manser, C., Grishin, E. V., Dolly, J. O., Ashley, R. H. et al. (2000) Latrophilin, neurexin, and their signaling-deficient mutants facilitate α-latrotoxin insertion into membranes but are not involved in pore formation. J. Biol. Chem. **275**, 41175–41183
18 Khvotchev, M. and Sudhof, T. C. (2000) α-Latrotoxin triggers transmitter release via direct insertion into the presynaptic plasma membrane. EMBO J. **19**, 3250–3262
19 Orlova, E. V., Rahman, M. A., Gowen, B., Volynski, K. E., Ashton, A. C., Manser, C., van Heel, M. and Ushkaryov, Y. A. (2000) Structure of α-latrotoxin oligomers reveals that divalent cation-dependent tetramers form membrane pores. Nat. Struct. Biol. **7**, 48–53
20 Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. Genetics **77**, 71–94
21 Thomas, J. H. (1990) Genetic-analysis of defecation in *Caenorhabditis elegans*. Genetics **124**, 855–872
22 Timmons, L., Court, D. L. and Fire, A. (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. Gene **263**, 103–112
23 Kamath, R. S. and Ahringer, J. (2003) Genome-wide RNAi screening in *Caenorhabditis elegans*. Methods **30**, 313–321
24 Fraser, A. G., Kamath, R. S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M. and Ahringer, J. (2000) Functional genomic analysis of *C-elegans* chromosome I by systematic RNA interference. Nature (London) **408**, 325–330
25 Reference deleted
26 Stein, L., Sternberg, P., Durbin, R., Thierry-Mieg, J. and Spieth, J. (2001) WormBase: network access to the genome and biology of *Caenorhabditis elegans*. Nucleic Acids Res. **29**, 82–86
27 Krasnoperov, V., Bittner, M. A., Holz, R. W., Chepurny, O. and Petrenko, A. G. (1999) Structural requirements for α-latrotoxin binding and α-latrotoxin-stimulated secretion – a study with calcium-independent receptor of α-latrotoxin (CIRL) deletion mutants. J. Biol. Chem. **274**, 3590–3596
28 Iwasaki, K., Staunton, J., Saifee, O., Nonet, M. and Thomas, J. H. (1997) aex-3 encodes a novel regulator of presynaptic activity in *C- elegans*. Neuron **18**, 613–622
29 Baylis, H. A., Matsuda, K., Squire, M. D., Fleming, J. T., Harvey, R. J., Darlison, M. G., Barnard, E. A. and Sattelle, D. B. (1997) ACR-3, a *Caenorhabditis elegans* nicotinic acetylcholine receptor subunit – molecular cloning and functional expression. Receptors Channels **5**, 149–158
30 Schaefer, A. M., Hadwiger, G. D. and Nonet, M. L. (2000) rpm-1, a conserved neuronal gene that regulates targeting and synaptogenesis in *C-elegans*. Neuron **26**, 345–356
31 Zhao, H. J. and Nonet, M. L. (2001) A conserved mechanism of synaptogyrin localization. Mol. Biol. Cell **12**, 2275–2289
32 Saeger, B., Schmitt-Wrede, H. P., Dehnhardt, M., Benten, W. P. M., Krucken, J., Harder, A., Von Samson-Himmelstjerna, G., Wiegand, H. and Wunderlich, F. (2001) Latrophilin-like receptor from the parasitic nematode *Haemonchus contortus* as target for the anthelmintic depsipeptide PF1022A. FASEB J. **15**, 1332–1334
33 Umbach, J. A., Grasso, A., Zurcher, S. D., Kornblum, H. I., Mastrogiacomo, A. and Gundersen, C. B. (1998) Electrical and optical monitoring of α-latrotoxin action at *Drosophila* neuromuscular junctions. Neuroscience **87**, 913–924
34 Sugita, S., Janz, R. and Sudhof, T. C. (1999) Synaptogyrins regulate Ca^{2+}-dependent exocytosis in PC12 cells. J. Biol. Chem. **274**, 18893–18901
35 Nonet, M. L., Grundahl, K., Meyer, B. J. and Rand, J. B. (1993) Synaptic function is impaired but not eliminated in *C-elegans* mutants lacking synaptotagmin. Cell (Cambridge, Mass.) **73**, 1291–1305
36 Take-uchi, M., Kawakami, M., Ishihara, T., Amano, T., Kondo, K. and Katsura, I. (1998) An ion channel of the degenerin/epithelial sodium channel superfamily controls the defecation rhythm in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. U.S.A. **95**, 11775–11780

Received 11 August 2003/14 October 2003; accepted 31 October 2003
Published as BJ Immediate Publication 31 October 2003, DOI 10.1042/BJ20031213

© 2004 Biochemical Society