Neuronal uptake of pesticides disrupts chemosensory cells of nematodes

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SUMMARY

Low doses of the acetylcholinesterase-inhibiting carbamate nematicides disrupt chemoreception in plant-parasitic nematodes. Fluorescein isothiocyanate (FITC)/dextran conjugates up to 12 kDa are taken up from the external medium by certain chemosensory neurons in *Caenorhabditis elegans*. Similar chemoreceptive neurons of the non-feeding infective stage of *Heterodera glycines* (soybean cyst nematode) fill with FITC and the nuclei of their cell bodies selectively stain with bisbenzimide. The widely used nematicide aldicarb disrupts the chemoreceptive response of *H. glycines* with 50% inhibition at very low concentrations (*ca* 1 pM), some 10^{-6}-fold lower than required to affect locomotion. Similarly, the anthelmintic levamisole had this effect at 1 nM. Peptides selected as mimetics of aldicarb and levamisole also disrupt chemoreception in *H. glycines* and *Globodera pallida* at 10^{-3}-fold or lower concentration than required to inhibit locomotion. We propose an uptake pathway for aldicarb, levamisole, peptide mimetics and other soluble molecules by retrograde transport along dendrites of chemoreceptive neurons to the cell bodies and synapses where they act. This may prove to be a general mechanism for the low-dose effects of some nematicides and anthelmintics.

Key words: aldicarb, *Heterodera glycines*, *Globodera pallida*, plant-parasitic nematode, axonal transport, nematicide, peptide mimetic.

INTRODUCTION

Plant-parasitic nematodes cause at least $100 billion per year in global crop losses (Sasser & Freckman, 1987). Carbamates, such as the oxime carbamate aldicarb, are widely used nematicides that inhibit acetylcholinesterase. In the field, aldicarb is effective against nematodes at low concentration. It does not lead to paralysis or death, but rather orientation to known stimuli is impaired (Trett & Perry, 1985; Perry, 1996). This implies an effect on chemoreception, although the mechanism of aldicarb uptake is ill-defined. This pesticide is active against a wide range of nematodes including the non-feeding infective stages of cyst nematodes, thus precluding an oral ingestion route. All uptake has therefore been presumed to occur by a transcuticular route – a process enhanced in certain animal-parasitic nematodes that excrete organic acids as end-products of a carbohydrate-dependent energy metabolism in microaerobic environments. This excretion provides an acidic cuticle pore microenvironment that favours uptake of certain anthelmintics (Sims *et al*. 1992). *Caenorhabditis elegans* does not excrete organic acids under aerobic conditions but relies on an aerobic lipid-dependent energy metabolism (Foll *et al*. 1999). Free-living stages of plant-parasitic nematodes also utilize lipid (Holz, Wright & Perry, 1997). Uptake of model compounds or drugs by nematodes in the millimolar to micromolar range results in effects like paralysis. Such work, particularly with nematodes excreting organic acids, does not adequately describe the effects of pico- and nanomolar concentrations of drugs or pesticides on plant-parasitic nematode behaviour. The present study suggests that, in nematodes, retrograde transport in chemosensory dendrites provides a route for uptake of soluble compounds present in the environment at low concentrations.

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MATERIALS AND METHODS

Nematodes

Caenorhabditis elegans (N2) were maintained in the laboratory on agar plates pre-seeded with *Escherichia coli* OP50, originally provided by Dr Ian Hope, University of Leeds. Plant-parasitic nematodes have been maintained in this laboratory for many years using appropriate host plants. *Heterodera glycines* (originally from University of Newcastle) and *Globodera pallida* (originally from Scottish Crops Research Institute) infective-stage larvae (J2) were obtained as described by Atkinson *et al*. (1988). Briefly, eggs released from cysts were placed on 30 μm nylon suspended in potato root diffusate (*G. pallida*) or sterile tap water (*H. glycines*). Hatched juveniles which had passed through the mesh were collected from the solution every 24 h, to be used in the experiments described below.

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Dye-filling and staining

C. elegans was incubated in FITC/dextrans (Sigma) of various sizes to ascertain the exclusion limits of the sensory neuron. Dextrans were dissolved in PBS at 1 mg/ml and nematodes were incubated in 0·45 μm mini-filter tubes for 16 h in the dark at room temperature. For *H. glycines*, a 50 μl aliquot of 5 mg/ml FITC in dimethylformamide was added to 200 μl of M9 buffer and nematodes were incubated for 1–16 h in the dark at room temperature. Incubations were carried out in 0·45 μm mini-filter tubes to facilitate stain removal and subsequent washing. Bisbenzamide staining was performed in a 1 mg/ml solution for 16 h at room temperature in the dark. Nematodes were mounted on glass slides and observed under a microscope using epilumination and filters for FITC fluorescence or a UV filter for bisbenzamide (400 ×, Leitz DMR B). Images were captured using a CCD camera (Cohu) attached to a PC running Leica QWin image analysis software.

Aldicarb

Aldicarb was removed from Temik™ granules (10% aldicarb w/w) by placing specific quantities into chloroform. Aldicarb dissolves in chloroform while the inert carrier does not. Aliquots of this chloroform/aldicarb solution were placed in Eppendorf tubes and allowed to evaporate in a fume hood. The aldicarb remained as a residue in the tubes and this was then dissolved in distilled H_2O to provide known concentrations when required.

Peptide mimetic selection

Two phage peptide-display libraries (New England Biolabs), a linear 12-mer and a constrained 7-mer were screened by incubating phage with *Torpedo* acetylcholinesterase (AChE) bound to agarose beads (Sigma). After extensive washing, bound phage were eluted with 5·25 μM aldicarb solution. Following 3 rounds, clones were selected using an ELISA with immobilized AChE and tested in an AChE inhibition assay. For levamisole, the constrained 7-mer library was biopanned for 3 rounds against homogenized *C. elegans* membrane fractions in MAN buffer (Lewis & Berberich, 1992). After washing, bound phage were eluted with 10 mM levamisole (Sigma). For both screens, selected clones were sequenced by dye-terminator chemistry using an ABI 373A and consensus peptide sequences derived. The following peptides were commercially synthesized for behavioural experiments, ACHE-I-7.1, a disulphide constrained 7-mer (CSINWRHHC), ACHE-I-12.1, a linear 12-mer (SVSVGMKPSPRP), both mimetics of aldicarb, and ACHE-R-7.1, a randomized sequence of ACHE-I-7.1 (CHNSHIRWC) for use as a control. LEV-I-7.1, a disulphide constrained 7-mer (CTTMHPRLC) was selected as a mimetic of levamisole.

Chemoreception assays

For chemoreception assays, plant-parasitic nematodes were incubated in small volumes of test or control solution in 0·45 μm mini-filter centrifuge tubes to simplify removal of solutions and washing. To prevent damage to nematodes, tubes were centrifuged at low speed (<300 ***g***). Initially, nematodes were incubated for 1, 2, 4, 6 or 16 h to define the timescales for uptake, and subsequently experiments were conducted using an incubation time of 16 h. The bioassay was modified from that of Grundler, Schnibbe & Wyss (1991), using 50 mM $CaCl_2$ as an attractant which gave more consistent results than root exudate. Briefly, 300 worms in a small volume (<50 μl) of water were placed in the centre of a 3·5 cm diameter Petri dish which contained a 1·5% agarose base. Worms were left to disperse for 1 h, then 6 mm agarose discs were placed at equal distances from the centre. One disc was coated with attractant and the other was a water control. Assays were performed for 1 h at 25 °C in the dark. Each day, control bioassay plates were set up and the difference between the number of nematodes under the H_2O and attractant discs were used as the 100% attraction figure for treatments that day. For untreated populations the number of nematodes under the attractant disc was approximately 2–4 times that under the H_2O control disc. Each experiment was replicated at least 8 times for each treatment. The nematodes under each disc were counted and the data were analysed using the statistical package SPSS, which provides proportionate responses based on probit values, therefore percentage response curves for each compound could be produced. This provided 50% and 90% inhibition concentrations.

RESULTS

The functions of 8 chemoreceptive neurons in the anterior sense organs (amphids) have been defined for the nematode *C. elegans* (Bargmann & Mori, 1997). These neurons 'fill' with the lipophilic dye fluorescein isothiocyanate (FITC) as do homologous neurons of animal-parasitic nematodes (Hedgecock *et al.* 1985; Ashton, Li & Schad, 1999). We have found that the chemoreceptory neurons of *C. elegans* also take up hydrophilic FITC/dextran conjugates of M_r 4·4 kDa and 12 kDa but not of M_r 19·5 kDa (Fig. 1A). Time-course studies showed that accumulation of FITC/dextrans to visible concentrations in *C. elegans* took at least 6 h. Certain sensory neurons of *H. glycines* filled with FITC beyond their cell bodies to commissures and the nerve ring (Fig. 1B). One of these cells appeared to have a projection directly into

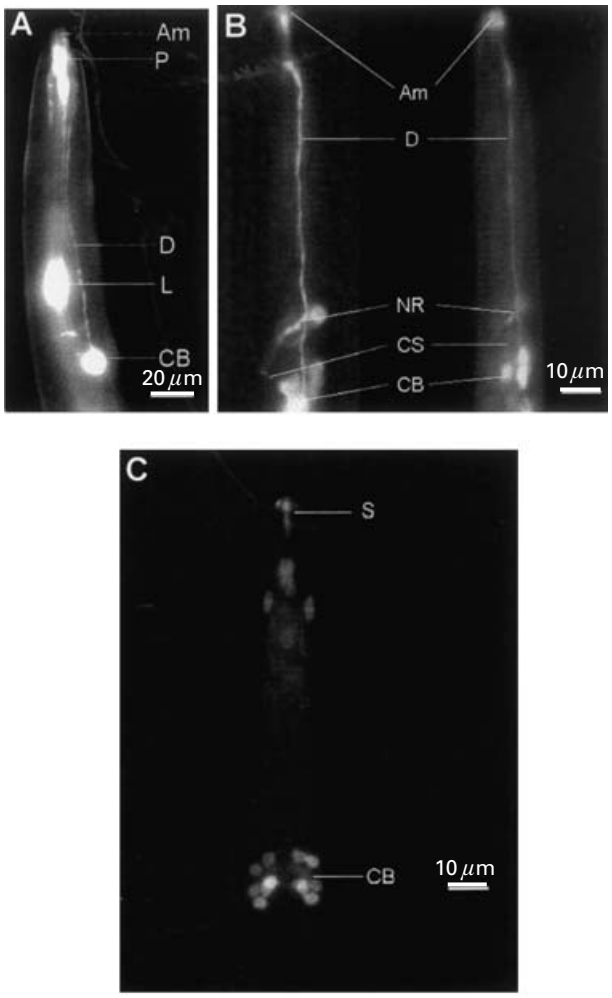

Fig. 1. Nematode sensory dendrite uptake of dyes and their accumulation at cell bodies (A) FITC/dextran conjugate of M_r 12 kDa (Sigma) is clearly visible from the amphids (Am) along the sensory dendrites (D) in *Caenorhabditis elegans*, reflecting transport of the compound to the cell bodies (CB). Ingested FITC/dextran is also visible in the anterior lumen of the pharynx (P) and more posteriorly in the lumen of one of the pharyngeal bulbs (L). (B) Sensory dendrites and cell bodies of 2 individual *Heterodera glycines* 'filled' with FITC. The dye extends down the neuron from the amphids (Am) to the cell body (CB), then via a commissure (CS). The axon enters the nerve ring (NR). In the right hand nematode the direct connection of the labelled cell body and commissure to the nerve ring can be seen. This connection is similar to the ADL neuron in *C. elegans*. The view is left lateral. (C) The infective stage of *H. glycines* following incubation in bisbenzimide. The chemosensory neural cell bodies (CB) are stained and clearly visible. The stylet (S) is visible due to autofluorescence.

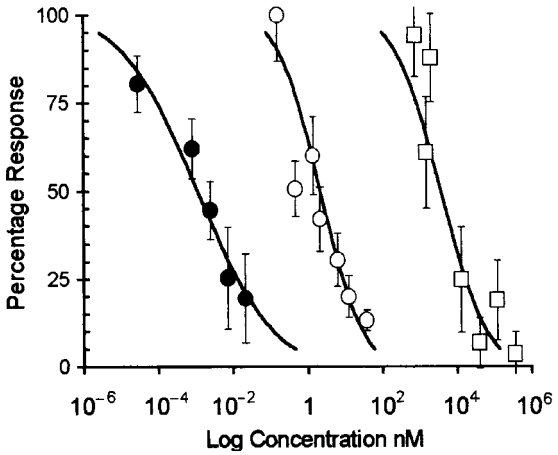

Fig. 2. Chemoreception assay responses. The dose-response curve of 2nd-stage juveniles of *Heterodera glycines* to an attractant disc following incubation in various concentrations of aldicarb (●), ACHE-I-7.1 (○) and ACHE-I-12.1 (□). Bars are S.E.M. values. Curves were fitted using predicted values provided by probit analysis in SPSS.

the nerve ring, comparable to ADL in *C. elegans*. However, accumulation was too low for visualization of the lower fluorescence yields provided by FITC/dextrans in *H. glycines*. Bisbenzimide, a nucleic acid vital stain, was taken up. It revealed 8 nuclei in neuronal cells on each side of the nematode (Fig. 1C). These cells may be comparable to the 8 amphidial neural cells ADF, ADL, ASE, ASG, ASH, ASI, ASJ and ASK in *C. elegans*, but a more detailed study is required to accurately define these cells in *H. glycines*. *C. elegans* ingests bisbenzimide and a wide range of nuclei are stained rather than just the limited number visualized in the non-feeding plant-parasitic nematode.

Probit analysis established a 50% loss in ability of *H. glycines* to respond to a chemoreceptive stimulus (Grundler *et al.* 1991) at $1{\cdot}1 \pm 3{\cdot}06$ pM aldicarb (Fig. 2). The analysis predicted a 90% inhibition of normal behaviour at 21 pM aldicarb whereas incubating nematodes in 1 μM was required to induce paralysis. At low concentrations of aldicarb, far below those required to cause paralysis, loss of chemoreception only occurred some 6 h after exposure.

ACHE-I-7.1 inhibited 50% of the chemoreceptive response of *H. glycines* at $2{\cdot}16 \pm 6{\cdot}54$ nM, while ACHE-I-12.1 was less effective with a corresponding value of $3{\cdot}68 \pm 33{\cdot}6$ μM. Much higher concentrations of both aldicarb ($>10^6$-fold higher) and peptides ($>10^3$-fold higher) were required to inhibit nematode movement. The ACHE-R-7.1 control had no effect on behaviour or locomotion at 1 mM concentration. Chemoreception was also inhibited in *G. pallida* when treated with ACHE-I-7.1 at 1 nM, with $55{\cdot}7 \pm 6{\cdot}19$ and $39{\cdot}6 \pm 3{\cdot}02$ nematodes under the control and attractant discs respectively. LEV-I-7.1 completely inhibited chemoreception in *H. glycines* at 1 μM, with $33{\cdot}0 \pm 4{\cdot}04$ and $31{\cdot}3 \pm 4{\cdot}18$ under the control and attractant discs respectively. It also inhibited *G. pallida* at the same concentration with

$34{\cdot}5 \pm 1{\cdot}32$ and $40{\cdot}8 \pm 3{\cdot}30$ under control and attractant discs respectively. There was no loss of locomotory activity after 16 h incubation for either genus at this concentration.

DISCUSSION

Transcuticular uptake of our peptides is highly unlikely based on observations by Sheehy *et al.* (2000) who examined the peptide AF2 (KHEYLRF-NH_2) and model hydrophobic peptides. They showed no transcuticular uptake of the excitatory AF2 peptide in *Ascaris* at 10^4-fold higher concentration than the peptides we show cause chemosensory effects. They also concluded that metabolism of peptides may prevent them from reaching their targets after uptake. The model peptides they showed that were taken up transcuticularly were all hydrophobic and neutral. Uptake was enhanced by methylation to reduce the hydrogen bonding potential with solvent, thus enhancing movement across the lipophilic hypocuticle (Sheehy *et al.* 2000). Our peptides are all positively charged at physiological pH and possess significant potential for hydrogen bonding with solvent, both factors that would reduce the possibility of efficient transcuticular uptake. Levamisole at $0{\cdot}3$–$10\,\mu$M is taken up across the cuticle of animal parasitic nematodes (Ho *et al.* 1994), and presumably both it and aldicarb can enter plant nematodes in this way. However, previous studies have only examined the effects of high concentrations (Marks, Thomason & Castro, 1968; Ho *et al.* 1994). The similar time frames (>6 h) for action of aldicarb, levamisole and our peptides at low concentrations suggest a common uptake mechanism. Our data lead us to propose that peptide mimetics of both aldicarb and levamisole are taken up from the environment by chemoreceptive sensillae, leading to retrograde dendritic transport to their site of action. One possible area for further research is that at very low concentrations, aldicarb and levamisole may also be preferentially transported along neurons rather than across the cuticle.

This mechanism may be distinct from that used by FITC due to the time difference observed and the differences in staining pattern for FITC and FITC/dextrans. Hedgecock *et al.* (1985) showed that FITC staining could be achieved in minutes, whereas our observations show several hours are required to see an effect on chemoreception. This delay is consistent with a period of transport and accumulation of the pesticide along the chemoreceptive neurons as observed with FITC/dextrans in *C. elegans*. This process may therefore require accumulation of pesticide to a threshold level at neuronal cell bodies before chemoreception is disrupted. Our observations of chemosensory disruption by levamisole and its mimetic at low concentrations also suggest an effect at a target other than the commonly studied receptor at neuro-muscular junctions (Fleming *et al.* 1997; Martin *et al.* 1997; Richmond & Jorgensen, 1999).

Retrograde transport of cholinesterase inhibitors presumably would result in their presence in neuronal cell bodies and at their axonal synapses. Similar interpretations may apply to ivermectin and *C. elegans*. At low concentrations this anthelmintic blocks glutamate-gated chloride ion channels of wild-type *C. elegans* and inhibits pharyngeal pumping. Its efficacy is not impaired if oral uptake is prevented (Smith & Campbell, 1996). However, mutations in 2 genes (*avr-1/che-3* and *avr-5/osm-3*) cause abnormal amphidial structure and confer resistance to ivermectin. *C. elegans* with mutations in these genes are defective in FITC-filling and they are chemotactically deficient (Starich *et al.* 1995; Johnson, Grant & Hunt, 1996; Blaxter & Bird, 1997). This phenotype is consistent with neuronal abnormality preventing uptake of molecules such as FITC and ivermectin and thus conferring resistance against this compound. *Che-3* encodes the cytoplasmic molecular motor dynein which is involved in retrograde neuronal transport (Signor *et al.* 1999). Current opinion suggests 2 possible models for the action of ivermectin on nematodes, based on the ivermectin-resistant mutants of *C. elegans*. Either it acts via a peri-amphidial receptor, or via neurons in the amphid that inhibit the pharyngeal muscle (Blaxter & Bird, 1997). In model one, the receptor becomes inaccessible due to anatomical changes in the amphid and in case two, the neurons responsible for inhibiting the pharynx in the wild-type are ablated. Our observations suggest a third possible model, namely that ivermectin is transported along the chemosensory neurons from the amphid to the cell bodies and synapses causing disruption of function at the nerve ring.

Retrograde dendritic transport of molecules and complexes along neurons occurs in many animals (Maratou, Theophilidis & Arsenakis, 1998; Ohka *et al.* 1998; Henriksson & Tjalve, 1998; Henriksson, Tallkvist & Tjalve, 1999). Pesticide uptake by retrograde neuronal transport may also prove to be a common mechanism and may explain some previous observations of their toxicity. Exposure of nematodes to aldicarb at 5–10 ppm is correlated with degradation of sensory dendrites in nematodes (Trett & Perry, 1985). It is surprising that accurate knowledge of uptake pathways does not form a part of the essential data required by regulatory authorities before environmentally hazardous pesticides are approved for use. We anticipate our findings will enable development of a new generation of specific, effective nematicides and anthelmintics that are neuronally transported by nematodes but not mammals.

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