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TRANSEPITHELIAL TRANSPORT OF NICOTINE AND VINBLASTINE IN ISOLATED MALPIGHIAN TUBULES OF THE TOBACCO HORNWORM (*MANDUCA SEXTA*) SUGGESTS A P-GLYCOPROTEIN-LIKE MECHANISM

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Summary

We have examined the accumulative transport properties of the Malpighian (excretory) tubules of the tobacco hornworm *Manduca sexta* to test the hypothesis that a P-glycoprotein-like multidrug transporter is active and is responsible for the excretion of dietary nicotine in this tissue. Isolated tubules were cannulated and exposed to radiolabelled forms of either nicotine (5 min exposure) or the P-glycoprotein substrate vinblastine (60 min exposure) in the bathing (basal surface) fluid. The luminal (apical) contents were then flushed, and lumen-to-bath ratios were measured. Although these ratios provide conservative estimates of the physiological ability of Malpighian tubules to move compounds from blood to lumen, tubules concentrated nicotine 10-fold from an initial bath concentration of 0.5 mmol l^{-1} and vinblastine threefold (from an initial concentration of 1 μmol l^{-1}). Vectorial transport of vinblastine and nicotine was eliminated by 25 μmol l^{-1} verapamil (a P-glycoprotein inhibitor) and was not dependent on the presence of a transepithelial electrical potential. Nicotine transport was inhibited by atropine (3 mmol l^{-1}), while nicotine (≥50 μmol l^{-1}) significantly reduced vinblastine transport. Verapamil was effective at reducing vinblastine transport when applied to the basal side alone, but not when applied to the apical side alone. Taken together, these results are consistent with the idea that the active excretion of nicotine and other alkaloids by the tobacco hornworm is mediated by a P-glycoprotein-like mechanism.

Key words: dillapiol, transepithelial transport, nicotine, vinblastine, Malpighian tubule, tobacco hornworm, *Manduca sexta*.

Introduction

The insect Malpighian tubule is often compared with the vertebrate kidney, since both perform the role of eliminating wastes from the body fluid. In both systems, non-specific filtration is supplemented by active transport mechanisms. The transporters responsible appear to be few in number but broad in specificity. For insects, one has been characterized as a multi-alkaloid transporter on the basis of mutually competitive transport of nicotine, atropine and morphine in the Malpighian tubules of *Rhodnius prolixus* (Maddrell and Gardiner, 1976), a blood-sucking bug (Hemiptera) unlikely to encounter any of these compounds in nature. The tobacco hornworm *Manduca sexta* (Lepidoptera: Sphingidae), which as a caterpillar can live entirely on tobacco, also has the facility to transport nicotine across its Malpighian tubules (Maddrell and Gardiner, 1976) and, significantly, to prevent influx of nicotine across its blood–brain barrier (Murray *et al.* 1994). Since both of these alkaloid-handling tissues are also immunopositive for P-glycoprotein (Murray *et al.* 1994), often called the multidrug pump, a P-glycoprotein homologue is a plausible candidate for the insect multi-alkaloid pump.

P-glycoprotein (P-gp) is a membrane protein of the ATP-binding cassette (ABC) transporter family conserved among taxa as diverse as protozoa, plants, insects and mammals (Higgins, 1992; Childs and Ling, 1996). Despite extensive study since its discovery as a mediator of multidrug resistance in tumour cells, its mode of action remains controversial (Roepe *et al.* 1996). What is clear is that, by direct or indirect means, P-gp expression can protect cells from a variety of unrelated toxic compounds by lowering intracellular concentrations of these drugs. A role in protecting organisms from xenobiotics is supported by a number of different studies. For example, 'knock-out' mice genetically deficient in P-gp are phenotypically normal except that they show an increased tissue, particularly brain, penetration by various drugs and a decreased elimination of these drugs, leaving them vulnerable compared with their wild-type counterparts. Such drugs include vinblastine and the centrally neurotoxic pesticide ivermectin (Schinkel *et al.* 1994, 1997; van Asperen *et al.* 1996). Likewise, when a P-gp gene is deleted in *Caenorhabditis elegans*, the nematode becomes sensitive to

colchicine and chloroquine (Broeks *et al.* 1995), and *Mdr49*-deleted *Drosophila melanogaster* show increased sensitivity to colchicine (Wu *et al.* 1991). Other lines of evidence implicating P-gp in protection from environmental toxins include P-gp-inhibitor-sensitive exclusion of multiple P-gp substrates from, for example, the gills of mussels *Mytilus* spp. (Cornwall *et al.* 1995; Kurelec, 1995) and from the larvae of the marine worm *Urechis caupo* (Toomey and Epel, 1993). In the tobacco budworm *Heliothis virescens* (Lepidoptera: Noctuidae), western blot analysis shows increased P-gp expression in the cuticle and fat body correlated with increased pesticide resistance (Lanning *et al.* 1996*b*). In the same insect, quinidine, a P-gp inhibitor, increases cuticular sensitivity to thiodicarb (Lanning *et al.* 1996*a*).

Evidence that P-gp functions as part of the excretory system comes from a number of different directions. In vertebrates, P-gp is highly expressed at secretory surfaces such as the bile canalicular face of hepatocytes, the intestinal surface epithelium and the apical surface of kidney proximal tubules (Thiebaut *et al.* 1987). Both intact renal proximal tubules (e.g. from killifish *Fundulus heteroclitus*; Miller, 1995) and mammalian renal cell lines (e.g. MDCK; Hunter *et al.* 1991) are capable of active outward transport of P-gp substrates such as daunomycin and vinblastine. A similar role for P-gp in whole-body elimination of xenobiotics in insects is suggested by the finding that Malpighian tubules are P-gp-immunopositive (Murray *et al.* 1994). If active transport of xenobiotics in the tobacco hornworm depends on P-gp, then the Malpighian tubules should meet the minimum criterion for such transport. Namely, they should be able to accumulate vinblastine, an archetypical P-gp substrate (and alkaloid), and this transport should be sensitive to the standard P-gp inhibitor verapamil. Furthermore, the transport of nicotine, the alkaloid of particular biological relevance to this insect, should also be sensitive to verapamil. We tested these ideas using an isolated Malpighian tubule preparation. The results suggest that a P-gp-like mechanism is indeed active in the insect Malpighian tubule and raise the intriguing possibility that nicotine is one of its substrates. If this is correct, it may have practical implications for such wide-ranging issues as multi-insecticide resistance (Denholm and Rowland, 1992), nicotine interference with drug clearance in clinical practice and nicotine tolerance among tobacco users.

Materials and methods

Animals

Tobacco hornworm (*Manduca sexta*) eggs were obtained from Carolina Biological Supply Company (Burlington, NC, USA) and raised at room temperature (22–23 °C) on a 16 h:8 h L:D photoperiod at ambient humidity. Larvae were raised individually on a nicotine-free artificial diet, based on either wheat germ (modified from Bell and Joachim, 1976) or cornmeal (Carolina Biological Supply). Fifth-instar larvae were used before they entered the wandering stage (determined by the appearance of the pulsating dorsal vessel).

Saline and chemicals

'*Manduca* saline', hereafter referred to as 'saline', was adapted from that of Maddrell and Gardiner (1976) and used for dissecting, bathing and perfusing the Malpighian tubule tissue and for final dilution of all applied drugs. Its composition, in mmol l^{-1}, was: NaCl, 15; KCl, 30; $CaCl_2{\cdot}2H_2O$, 2; $MgCl_2{\cdot}6H_2O$, 30; Hepes, 5; D-glucose, 10; maltose, 10; sodium citrate, 5; and glycine, 10. It was adjusted to pH 7.2 with 5 mol l^{-1} KOH, filter-sterilized and stored at 4 °C.

For the nicotine transport assay, [*N*-methyl-^{3}H]nicotine (Amersham, Oakville, Ontario, Canada; 84 Ci $mmol^{-1}$, 70 nmol l^{-1} final concentration) was mixed with unlabelled nicotine hydrogen tartrate (Sigma-Aldrich, Oakville, Ontario, Canada), adjusted to pH 7 with KOH, to give solutions of 0.05, 0.5 and 5 mmol l^{-1} nicotine in saline. When unlabelled nicotine was used in solutions with radiolabelled vinblastine, the pH was adjusted to 7.2 with 5 mol l^{-1} KOH, and the solutions were used within 5 days.

For the vinblastine transport assay, [G-^{3}H]vinblastine sulphate (Amersham; 11.4 Ci $mmol^{-1}$ in methanol) was mixed with unlabelled vinblastine sulphate (Sigma; made as 17 mmol l^{-1} stock in 2:1 ethanol:saline) to make a 2× working solution of vinblastine (2 μmol l^{-1}, 0.75 Ci $mmol^{-1}$). The final concentration applied to the tubules was thus 1 μmol l^{-1} vinblastine with 0.3 % methanol and 0.004 % ethanol.

Stock solutions of (±)verapamil hydrochloride (Sigma) and atropine (Sigma) were made in water and diluted to their final concentration in saline.

Dillapiol [1-allyl-2,3-dimethoxyl-4,5-(methylenedioxy)-benzene] was purified (99 % as determined by nuclear magnetic resonance spectra) as an oil from natural sources by T. Durst (University of Ottawa), and a 100 mmol l^{-1} stock was made in ethanol:water (5:4). Even after vortexing, tiny oil droplets were visible, so the stock solution and dilutions in saline were sonicated (Bransonic 12, Branson Cleaning Equipment Co., Shelton, CT, USA) for 1 min immediately prior to use. The final concentration of ethanol was less than 0.06 %.

Transport assay

Larvae were sedated by refrigerating at 4 °C, decapitated with scissors, then pinned, dorsal side up, to a dissection tray and covered with ice-cold saline. The dorsal surface was cut lengthwise, exposing four of the six Malpighian tubules. A 3–4 cm section of a single Malpighian tubule was carefully dissected free of tracheal connections and removed. The regions of tubule defined as proximal (nearest the entry point to the gut) and medial (Nijhout, 1975) or as descending and ascending straight segments (Moffett, 1994) were used. Maddrell and Gardiner (1976) showed that either the proximal region alone or the proximal plus medial region exhibited nicotine transport. For our nicotine transport experiments, the 3–4 cm sections overlapped the two regions; for the vinblastine transport experiments, we reduced the number of variables by using only the proximal region.

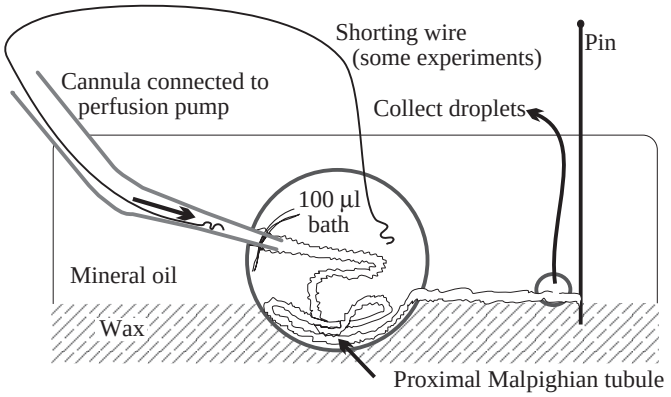

Fig. 1. Diagram illustrating the procedure for measuring the transport of vinblastine or nicotine across the Malpighian tubule epithelium. A 3–4 cm piece of the proximal Malpighian tubule was cannulated and incubated in a 100 µl bath droplet containing radiolabelled drug. Cannulation enabled the rapid collection of the luminal contents from a cut in the tubule and also allowed for drugs to be selectively applied to the apical (luminal) or basal (bath) side of the tissue or to both sides simultaneously. For some experiments, a chlorided silver shorting wire was inserted, as shown, to dissipate any transepithelial electrical potential.

In a modification of the method described by Maddrell and Overton (1990), the isolated Malpighian tubule was immersed in a 100 µl bath droplet in a wax-lined, Petri dish filled with mineral oil and cannulated to allow artificial perfusion of the tubule, as shown in Fig. 1.

Cannulae were prepared by pulling glass, non-heparinized microhaematocrit capillary tubes (Fisherbrand, internal diameter 1.1–1.2 mm) with a pipette puller (List-Medical, model L/M-3P-A) to a fine tip, breaking the tip to 30–60 µm outer diameter and then removing the jagged edges by fire-polishing close to a red-hot wire. The cannula was bent so that it would enter the bath droplet at a convenient angle when mounted on a micromanipulator. The latter held the cannula in a pipette holder, and flexible tubing connected the cannula to a 1 ml syringe driven by a perfusion pump (Harvard Apparatus, South Natick, MA, USA; pump 22).

One end of the Malpighian tubule segment was pulled onto the cannula using two pairs of fine forceps and secured with a short piece of waxed dental floss (3–5 individual strands). The other end was pulled out of the bath droplet and pierced with a sharp implement to allow fluid to escape. To hold the tissue in place, a fine pin was pushed through the end, into the wax.

Because fluid secretion is naturally slow in this segment, the orientation of the tubule was not considered critical. However, in an effort to improve reproducibility, for the vinblastine experiments only the more distal end was cannulated and fluid was collected from the proximal end, mimicking the natural movement of wastes towards the hindgut (Nijhout, 1975).

After cannulation, saline (or saline plus drugs) was perfused for several minutes at 2.5 µl min^{-1} through the tubule, refilling it and flushing out the contents. This step was necessary because the proximal tubules became deflated during dissection and natural fluid secretion is very slow (Maddrell and Gardiner, 1976). A control sample, for background radioactivity, was taken from the luminal contents at this time. Once it had been established that there were no leaks in the tissue, on the basis of the throughput at the expected rate of 2.5 µl min^{-1}, artificial perfusion was halted, and the radiolabelled drug (with or without inhibitors) was added to the basal (bath) side of the tissue. For the nicotine transport experiments, the entire bath droplet was removed and replaced with a new droplet containing drugs at their desired final concentration. For vinblastine transport experiments, half the bath fluid was replaced with drugs at twice the desired final concentration.

For testing interactions with nicotine transport, verapamil (25 µmol l^{-1}) and atropine (3 mmol l^{-1}) were included both in the bath droplet, exposing the basal surface of the tubule cells, and in the perfusate, exposing the apical surface, and thus pre-exposing the apical surface for approximately 2 min. In studies of vinblastine transport inhibition, verapamil (25 µmol l^{-1}) and dillapiol (50 and 100 µmol l^{-1}) were likewise applied to both surfaces, but the preincubation period was approximately 20 min. When nicotine (5–500 µmol l^{-1}) was tested as a potential inhibitor of vinblastine transport, it was added simultaneously with the vinblastine at the basal side only. To characterize the action of verapamil on vinblastine transport more fully, experiments were performed in which verapamil was applied to only one surface of the Malpighian tubule. To expose the apical surface, the tubule was cannulated and pre-perfused for 10 min with 25 µmol l^{-1} verapamil, flushing through at least 15 µl. During this time, 50 µl (50 %) of the bath was replaced three times with fresh saline in case some verapamil had entered the bath droplet from the cannula prior to its insertion into the lumen. For basal application, the tubule was also pre-incubated for 10–12 min.

For experiments to determine whether a transepithelial electrical potential might be driving the movement of (charged) drugs, a 0.25 mm chlorided silver wire was passed from inside the cannula to the bath (see Fig. 1) to short-circuit any electrical potential between the bath and the lumen.

Sampling

Once the apical and basal solutions had been established as above, the fluids were left undisturbed for the duration of the experiment, and the lumen was then flushed and samples collected after an appropriate period. For labelled nicotine, a single 5 µl sample was collected by flushing after 5 min. For labelled vinblastine, accumulative transport occurred much more slowly and so, typically, 2.0 µl samples of the bathing solution were taken 1, 15, 30 and 45 min after addition of the labelled drug. At 60 min, a 5.0 µl sample of the bathing solution was taken and the perfusion pump was restarted (at 2.5 µl min^{-1}) to flush the contents of the lumen. The first of three 5.0 µl samples collected sequentially from the cut end of the Malpighian tubule was compared with the sample from the bath.

Samples were dispensed into 3 ml of scintillation cocktail [EcoLite (+), ICN, Costa Mesa, CA, USA) and counted in a liquid scintillation counter (Beckman LS1701). Background counts were negligible compared with all samples and were not subtracted.

It was found that a considerable amount (average approximately 15 % of total activity) of radiolabelled vinblastine adhered to the plastic pipette tips and was thus not normally counted. However, there was no difference in the lumen-to-bath ratios obtained when this was taken into account.

Statistical treatment

Results are presented as the mean ± standard error of the mean (number of samples). Two-tailed Student's *t*-tests at the 5 % level of significance were performed to test for differences in means.

Results

Nicotine accumulates in the lumen of Malpighian tubules

Because our assay differed from that used by Maddrell and Gardiner (1976) (e.g. the tubules were not continually perfused but rather flushed out after 5 min), we first confirmed that the nicotine transport reported by them could be observed in our system.

When the initial bath nicotine concentration was 0.05 mmol l^{-1}, the luminal concentration reached 0.12±0.02 mmol l^{-1} (*N*=5) over the course of 5 min. Increasing the bath concentration to 0.5 mmol l^{-1} increased the luminal concentration to 2.0±0.2 mmol l^{-1} (*N*=6), and increasing the bath concentration to 5 mmol l^{-1} increased the luminal concentration to 11.1±0.8 mmol l^{-1} (*N*=4). Because there was significant (up to 60 %) depletion of the bath nicotine, this movement of nicotine into the lumen actually resulted in lumen-to-bath ratios of 4.0±0.6 at 0.05 mmol l^{-1} bathing nicotine, 10.1±1.5 at 0.5 mmol l^{-1} and 3.2±0.4 at 5 mmol l^{-1} (Fig. 2). These values give a clear indication of accumulative transport from bath to lumen, as initially reported by Maddrell and Gardiner (1976).

Whereas the Malpighian tubule of a living insect would be subject to agitation from body movements, the artificial assay allows unstirred layers to develop at both apical and basal surfaces. To test the idea that the resulting unfavourable concentration gradients inhibited maximal throughput of nicotine, some Malpighian tubules were gently agitated in the bath during the 5 min of incubation in 0.05 mmol l^{-1} nicotine. This stirring increased the lumen-to-bath ratio threefold to 12.3±1.3 (*N*=3) (Fig. 2, jiggle). This is a striking indication that values obtained from the assay probably underestimate the nicotine-transporting ability of the Malpighian tubule (see Discussion).

Nicotine accumulation is inhibited by atropine and by verapamil

Although 3 mmol l^{-1} atropine inhibits nicotine transport in larval *Rhodnius prolixus* Malpighian tubules (Maddrell and Gardiner, 1976), the effect of atropine on nicotine transport in *Manduca sexta* has not been tested. We therefore examined the effect of 3 mmol l^{-1} atropine (present in both the bath and perfusing fluids, resulting in 2 min of pre-exposure for the apical surface) on the transport of radiolabelled nicotine into the lumen (Fig. 2). With an initial bath concentration of 0.5 mmol l^{-1} nicotine, the luminal concentration reached only 0.36 mmol l^{-1} after 5 min, giving a mean lumen-to-bath ratio of 0.9±0.2 (*N*=3), significantly lower (11-fold) than in the absence of atropine.

Verapamil, an inhibitory ligand for P-gp-based transport, applied at 25 µmol l^{-1} to both sides of the tissue, also had a dramatic effect on nicotine transport (Fig. 2). After 5 min, the lumen-to-bath ratio was only 1.2±0.2 (*N*=5), again significantly (eightfold) lower than in the absence of verapamil.

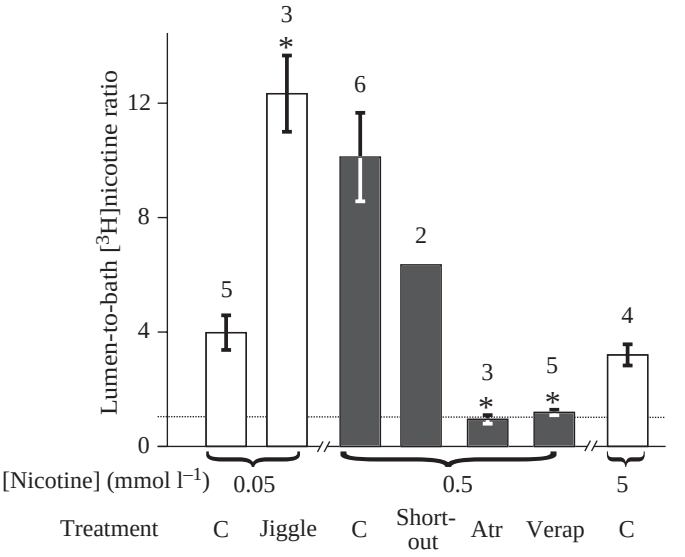

Fig. 2. Properties of nicotine active transport. Transport is sensitive to atropine (Atr, 3 mmol l^{-1}) and verapamil (Verap, 25 µmol l^{-1}), is not dependent on a transepithelial electrical potential (short-out) and is most dramatically seen using an initial bath concentration of 0.5 mmol l^{-1} nicotine. The 'jiggle' treatment is a gentle agitation explained in the text. The dotted line indicates a lumen-to-bath ratio of 1, which would be expected for equilibration of the two compartments. An asterisk indicates a value significantly (P<0.05) different from the control (*C*) at the corresponding initial concentration of nicotine. Values are means ± S.E.M. Values of *N* are given above the columns.

Vinblastine accumulates in the lumen of Malpighian tubules

While the accumulation of nicotine in the lumen of Malpighian tubules was readily evident after only 5 min, preliminary trials showed that vinblastine (M_r 811) accumulated much more slowly than nicotine (M_r 162). This necessitated a change in the experimental protocol; instead of perfusing and collecting the perfusate 5 min after the addition of the radiolabelled alkaloid, collection was delayed until

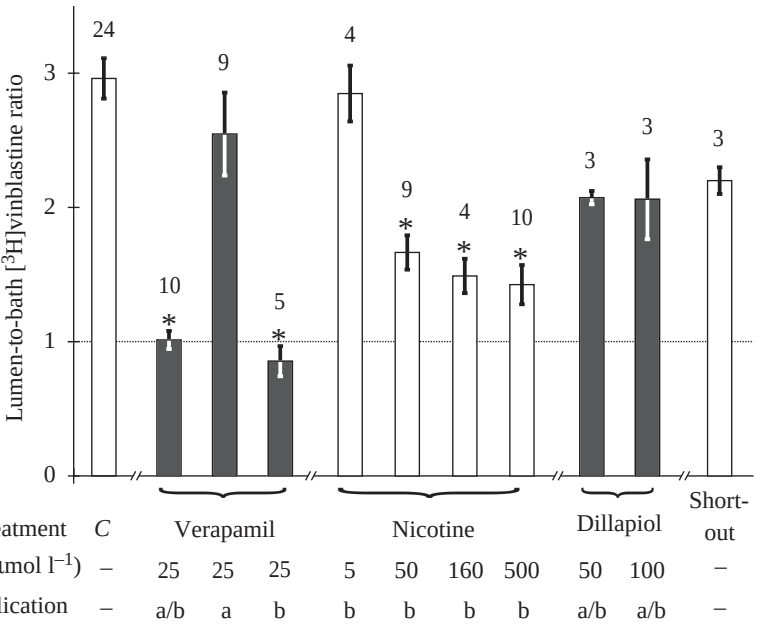

Fig. 3. Vinblastine is actively transported across the Malpighian tubule. This transport is sensitive to verapamil applied to the basal side or to both sides of the tissue, and to nicotine at concentrations $\geqslant 50\,\mu mol\,l^{-1}$, but is not significantly affected by dillapiol at 50 or $100\,\mu mol\,l^{-1}$, nor is it the result of a transepithelial potential (results for tubules in the absence of a transepithelial electrochemical potential difference, short-out tubules, are not significantly different from control values). The initial concentration of vinblastine in the bath was $1\,\mu mol\,l^{-1}$. The dotted line indicates a lumen-to-bath ratio of 1, which would be expected for equilibration of the two compartments. An asterisk indicates a value significantly ($P<0.05$) different from the control (*C*); a, apical application of inhibitor; b, basal application of inhibitor; a/b, inhibitor added to both sides of tissue, as described in Materials and methods. Values are means ± S.E.M. Values of *N* are given above the columns.

60 min. For vinblastine, therefore, our principal measurement of uptake into the lumen was the ratio of lumen radiolabel in the first 5 µl sample collected at 60 min to that in a 5 µl sample of the bath taken at the same time. The mean ratio (lumen-to-bath) was 3.0±0.2 (*N*=24) (Fig. 3). The mean concentration of vinblastine in the first 5 µl collected from the lumen was $1.3\pm0.1\,\mu mol\,l^{-1}$ (Fig. 4). Fig. 4 also illustrates that the first 5 µl sample collected most, but not all, of the luminal contents, since the next two 5 µl samples pushed through each contained some activity. The fraction of the total luminal activity collected in the first 5 µl sample would have depended on the size and, presumably, on the morphology of the tubule.

The levels of vinblastine in the bathing solution, monitored by sampling 2 µl of the bathing droplet at 15 min intervals during the experiment, were variable for any given preparation, but from the whole set of tubules (*N*=24) it became evident that, over the course of the 60 min, the bath droplet concentration fell significantly (by 32±2 %) (Fig. 4).

Verapamil inhibits the accumulation of vinblastine when applied to the basal surface

When $25\,\mu mol\,l^{-1}$ verapamil was applied to both surfaces of the Malpighian tubule, the accumulative transport of vinblastine was effectively inhibited (Fig. 3). The mean lumen-to-bath ratio of [^{3}H]vinblastine under these conditions (a pre-incubation period of approximately 20 min for both apical and basal surfaces) was 1.0±0.1 (*N*=10). When verapamil was applied only to the basal (bath) side of the tubule (with a pre-incubation period of approximately 10 min), a similar result (0.9±0.1, *N*=5) was obtained. In contrast, when verapamil was applied to the apical (lumen) side only, by perfusing it into the lumen (with a pre-exposure of approximately 10 min), the resulting vinblastine transport (lumen-to-bath ratio 2.5±0.3, *N*=9) was not significantly different from that without any verapamil (Fig. 3).

Thus, verapamil inhibited accumulative vinblastine transport threefold when applied from the haemolymph side,

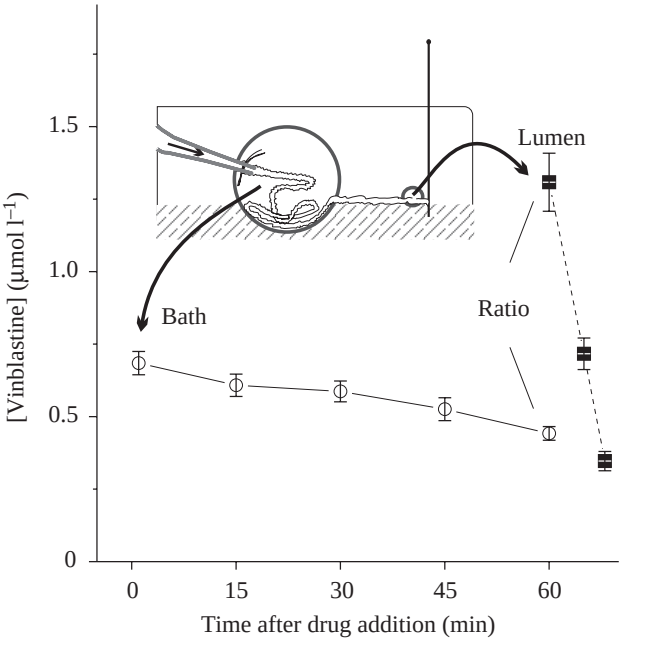

Fig. 4. Vinblastine concentrations in bath (open circles) and lumen (black squares) samples, showing that vinblastine moves from the bath into the lumen over the course of 60 min (*N*=24). As shown (Ratio), the first 5.0 µl sample flushed from the lumen at 60 min was compared with the 5.0 µl bath sample at 60 min to give the values shown in Fig. 3. This illustrates that the first 5.0 µl sample collects appreciably less than the total luminal radioactivity. Values are means ± S.E.M.

but was ineffective when applied at the same concentration from the luminal side.

Nicotine inhibits the accumulation of vinblastine

At concentrations greater than or equal to 50 μmol l^{-1}, nicotine (applied basally as described in Materials and methods) significantly reduced the lumen-to-bath ratio of [^{3}H]vinblastine (Fig. 3). Nicotine at 5 μm, however, had no effect on [^{3}H]vinblastine accumulation. Evidently, the effect of nicotine was already saturating at 50 μm, since there was no significant difference in the inhibition caused by 50, 160 or 500 μmol l^{-1} nicotine.

Verapamil was a more effective inhibitor of vinblastine transport than was nicotine; the level of inhibition achieved by 25 μmol l^{-1} verapamil exceeded that achieved by nicotine at any concentration.

Dillapiol has no effect on the accumulation of vinblastine

Dillapiol is a natural product of Indian dill (*Anethum sowa*) and other plants that has been shown to have a synergistic action for pyrethrum and carbamate pesticides (Tomar *et al.* 1979). Since a possible mode of synergism could be interference with excretion of these xenobiotics, we postulated that dillapiol might interfere with vinblastine transport. When the [^{3}H]vinblastine assay was performed in the presence of dillapiol (on both sides of the tissue), the mean lumen-to-bath ratios were 2.1±0.05 (N=3) in the presence of 50 μmol l^{-1} dillapiol and 2.1±0.3 (N=3) in the presence of 100 μmol l^{-1} dillapiol, not significantly different from the control.

Transport of nicotine or vinblastine is not the result of a transepithelial potential

Both nicotine and vinblastine in aqueous solution are predominantly cationic at the pH used in this study, and it is therefore possible that the observed accumulation was due to an electrical potential (negative inside) set up across the epithelium. To investigate this issue, experiments were performed in which a chlorided silver wire was passed from inside the cannula to the bath (see Fig. 1) to short-circuit any electrical potential between the bath and the lumen. Using 0.5 mmol l^{-1} nicotine, lumen-to-bath ratios of radiolabelled nicotine for two samples were 5.8 and 6.9. While these fall below control (no short-circuit) values (Fig. 2), they still represent accumulative transport. Similarly, accumulative vinblastine transport was observed with a shorting wire in place (Fig. 3); for vinblastine, the mean lumen-to-bath ratio was 2.2±0.1 (N=3), not significantly different from that in experiments without the short circuit. From these small samples, we do not claim that there is no effect of transepithelial potential, only that accumulative uptake persists in the presence of a short circuit.

Discussion

Using isolated and cannulated Malpighian tubules from the tobacco hornworm, we have confirmed and expanded Maddrell and Gardiner's (1976) finding that 'blood' nicotine rapidly accumulates in the Malpighian tubule lumen. Our assay was simplified in that the tissue was not continuously perfused, but flushed out only once after an appropriate incubation period. After only 5 min, nicotine was concentrated in the lumen by up to 10-fold. By demonstrating that nicotine transport can be inhibited by atropine in *Manduca sexta*, we extended the generality of the observation previously seen only in *Rhodnius prolixus* (Maddrell and Gardiner, 1976). Specifically, a sixfold excess of atropine caused an 11-fold decrease in the nicotine lumen-to-bath ratio, consistent with competition for a common transport mechanism. Why vinblastine accumulated so much more slowly than nicotine is a matter of conjecture; differences in lipid solubility, in absolute substrate concentration and in molecular mass may all be factors. It is also possible that the medial-plus-proximal region used for nicotine experiments was more effective than the proximal-only region used for vinblastine experiments, although there is nothing to suggest this from the earlier work of Maddrell and Gardiner (1976).

Both nicotine, with a pKa of 7.9, and vinblastine, with a pKa of 7.4, would be predominantly (>60 %) cationic in the pH 7.2 solutions used in this study. Although Malpighian tubules usually maintain the lumen positive with respect to the basal side (O'Donnell *et al.* 1996), it is conceivable that an unusual transepithelial potential (negative inside) non-selectively drove the alkaloid accumulation we observed. This conjecture can be ruled out, however, since placement of a wire connecting the bath and lumen, to dissipate any transepithelial potential (Fig. 1), did not prevent concentrative uptake into the lumen (Figs 2, 3; short-out). Non-selective accumulation could also occur if drugs passively penetrated the epithelium in their lipophilic neutral form but were protonated and hence trapped once in the lumen. While this could be a factor *in vivo* for the proximal region, where luminal pH is evidently below 6.8 in *M. sexta*, it would not apply for the medial region, where pH is reported to be greater than 8.4 (Moffett, 1994). Moreover, in our experiments, both bath and lumen fluids were replaced with Hepes-buffered saline at pH 7.2; the solutions used by Maddrell and Gardiner (1976) were also well-buffered. Finally, there is no ready explanation for how pH-trapping would be abolished by verapamil, atropine and nicotine, drugs shown here and by Maddrell and Gardiner (1976) to inhibit vinblastine and/or nicotine transport.

Our aim in these experiments was to determine whether uphill transport of xenobiotic substrates was occurring and we therefore looked for accumulation against a gradient, rather than measuring the rate of appearance of substrate in the lumen (estimated by continuously sampling tubule perfusate, e.g. Maddrell and Gardiner, 1976). We chose not to measure the rate of transport for two reasons. First, unidirectional transport rates alone do not establish whether transport is energetically uphill and, indeed, by maintaining a favourable concentration gradient can emphasize the downhill components of epithelial transport (an unknown fraction of which may be *via* transporters). Second, while instantaneous rates are useful for within-tubule comparisons of pharmacological agents, they are

most meaningful when transport is from an infinite pool. In our experiments, the substrate pool (bath droplet) became appreciably depleted of substrate during the course of the experiment. While a single bath sample allowed us to compensate for this depletion in ratio experiments, multiple samples would be needed for rate experiments, further exacerbating the depletion.

Although accumulative transport was unequivocally established for vinblastine and nicotine, our lumen-to-bath ratios probably underestimate the physiological ability of tubules to move these xenobiotics from blood to lumen. An obvious factor leading to underestimates of accumulation is that part of the tubule was in oil and so was not exposed to tracer (see Fig. 1), while the entire luminal contents were sampled when the lumen was flushed out. A second factor is that unstirred layers at both apical and basal surfaces (O'Donnell *et al.* 1982) create unfavourable local concentration gradients against which the transport system must work. These effects are presumably peculiar to the *in vitro* model since continual body movements *in vivo* should keep unstirred layers to a minimum. Illustrating this point directly is the observation that, simply by gently agitating the tissue, we increased uptake ratios of nicotine from 4 to 12, a threefold improvement. Additionally, with vinblastine, we found that a substantial fraction of lumen radioactivity was only flushed out in the second and third sequential samples (Fig. 4). The difficult-to-remove tracer may in part represent tracer 'trapped' between the microvilli and among the folds and pockets of the diverticula. If the counts from the second and third lumen samples are added to those from the first, the ratio at 60 min becomes 5.5 instead of 3.0.

The verapamil-sensitive accumulative transport of vinblastine shown here is currently the best evidence for the operation of a P-gp-like xenobiotic efflux mechanism in the tobacco hornworm Malpighian tubule. Vinblastine, an alkaloid from *Catharanthus roseus*, is important in cancer chemotherapy (Rowinsky and Donehower, 1991) and a well-characterised P-gp substrate. It is vectorially transported across P-gp-expressing epithelia, including those of renal cells (Horio *et al.* 1990), it stimulates the ATPase activity of recombinant P-gp (e.g. Dong *et al.* 1996) and it interacts directly with P-gp in photoaffinity labelling studies (Akiyama *et al.* 1988; Bruggemann *et al.* 1992). Verapamil, a widely studied inhibitor of P-gp activity, interferes with drug transport independently of its action on Ca^{2+} channels (Bosch and Croop, 1996; Huet and Robert, 1988; Tsuruo, 1991). Photoaffinity labelling suggests that, like vinblastine, verapamil interacts directly with P-gp, perhaps by competing for binding sites (Safa, 1988). The relevance of any of these interactions is, however, disputed, largely because of disagreements on how P-gp actually operates.

Our discovery that verapamil exerted its effect from the basal side was somewhat unexpected because, in polarized epithelia, such as the mammalian kidney proximal tubule, P-gp is predominantly located on the apical surface (Thiebaut *et al.* 1987). In studies of transepithelial vinblastine transport, verapamil has typically been applied to both sides simultaneously (e.g. Horio *et al.* 1990), but Hunter *et al.* (1991), using intestinal adenocarcinoma cell layers, noted that at high concentrations (200 μmol l^{-1}) verapamil inhibition of vectorial transport of vinblastine was similar with verapamil on either side. The same authors subsequently reported, however, that the effectiveness of verapamil as a chemosensitizer (inhibitor) fell as access to the basolateral surface decreased (Hunter *et al.* 1993). Consistent with our finding, this suggests that the site of verapamil inhibition is more accessible from the basal side. The immunostaining of Malpighian tubules of *M. sexta* (Murray *et al.* 1994) and *R. prolixus* (Murray, 1996) showed that P-gp was not limited to apical regions. In some human tumour cells, P-gp immunolocalizes to intracellular sites (Broxterman *et al.* 1989; Molinari *et al.* 1994; Baldini *et al.* 1995), a point of particular interest, since alkaloid efflux studies at the insect blood–brain interface suggested temporary sequestration at intracellular sites (Morris, 1983*a*,*b*). A remote possibility for the complete lack of effect of apically applied verapamil is that the preincubation period is critical; application of verapamil through the cannula may not be as thorough as replacement of the bathing medium on the basal side.

In insects, P-gp might be responsible for the transport of alkaloids present in the diet. P-gp occurs in all major taxa (Childs and Ling, 1996) and appears to protect many organisms from xenobiotics (e.g. Kurelec, 1992; Broeks *et al.* 1995). Plants and phytophagous insects have an evolutionary history of 'chemical warfare' and any 'multi-drug pump' should be welcome in the insect arsenal. In *R. prolixus* Malpighian tubules, the alkaloids nicotine, atropine and morphine seem to share a common transport mechanism (Maddrell and Gardiner, 1976). By virtue of their chemical structure, all three are candidate substrates for P-gp, i.e. they are heterocyclic, relatively hydrophobic and possess a tertiary nitrogen cationic group. Atropine enhances the cytotoxicity of *Vinca* alkaloids in a multidrug-resistant human leukaemic cell line (Zamora *et al.* 1988), although it does not inhibit photolabelling of P-gp by a vinblastine analogue (Akiyama *et al.* 1988). Morphine accumulates threefold less in multidrug-resistant CHO-B30 cells than in controls and binds specifically and saturably to CHO-B30 plasma membranes in a verapamil- and vinblastine-sensitive fashion (Callaghan and Riordan, 1993). In *M. sexta*, the blood–brain barrier to nicotine co-localizes with P-gp immunostaining (Murray *et al.* 1994). At a biochemical level, nicotine/P-gp interaction is suggested by the observation that nicotine stimulated the ATPase activity of plasma membrane vesicles from the multidrug-resistant Chinese hamster ovary cell line $CH^{R}B30$ (H. McDiarmid and F. J. Sharom, personal communication).

We now add to the weight of these suggestions that P-gp is the insect alkaloid transporter the pharmacological findings that nicotine transport is inhibited by verapamil and atropine and that vinblastine transport is inhibited by verapamil and nicotine. This is consistent with P-gp activity being directly or indirectly responsible for the luminal accumulation of nicotine

and atropine. At this point, however, we cannot rule out several alternatives. In vertebrate kidney, xenobiotics are also excreted by an organic cation transport system whose model substrates are quaternary ammonium compounds (Pritchard and Miller, 1996). While the 'classic' cation and P-gp transport pathways involve distinct proteins (Dutt *et al.* 1992; Gründemann *et al.* 1994), their substrates are chemically similar and some appear to interact with both; for example, vinblastine reduces tetraethylammonium uptake into renal brush-border membrane vesicles (McKinney and Hosford, 1993; Dutt *et al.* 1994). Uncertainties about mechanisms of drug efflux in whole tissue are likely to persist until there is more agreement about mechanisms in cellular models. Although P-gp is described as 'the multidrug transporter' (Gottesman and Pastan, 1988), there is no consensus on how it acts. Moreover, for charged and pH-sensitive drugs, there is evidence that the multidrug-resistance phenotype can be brought about, with or without P-gp, by altered electrical or pH gradients across plasma and intracellular membranes (Simon and Schindler, 1994; Roepe *et al.* 1996).

If nicotine is indeed transported by P-gp in the insect Malpighian tubule, as our results suggest, it raises the intriguing possibility that P-gp could also affect the distribution of nicotine in humans, *via* its activity both at the blood–brain barrier (Schinkel *et al.* 1994) and in the kidney (Pritchard and Miller, 1996). This could have implications for nicotine addiction, since delivery of the drug to the central nervous system and its rate of elimination from the body are important factors in determining self-administration, tolerance and physical dependance (Busto *et al.* 1989).

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