Characterization of tetraethylammonium uptake across the basolateral membrane of the Drosophila Malpighian (renal) tubule

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Abstract

Basolateral transport of the prototypical type I organic cation tetraethylammonium (TEA) by the Malpighian tubules of *Drosophila melanogaster* was studied using measurements of basolateral membrane potential ($V_{bl}$) and uptake of $[^{14}\text{C}]$ labeled TEA. TEA uptake was metabolically dependent and saturable ($J_{max}$, 0.87 pmol tubule$^{-1}$min$^{-1}$; $K_t$, 24 µmol l$^{-1}$). TEA uptake in Malpighian tubules was inhibited by a number of type I (e.g. cimetidine, quinine and TEA) and type II (e.g. verapamil) organic cations and was dependent on basolateral membrane potential ($V_{bl}$). TEA uptake was reduced in response to conditions which depolarized $V_{bl}$ (high-$K^+$ saline, Na$^{+}$-free saline, NaCN) and increased in conditions which hyperpolarized the $V_{bl}$ (low- $K^+$ saline). Addition of TEA to the saline bathing Malpighian tubules rapidly depolarized the $V_{bl}$, indicating that TEA uptake was electrogenic. Blockade of $K^+$ channels with Ba$^{+2}$ did not block effects of TEA on $V_{bl}$, or TEA uptake indicating that TEA uptake does not occur through $K^+$ channels. This is the first study to provide physiological evidence for an electrogenic carrier mediated basolateral organic cation transport mechanism in insect Malpighian tubules. Our results also suggest that the mechanism of basolateral TEA uptake by Malpighian tubules is distinct from that found in vertebrate renal tubules.

Keywords: *Drosophila melanogaster*, Malpighian tubule, tetraethylammonium, organic cation transport, comparative renal physiology
Introduction

Organisms must constantly deal with exposure to potentially toxic endogenous compounds and xenobiotics that require rapid elimination from the body. Xenobiotics may include environmental pollutants, plant alkaloids, animal toxins and drugs. Many of these compounds are organic cations (OCs). In addition, both xenobiotic and endogenous compounds are often metabolized into OCs. Excretion of a wide range of organic compounds by an OC transport system appears to be a common characteristic of the renal tissues of both invertebrates and vertebrates. In 1993 Pritchard and Miller (31) reviewed the physiological evidence for the “classical” organic cation transport pathway. This “classical” transport included a carrier-mediated potential-driven uptake of OCs through a single pathway at the basolateral membrane, intracellular sequestration of the cation, and luminal exit through OC-proton exchange or by a p-glycoprotein. More recent findings suggest that this “classical” model is an oversimplification of the OC transport pathway. Studies of OC secretion in hepatic tissue has led to the classification of OCs into two types (24). Type I OCs, such as tetraethylammonium (TEA) and N\textsuperscript{1}-methylnicotinamide (NMN) are typically monovalent with a molecular weight of $< 400$. Type II OCs, such as d-turbocurarine and vecuronium, are typically polyvalent with a molecular weight $> 500$. A review by Wright and Dantzler (39) incorporating recent physiological and molecular evidence indicates that multiple OC transporters with different selectivities and substrate affinities for type I and type II OCs exist at both the basolateral and luminal membranes of renal cells for excretion of OCs.

In *Drosophila*, the Malpighian tubules and hindgut are functionally analogous to the vertebrate renal tubule. Although mechanisms of inorganic ion transport at both the
basolateral and luminal membranes along with the hormones and second messengers that regulate inorganic ion secretion have been extensively studied and reviewed (8, 30), there are only a few studies of OC transport by the MTs of insects. MTs of the tobacco hornworm Manduca sexta excrete the basic (cationic) dyes methyl green and methylene blue (27). Insect MTs also exhibit mutually competitive transport of nicotine, atropine and morphine, suggesting the presence of a multi-alkaloid transporter (21). Studies using isolated tubules from Manduca larvae suggest that a p-glycoprotein-like mechanism is involved in alkaloid transport (9). In our previous study (32) we demonstrated transepithelial secretion of the prototypical type I organic cation TEA by the MTs and gut of Drosophila.

In this study we used electrophysiological and radiolabeled tracer techniques to examine the uptake of the prototypical type I organic cation TEA across the basolateral membrane of the Malpighian tubules of Drosophila melanogaster. Kinetics of type I OC uptake across the basolateral membrane were determined using [14C] TEA and the effects of various type I or type II OCs and organic anions on TEA uptake were used to characterize the substrate selectivity of the basolateral OC transport pathway. Experimental conditions which depolarized or hyperpolarized the basolateral membrane potential (V_{bl}) were used to determine if TEA uptake is potential dependent. In addition, measurements of basolateral membrane potential were used to determine if TEA transport was electrogenic and if it was mediated by K^+ channels.
Materials and Methods

Animals

*Drosophila melanogaster* Meigen (Oregon R. strain) were obtained from laboratory cultures maintained in the Department of Biology, McMaster University according to procedures described previously by Ashburner (2). All experiments were carried out at room temperature (21-25 °C) and ambient humidity. Animals 3-7 days post-emergence were used in all experiments.

Preparation and isolation of Malpighian tubules

Malpighian tubules were isolated from adult females under control saline (see Table 1) as described previously (7). All salines were titrated with NaOH or HCl to pH 7. The addition of L-glutamine has been found to maintain higher and stable rates of fluid secretion for prolonged periods (> 2 hours; see Ref. 12). The anterior pair of Malpighian tubules were used because they are easily dissected, and females were used because they are larger. Tubules were isolated under a dissecting microscope by gripping the dorso-ventral margin of the abdomen with two pairs of forceps to tear open the body wall and uncoil the alimentary canal. The anterior and posterior pair of MTs are connected to the hindgut through a short ureter. Anterior tubules were cut and removed at the junction of their common ureter with the alimentary canal.
**Basolateral uptake of TEA by isolated tubules**

Pairs of isolated anterior tubules were transferred using fine glass probes to 50µl droplets of bathing saline containing [14C] TEA (10 - 100 µmol l⁻¹). Higher concentrations of bathing saline TEA were made by combining 100 µmol l⁻¹ [14C] TEA and unlabeled TEA. For measurements of TEA uptake over time (10 seconds - 10 minutes), tubules were incubated in bathing saline containing 100 µmol l⁻¹ [14C] TEA. For uptake versus concentration experiments tubule pairs were incubated in TEA (10 µmol l⁻¹ – 2 mmol l⁻¹) for 40 seconds. The bathing droplets were positioned in depressions in Sylgard on the bottom of a 100 x 20 mm glass Petri dish. Each bathing droplet incubated 10 pairs of tubules successively. Each tubule pair was then dragged with fine glass probes through its own set of three 10 µl rinse droplets of standard bathing saline containing an excess of cold TEA (1 mmol l⁻¹), in order to displace any remaining surface bound [14C] TEA and minimize any efflux of TEA from the tissue. Ten tubule pairs were lysed in a 50 µl droplet of distilled water under oil for 15 minutes before the water and tubule were transferred into 4 ml of scintillation fluid for β counting in a LKB Wallac 1217 Rackbeta liquid scintillation counter. Pharmacological agents were added to the saline either for 30 min prior to addition of TEA or at the same time as TEA.

**Electrophysiology**

The apparatus was mounted on a vibration damping platform (TMC, Peabody, MA) within a custom made Faraday cage. Basolateral membrane potential (V₉l) was measured in individual principal cells of the lower segment of the Malpighian tubule maintained in a custom built 0.2 ml - 0.6 ml superfusion chamber. Measurements were
made in the cells of the lower segment because our previous study (32) identified this region of the tubules as having greatest area-specific rate of TEA transport. Moreover, blocking potassium channels with Ba$^{2+}$ had no effect on the $V_{bl}$ of lower segment (see below); whereas it has been demonstrated to hyperpolarize the $V_{bl}$ of the main segment (15, 29). Superfusion of the fluid bathing the tubule was accomplished via a gravity fed inflow and a suction outflow. The rate of perfusion of the chamber was 6 ml min$^{-1}$, sufficient to exchange the chamber’s volume in ~ 5 seconds.

Microelectrodes were fabricated from 2.0 mm diameter theta glass (Harvard Apparatus, UK) pulled to submicron tips with a Narishige vertical puller. Both barrels of the microelectrodes were filled with 0.5 mol l$^{-1}$ KCl and one barrel was connected through a chlorided silver wire to the electrometer. Microelectrode resistance was typically 20-40 megaohms. Basolateral membrane potential was measured using an A-M Systems model 2000 electrometer (Carlsborg, WA). A reference ground for the voltage sensing microelectrode was made via a 3 mol l$^{-1}$ KCl - 4% agar bridge placed in the saline bathing the tubule. Data were recorded using a PC-based data acquisition system (Axotape; Axon Instruments, Burlingame, CA).

For each experiment, freshly dissected tubules were placed in Petri dishes in which 80 µl drops of 125 mg/ml poly-L-lysine (70,000 – 150,000 mol. wt., Sigma) had previously been placed and allowed to air-dry. Tubules readily adhered to the bottom of these dishes and did not move when the microelectrode tip was advanced against the tubule wall. Tubules were observed under a Wild 3M dissecting microscope as microelectrodes were advanced at an oblique angle using a hydraulic micromanipulator.
A sudden shift in potential indicated that the basolateral membrane of a principal cell had been impaled.

**Chemicals**

$[^{14}\text{C}]$ TEA (55.6 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). All other chemicals were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Chemicals were dissolved in standard bathing saline, ethanol or dimethylsulphoxide (DMSO). The final concentration of DMSO or ethanol did not exceed a final concentration of 1%. Previous studies (e.g. 29) have shown that tubule function is unaffected by these solvents at $\leq 1\%$. Preliminary experiments (data not shown) have demonstrated that TEA uptake is unaffected by these solvents at $\leq 1\%$.

**Calculations and statistics**

Measurements of TEA uptake using $[^{14}\text{C}]$ TEA values are expressed as mean $\pm$ SEM for the indicated (N) number of replicates for (n) number of tubules per replicate. Measurements of V_{bl} values are expressed as mean $\pm$ SEM for the indicated (n) number of tubules. Two-sample $F$-tests were used to compare the variances of the data for the control and experimental groups. Depending on the outcome of each $F$-test, differences between experimental and control groups were compared using unpaired Student’s $t$-tests assuming either equal or unequal variances. The responses of the same group of tubules before and after an experimental treatment were compared using a paired $t$-test. Where appropriate, data were analysed by one-way analysis of variance (ANOVA).
differences were considered significant if $P < 0.05$. Concentration-response curves relating TEA uptake to bathing saline TEA concentration were fitted using a commercial graphics and analysis package (Sigmaplot, SPSS Inc., Chicago, IL, USA). The iterative procedure allowed estimation of the kinetic parameters $J_{\text{max}}$ and $K_t$ (see below) for TEA uptake.

**Results**

*Time dependent TEA uptake into isolated Malpighian tubules.*

The cumulative uptake of $[^{14}\text{C}]$ TEA by isolated MTs (Fig. 1) increased with time and approached a steady state after ~2 min. Uptake was approximately linear for an initial period of 60 s. This allows us to calculate an initial constant rate of mediated TEA uptake of ~1 pmol tubule$^{-1}$ min$^{-1}$. The uptake rate decreased as incubation time was extended beyond 1 min. The 10 min (steady state) tissue accumulation of TEA was 1.85 ± 0.14 pmol tubule$^{-1}$. The addition of 1 mmol l$^{-1}$ cimetidine reduced the uptake of $[^{14}\text{C}]$ TEA by ~95 % at all time points examined. The y-intercept of the uptake versus time plot in the presence of cimetidine was not significantly different from zero, a finding which is consistent with carrier mediated, and therefore saturable, uptake of TEA. The data in Fig. 1 indicated that measurements at 40 seconds were well within the linear portion of the uptake versus time plot and this duration was therefore used in subsequent kinetic experiments.

*Concentration dependence of TEA uptake into isolated Malpighian tubules*
Figure 2 shows the effect of increasing concentrations of bath TEA on the rate of total TEA uptake in isolated *Drosophila* Malpighian tubules. Isolated tubules were exposed to TEA (10 – 400 µmol l⁻¹) for 40 seconds. Over this concentration range the relationship is curvilinear and the kinetics of TEA uptake were adequately described by an equation that included both saturable (Michaelis-Menten) and a non-saturable (linear) terms:

\[
J = \frac{J_{\text{max}} [\text{TEA}]}{K_t + [\text{TEA}]} + D[\text{TEA}]
\]

where \( J \) is the rate of [¹⁴C] TEA uptake into the isolated tubule from an extracellular concentration of TEA; \( J_{\text{max}} \) is the maximal rate of mediated TEA uptake by all potential transport processes, reflecting the total transport capacity of the membrane; \( K_t \) is the concentration of TEA at \( \frac{1}{2} J_{\text{max}} \); and \( D \) is a coefficient that represents the component of total TEA uptake that was not saturable over the entire range of extracellular TEA concentrations studied. This non-saturable component reflects a combination of diffusion, surface binding, and/or carry-over of radiolabel in the boundary layer during rinsing of the tissue. The values of \( J_{\text{max}} \) and \( K_t \) and \( D \) generated by a Michaelis-Menten analysis were 0.87 ± 0.09 pmol tubule⁻¹ min⁻¹ and 24.3 ± 6.7 µmol l⁻¹, and 0.0053 ± 0.0002 respectively (N = 5 replicates of n = 20 tubules per replicate, \( r^2 = 0.99 \pm 0.03 \)). For concentrations over the range of >200 µmol l⁻¹ (Figure 2, inset), the TEA uptake versus bath concentration plot yielded a linear relationship with a slope of \( 4.16 ± 0.14 \) pmol tubule⁻¹ min⁻¹ mmol⁻¹, and a y-intercept of 1.21 ± 0.16 pmol tubule⁻¹ min⁻¹. Over the entire concentration range studied (10 µmol l⁻¹ – 2 mmol l⁻¹) the relationship between TEA uptake and bath TEA concentration cannot be described solely by a single saturable transport process or by simple diffusion. We interpret the linear portion of the TEA
uptake versus bath concentration curve (Figure 2, inset) to mean that no very low affinity mediated process is available for TEA uptake.

Figure 3 shows a double reciprocal plot of mediated uptake vs. TEA concentration. Mediated uptake was calculated by subtracting the diffusional component, given in equation 1, from the total TEA uptake (Figure 2). This plot yielded a single line with a non-zero intercept. Using this plot we estimated a maximum velocity ($J_{\text{max}}$) and $K_t$ for TEA uptake through the mediated pathway of 0.87 pmol tubule$^{-1}$ min$^{-1}$ and 24 µmol l$^{-1}$, respectively. These values are identical to those determined in Fig. 2 using non-linear regression analysis. Additionally, this value of $J_{\text{max}}$ in this plot is not significantly different from the y-intercept of the TEA uptake versus bath concentration line at high bath concentrations (Figure 2, inset); this intercept gives an independent estimate of $J_{\text{max}}$. Taken together our data suggest that TEA uptake by Drosophila tubules is via both saturable and diffusive pathways.

Figure 3 also shows the effects of 0.1 and 1 mmol l$^{-1}$ cimetidine on basolateral uptake of TEA in isolated Drosophila MTs. Previous studies in vertebrate renal tissues have demonstrated that cimetidine is both a substrate of the basolateral OCT (4) and potent competitive inhibitor of basolateral TEA uptake (16). Figure 3 shows that at all TEA concentrations, uptake decreased with increasing cimetidine concentrations of the medium. Moreover, after correction for non-mediated uptake, as described above, the TEA uptake data for cimetidine treated tubules also yielded double reciprocal plots that could be described by a single line. Thus, increasing cimetidine concentration did not affect the $J_{\text{max}}$ of TEA transport, but did increase the apparent $K_t$ from 24 µmol l$^{-1}$ for
control tubules to 77 and 411 µmol l⁻¹ for 0.1 and 1 mmol l⁻¹ cimetidine treated tubules respectively. This is the precisely the pattern expected for competitive inhibition.

**Effects of ion substitution and NaCN on \(V_{bl}\).**

The average steady-state \(V_{bl}\) in control saline was -50.8 mV and -50.1 mV for ion substitution and NaCN experiments respectively (Table 2, legend). These values are very similar to the basolateral membrane potential of the main segment of *Drosophila* MTs (27). In most animal cells membrane potential is primarily \(K^+\)-dependent (14). Incubation in low-\(K^+\) medium therefore hyperpolarizes membrane potential whereas incubation in high-\(K^+\) depolarizes. Figure 4A and Table 2 show the effects of changes in bathing saline \(K^+\) concentration on \(V_{bl}\). A fivefold reduction in the bathing saline potassium concentration from 20 to 4 mmol l⁻¹ reversibly hyperpolarized \(V_{bl}\) by 25 mV in less than 2 minutes. A subsequent fivefold increase in bathing saline \(K^+\) concentration from 20 to 100 mmol l⁻¹ reversibly depolarized \(V_{bl}\) by 36 mV. A purely \(K^+\)-selective membrane would hyperpolarize or depolarize by 41 mV in response to a fivefold reduction or increase in bathing saline \(K^+\) concentration respectively, provided that the intracellular \(K^+\) level remained constant. However, a gradual reduction in intracellular \(K^+\) level in response to a reduction in bath \(K^+\) concentration would result in a corresponding gradual reduction in the magnitude of the hyperpolarization of \(V_{bl}\), as observed previously (29). The opposite would also hold true for the effects of increasing bathing saline \(K^+\) on \(V_{bl}\). As a result, both the hyperpolarizing effects of low-\(K^+\) and depolarizing effects of high-\(K^+\) saline are underestimated.
Figure 4B and Table 2 show the effects of Na⁺-free saline on the $V_{bl}$ of the lower segment of the *Drosophila* Malpighian tubule. Replacement of control saline with a nominally Na⁺-free solution resulted in a reversible slow depolarization of $V_{bl}$.

A previous study by Miller and Holohan (25) has demonstrated that the general metabolic inhibitor NaCN inhibited TEA uptake by isolated flounder proximal tubules. This effect was presumed to reflect slow decreases of intracellular ion gradients and a resultant depolarization. The addition of 1 mmol l⁻¹ NaCN resulted in a 45.9 mV depolarization of $V_{bl}$ in the lower segment of *Drosophila* tubules (Table 2). Depolarization due to NaCN was slow and a new steady state $V_{bl}$ was reached within 7-12 minutes. The effect of NaCN on $V_{bl}$ was not reversible.

*Effects of ion substitution and NaCN on TEA Uptake isolated Drosophila Malpighian tubules.*

The mean value of TEA uptake for control tubules was $1.69 \pm 0.07$ pmol tubule⁻¹ min⁻¹ ($N = 11$ replicates of $n = 20$ tubules per replicate). Figure 5 shows the effects of various bathing media (Table 1) on initial uptake rates of 100 µmol l⁻¹ [¹⁴C] TEA by isolated *Drosophila* MTs expressed as a percent of the uptake observed in tubules incubated in control saline. Uptake of TEA was reduced by 74% in high-K⁺ ($[K^+] = 100$ mmol l⁻¹) saline ($N = 5$ replicates of $n = 20$ tubules per replicate, unpaired t-test, $P<0.05$). Reduction in bathing saline K⁺ concentration to 2 mmol l⁻¹ resulted in a 45% increase in TEA uptake ($N = 5$ replicates of $n = 20$ tubules per replicate, unpaired t-test, $P<0.05$).

Figure 5 also shows the effects of short term (40 sec) exposure to Na⁺-free media and 30 minute pre-incubation in Na⁺-free media on TEA uptake by *Drosophila* MTs.
Short term exposure to Na\(^+\)-free media significantly reduced basolateral TEA uptake by 28% (N = 5 replicates of n = 20 tubules per replicate, unpaired t-test, P<0.05). Pre-incubation in Na\(^+\)-free media for 30 minutes prior to measuring TEA uptake resulted in an even larger 68% reduction of basolateral TEA uptake (N = 3 replicates of n = 20 tubules per replicate, unpaired t-test, P<0.05). Again, a treatment which depolarizes V\(\text{bl}\) (see above) resulted in a reduction in TEA uptake by Drosophila MTs. The difference between TEA uptake values in short term Na\(^+\)-free exposures versus pre-incubation in Na\(^+\)-free saline will be discussed further below.

Figure 5 also shows the effects of pre-incubation in 1 mmol l\(^{-1}\) NaCN on TEA uptake by Drosophila MTs. Pre-incubation with NaCN completely inhibited fluid secretion by MTs (data not shown) and resulted in a 90% inhibition of TEA uptake from a value of 0.99 ± 0.05 pmol tubule\(^{-1}\) min\(^{-1}\) in control tubules (N = 3 replicates of n = 20 tubules per replicate) to 0.09 ± 0.02 pmol tubule\(^{-1}\) min\(^{-1}\) in NaCN exposed tubules (N = 3 replicates of n = 20 tubules per replicate). Taken together, the results of Figure 5 show that TEA uptake decreased when V\(\text{bl}\) depolarized and TEA uptake increased when V\(\text{bl}\) hyperpolarized.

**Electrogenic effects of TEA on V\(\text{bl}\) of isolated Drosophila MTs.**

The dependency of basolateral TEA uptake on membrane potential implies that TEA transport is electrogenic, and that uptake of TEA should therefore produce a depolarization of the V\(\text{bl}\). Figure 6 shows a representative recording from an experiment done to test this hypothesis. Addition of 1 mmol l\(^{-1}\) TEA depolarized the membrane potential by ~15 mV within 30 seconds and the effect was reversed with approximately
the same time course when TEA was removed. Repeated exposures of the same tubule to TEA produced repeated depolarizations that were within 1 mV of the initial response and completely reversible. The mean $V_{bl}$ for all tubules used in these experiments was $-51.2 \pm 1.6$ mV ($n = 9$ tubules) and the addition of 1 mmol$^{-1}$ TEA caused a mean depolarization of $13.6 \pm 1.1$ mV.

*Electrogenic effects of TEA on $V_{bl}$ in the presence of $Ba^{2+}$.*

It could be argued that the effect of TEA on $V_{bl}$ might be due to the blockage of basolateral potassium channels in the lower tubule or entry of TEA through these same potassium channels. If the route of TEA entry across the basolateral membrane was not via potassium channels, it would follow that prior addition of $Ba^{2+}$, a known blocker of basolateral potassium channels in *Drosophila* MTs (15), should abolish the hyperpolarization of the $V_{bl}$ in response to a tenfold reduction in bathing saline $K^+$ concentration but not the depolarization observed by treatment of tubules with 1 mmol l$^{-1}$ TEA. Figure 7 is an example of an experiment designed to test this hypothesis. In these experiments $V_{bl}$ in control saline ($[K^+] = 20$ mmol l$^{-1}$) was $-52.4 \pm 1.1$ mV ($n = 8$ tubules). A tenfold reduction in bath $K^+$ concentration caused a further reversible hyperpolarization of $30.5 \pm 1.7$ mV. Addition of 6 mmol l$^{-1}$ $Ba^{2+}$ to the bathing saline had no significant effect on the $V_{bl}$ ($n = 8$ tubules, paired t-test, $P > 0.05$). $NaH_2PO_4$ was omitted from the salines containing $Ba^{2+}$ to prevent precipitation of barium phosphate. The inclusion of $Ba^{2+}$ in the bathing saline completely abolished the response of the $V_{bl}$ to a tenfold reduction in bathing saline $K^+$ ($n = 8$ tubules, paired t-test, $P < 0.05$), consistent with a reduction of $K^+$ conductance. In contrast, the addition of 1 mmol l$^{-1}$
TEA in the presence of Ba$^{2+}$ caused a depolarization of $V_{bl}$ of 18.3 ± 0.6 mV, consistent with the hypothesis that basolateral TEA uptake does not occur through potassium channels ($n = 8$ tubules). The response of the basolateral membrane potential to TEA in the presence of Ba$^{2+}$ was greater than that observed in control saline. This presumably reflects an increased resistance in the basolateral membrane due to a reduced permeability of the basolateral membrane to K$^+$. Effects of Ba$^{2+}$ on K$^+$ conductance were completely reversible upon return to control saline ($n = 8$ tubules).

**Effects of Ba$^{2+}$ on TEA uptake by isolated tubules.**

We examined effect of 6 mmol l$^{-1}$ Ba$^{2+}$ on uptake of 100 µmol l$^{-1}$ [14C] TEA by isolated *Drosophila* MTs (data not shown). The uptake of TEA by control tubules and tubules exposed to Ba$^{2+}$ were 0.65 ± 0.16 pmol tubule$^{-1}$ min$^{-1}$, and 0.65 ± 0.12 pmol tubule$^{-1}$ min$^{-1}$ respectively ($N = 5$ replicates of $n = 20$ tubules per replicate). Ba$^{2+}$ had no significant effect on basolateral TEA uptake by isolated *Drosophila* tubules (unpaired t-test, $P<0.05$), indicating that TEA uptake across the basolateral membrane of tubules does not occur through Ba$^{2+}$-sensitive K$^+$ channels.

**Effects of tetraalkylammonium compounds on TEA uptake by isolated tubules.**

Previous studies conducted in microperfused rat kidneys (35) and isolated rabbit renal proximal tubules (11) have shown that various tetraalkylammonium ($n$-TAA) compounds inhibit the basolateral uptake of organic cations, such as TEA and NMN, to a greater extent as the hydrophobicity (i.e. alkyl chain length) of the compound increases. Figure 8 shows that the addition of 1 mmol l$^{-1}$ tetramethylammonium (TMA),
tetrapropylammonium (TPrA), tetrabutylammonium (TBA) and tetrapentylammonium (TpeA) to the bathing saline all inhibited basolateral TEA uptake by isolated *Drosophila* MTs. Moreover it also shows that TPeA, the most hydrophobic compound, resulted in greater inhibition of TEA uptake than TMA, the least hydrophobic compound (P<0.05; one-way ANOVA and Tukey-Kramer multiple comparisons).

**Effects of organic ion transport inhibitors on TEA uptake by isolated MTs.**

Figure 9 shows the effects of various pharmacological compounds on the mediated basolateral uptake of[^14C]TEA by isolated *Drosophila* MTs (N = 3-6 replicates of n = 20 tubules per replicate). All data were corrected for diffusion by subtracting the TEA diffusion component calculated from Equation 1.

Addition of increasing concentrations of the type I organic cation transporter substrates cimetidine, quinine and TEA all inhibited uptake of TEA in a dose dependent manner. Using these values we calculated an IC$_{50}$ of 33 µmol l$^{-1}$ for cimetidine and 6 µmol l$^{-1}$ for quinine on the uptake of 100 µmol l$^{-1}$ TEA by isolated tubules. These IC$_{50}$ values were lower than the approximated 50% inhibition of[^14C]TEA uptake by 100 µmol l$^{-1}$ cold TEA itself. In contrast, a tenfold higher concentration of the prototypical type I organic cation N$^{1}$-methylnicotinamide did not significantly inhibit basolateral TEA uptake (one-way ANOVA, P>0.05). The effect of the type II organic cation verapamil on TEA uptake was also examined, and appeared to have inhibitory effects that were concentration dependent. Cimetidine, quinine and verapamil at concentrations of 1mmol l$^{-1}$ do not inhibit the rate of fluid secretion by *Drosophila* MTs indicating that they do not reduce tissue viability (data not shown). The type I organic anion substrate para-
aminohippurate (PAH) and probenecid, a potent organic anion transport inhibitor, also exhibited inhibitory effects on TEA uptake. However, a 10-fold increase in the concentration of PAH from 0.1 to 1 mmol l⁻¹ did not appear to have an increased inhibitory effect on TEA uptake. The addition of the leukotriene LTD4 receptor antagonist and specific blocker of multi-drug resistant protein transporter 1 (MRP1) MK571 (9) inhibited TEA uptake by isolated tubules at all concentrations. However concentrations of 10 and 100 µmol l⁻¹ MK571 did not exhibit any difference in the extent of inhibition. An increased inhibition was only observed when a 1 mmol l⁻¹ concentration of MK571 was used. The addition of 1 mmol l⁻¹ nicotine, a plant alkaloid, inhibited TEA uptake by 82%. The organic cation/carnitine transporter substrate L-carnitine had no effect on TEA uptake by isolated Drosophila tubules at a concentration 50-fold higher than that of TEA.

Discussion

A recent study by the authors has shown for the first time that insect Malpighian tubules, midgut and ureter actively transport the prototypical type I organic cation TEA (32). Moreover it also demonstrated that the lower segment of the Malpighian tubule is the primary site for active secretion of TEA. Previous studies have demonstrated that the lower Malpighian tubule is involved in the acidification of the urine, active secretion of Ca²⁺, and reabsorption of K⁺ and Cl⁻ (28). Active secretion of organic cations, such as TEA, by an epithelium requires transport across the basolateral membrane, an intracellular compartment and an apical membrane in series. Therefore a thorough
understanding of transepithelial secretion requires a description of transport events occurring at both membranes.

This study used both radioisotopic and electrophysiological techniques to provide the first evidence that the Malpighian tubules of *Drosophila melanogaster* possess a carrier-mediated and potential-dependent mechanism for the uptake of organic cations across the basolateral membrane. This preparation contains separate interstitial, intracellular and luminal fluid spaces. Therefore, solutes transported into the tissue may be distributed over both the cellular and luminal compartments. However, with short-term incubations which approximate initial uptake rates, we can be confident that we are reporting only transport from the bathing medium into the cellular compartment and not into the lumen. Thus all the data presented here supports a mechanism of entry at the basolateral surface of the tubular epithelium.

**Kinetics of TEA Uptake.**

Initial rate studies show that TEA uptake into *Drosophila* MTs occurs through two types of pathways, one displaying diffusion kinetics and the other a saturable carrier mediated pathway with a $J_{\text{max}}$ of 0.87 pmol tubule$^{-1}$ min$^{-1}$ and a $K_t$ of 24 µmol l$^{-1}$. Our half maximal TEA uptake value is consistent with those previously observed in rabbit (~30-75 µmol l$^{-1}$; see Ref 38), rat (63 µmol l$^{-1}$; see Ref 3) and snake renal proximal tubules (~18 µmol l$^{-1}$; see Ref 17). We previously determined the $J_{\text{max}}$ and $K_t$ for transepithelial transport of TEA to be 1.52 pmol tubule$^{-1}$ min$^{-1}$ and 180 µmol l$^{-1}$ respectively (32). Using these kinetic values and the kinetic values from our present study we have calculated that the transport efficiency ($J_{\text{max}}/K_t$) for basolateral TEA
uptake is 4.3 fold higher than the transport efficiency for transepithelial TEA secretion. Therefore, this higher efficiency for basolateral uptake versus transepithelial transport suggests that transport across the apical membrane is rate limiting for transepithelial secretion of TEA in the Drosophila MTs. Although our data are consistent with a single carrier mediated uptake pathway for TEA, it must be noted that our data could represent the combined effects of multiple carrier mediated processes in parallel which have relatively similar affinities for TEA (26). Such a situation has been found for transport of organic cations in the basolateral membrane of mammalian renal tissues. Although studies in rat renal tissue by Ullrich et al. (35) are consistent with a single mediated pathway for the basolateral transport of OCs, there is ample physiological, immunohistochemical and molecular evidence for the parallel roles of multiple OCTs in the basolateral membrane of mammalian renal tissues (16, 38, 39).

Potential dependence of TEA transport

Using a combined radiolabeled and electrophysiological approach we have found strong evidence for electrogenic, carrier-mediated transport of the prototypical organic cation TEA at the basolateral membrane. Experimental conditions that depolarized the basolateral membrane, i.e., high-K⁺ saline, Na⁺-free saline and NaCN (Figure 4, Table 2), led to the inhibition of TEA uptake (Figure 5). Moreover, we observed that the effects of high-K⁺ on basolateral membrane potential were very rapid (<30 sec) and were reflected in the immediate inhibition of TEA uptake observed in acute short term exposure to high-K⁺ saline. However, the depolarizing effects of Na⁺-free saline and NaCN on basolateral membrane potential occurred much more slowly, over the course of ~10 minutes.
Similarly, it could be seen that the inhibition of TEA uptake was enhanced by pre-incubation in Na\(^+\)-free saline relative to a short term exposure to Na\(^+\)-free saline. In contrast, experimental conditions that hyperpolarized the basolateral membrane, i.e., low-K\(^+\) saline (Figure 4, Table 1) led to an increase in TEA uptake (Figure 5). In addition the effects of low-K\(^+\) saline on basolateral membrane potential were very rapid (<30 sec) and were reflected in the immediate stimulation of TEA uptake observed in acute short term exposure to low-K\(^+\) saline. These are precisely the trends that we would expect to find if basolateral TEA transport is via a facilitated, potential dependent transport process.

Addition of TEA (1 mmol l\(^{-1}\)) to the bathing saline caused a ~14 mV depolarization of the V\(_{bl}\) (Figure 6), consistent with electrogenic uptake of TEA. Previous studies on teleost (33) and snake (17) renal proximal tubules showed depolarizations of the V\(_{bl}\) of ~7 and ~10 mV respectively when 1 mmol l\(^{-1}\) TEA was added the bathing medium. In both vertebrate studies the depolarization occurred slowly over 6-8 minutes. Kim and Dantzler (17) concluded, on the basis of this slow response that the depolarization observed was due to some effect of TEA on K\(^+\) channels and not the electrogenic transport of TEA. In contrast, this study showed that the depolarization of the basolateral membrane potential in response to 1 mmol l\(^{-1}\) TEA occurred rapidly, within less than 30 seconds, and was washed off with a similar time course (Figure 6). More importantly the response time of the basolateral membrane potential to TEA is similar to the response times observed for changes in bathing K\(^+\) concentration. To the best of our knowledge, this study is the first to test the hypothesis of Kim and Dantzler (17) that the effect of TEA on basolateral membrane potential is due to the blockage of a K\(^+\) channels. TEA is an impermeant but potent extracellular and intracellular blocker of
K⁺ channels in metazoan neurons and muscle fibers (1, 13). In order to test if TEA could be transported from the bathing saline into tubules through basolateral K⁺ channels we used Ba²⁺, which has been previously demonstrated to block basolateral K⁺ channels in the K⁺-secreting main segment of Drosophila tubules (15, 29). In contrast, addition of Ba²⁺ to the bathing medium had no effect on the Vₐ of the lower K⁺-reabsorptive segment of Drosophila tubules in control saline. Ba²⁺ eliminated the response of the V_b to changes in bathing saline K⁺ concentration without abolishing the depolarizing effect of TEA, indicating that TEA uptake is not mediated by K⁺ channels. In fact, the depolarizing response of the V_b to TEA in the presence of Ba²⁺ appeared larger than in control tubules, presumably since a reduction in membrane K⁺ permeability in response to blockade of K⁺ channels unmasks the contribution that TEA makes to V_b. Moreover, our findings demonstrated that there was no observable effect of Ba²⁺ on [¹⁴C] labeled TEA uptake by Drosophila tubules. This is in stark contrast to the inhibitory effect previously reported for Ba²⁺ on TEA uptake in snake renal proximal tubules (17, 18). Taken together our findings indicate that K⁺ channels play no role in mediating TEA uptake across the basolateral membrane of Drosophila Malpighian tubules.

**Pharmacology**

Previous studies on microperfused rat kidney (35) and rabbit renal proximal tubule (11) have demonstrated the increasing inhibition of TEA uptake with increasing hydrophobicity (i.e. alkyl chain length) of n-TAA compounds. Here we have extended this observation to TEA uptake in the Malpighian tubules of Drosophila. In this study inhibition of TEA uptake increased as alkyl chain length of n-TAA compounds increased.
It should be noted that most vertebrate studies on the interaction of \( n \)-TAA compounds have assessed only the effectiveness of these compounds as inhibitors of OC transport but have not tested whether these compounds themselves are transported. In contrast, our previous study (32) using the TEA self-referencing microelectrode technique has demonstrated that tetramethylammonium, tetrapropylammonium, and tetrabutylammonium, are all transported by Malpighian tubules of \textit{Drosophila}, albeit at lower rates than TEA itself. It should also be noted that as alkyl chain length increased the transport rate of the \( n \)-TAA compounds also increased. Taken together these results suggest that the inhibition of TEA uptake by \( n \)-TAA compounds observed in the present study is likely due to competitive inhibition.

In this study a number of type I organic cations, type II organic cations, organic anions and known p-glycoprotein and MRP inhibitors had inhibitory effects on TEA uptake by the Malpighian tubules. The type I organic cations cimetidine, quinine, and TEA, but not NMN inhibited TEA uptake in a concentration dependent manner. Cimetidine has been shown previously to be transported by the rabbit proximal tubules (23) and to be a discriminating inhibitor of OCT2 in the S2 segment of rabbit renal proximal tubules (40). Quinine has been shown to inhibit TEA uptake in teleost renal tubules (25). In the present study, inhibition of TEA uptake by cimetidine or quinine was determined to be competitive. The IC\(_{50}\) values for cimetidine (33 \( \mu \)mol l\(^{-1}\)) and quinine (6 \( \mu \)mol l\(^{-1}\)) are lower than the half maximal inhibition of \([^{14}\text{C}]\)TEA uptake by cold TEA itself (~ 100 \( \mu \)mol l\(^{-1}\)), suggesting that cimetidine, and quinine are better substrates for the basolateral organic cation transporter of Malpighian tubules than TEA. The lack of inhibition of TEA uptake by NMN suggest that it is not a substrate for the basolateral
organic cation transport pathway in *Drosophila* tubules or alternatively this suggests that there may be a second transporter for NMN uptake that cannot be determined by our current data. The functional separation of TEA and NMN transport pathways in snake renal tubules has been reported previously by Dantzler and Brokl (5).

We also observed that the addition of verapamil, a type II organic cation, produced a concentration dependent inhibition of TEA uptake. Previous studies on snake renal proximal tubules have demonstrated that verapamil competes with TEA for the basolateral organic cation uptake pathway (18). Also, the plant alkaloid, nicotine, a demonstrated p-glycoprotein substrate in insect MTs (9), inhibited basolateral TEA uptake. Taken together these results suggest a broad overlap in specificity of the basolateral organic cation transport pathway for type I and type II organic cations.

In addition, the organic anion transport pathway substrate PAH and probenecid, an inhibitor of the organic anion transport pathway in insects (20), reduced basolateral TEA uptake. However, the inhibition caused by PAH did not appear to be concentration dependent. The results for PAH and probenecid observed in our study are characteristically similar to the effects of PAH and probenecid on the inhibition of the secretion of the organic cations cimetidine (23) and uptake of choline (6) by rabbit renal proximal tubules, and cimetidine uptake in isolated rat proximal tubule cells (3). Furthermore MK571, a specific inhibitor of the MRP1 pathway also showed an inhibition of TEA uptake that did not appear to be concentration dependent. A number of organic cations, anions or zwitterions have been found that interact to some degree with both basolateral organic cation and organic anion transport pathways. Ullrich et al. (36, 37), has termed these substrates “bisubstrates”, and has tested the interaction of a number of
these compounds with rat renal basolateral organic cation and organic anion transport pathways. His findings suggest that hydrophobic compounds, regardless of charge may have the ability to interact with hydrophobic domains of organic cation or organic anion transporters in a non-competitive manner. Such interaction may explain the effects of the OA pathway substrates PAH, probenecid, and MRP1 inhibitor MK571 observed in our study.

It is worth noting that an orthologue of the basolateral OCTs of vertebrate kidney, designated Orct, has been cloned from a larval *Drosophila* cDNA library (34). Tissue specific expression patterns, membrane localization and substrate affinities of Orct remain to be elucidated in both adult and larval *Drosophila*. However, we propose that the results of this study may provide a basis in which to examine the physiological characteristics of Orct in a suitable expression system.

*Drosophila* MTs share both similarities and differences with mechanisms of TEA uptake in vertebrate renal proximal tubules. This study demonstrates that TEA uptake in *Drosophila* MTs shares several characteristics with TEA uptake in vertebrate renal proximal tubules: the process is dependent on membrane potential, has kinetics indicating a saturable mediated transport pathway, is inhibited by quaternary ammonium ions of increasing alkyl chain length, and exhibits a broad specificity for both type I and type II organic cations. However, there are also distinct differences reported here for TEA uptake by *Drosophila* MTs versus vertebrate renal proximal tubules. The depolarizing effect of TEA on $V_{bl}$ is very rapid in *Drosophila* MTs and is not due to blockage of $K^+$ channels, whereas the depolarizing effect of TEA on $V_{bl}$ of renal proximal tubules is relatively slow and hypothesized to be an effect on basolateral $K^+$ channels. Also, $Ba^{2+}$
does not block TEA uptake in *Drosophila* MTs, but inhibits TEA uptake in vertebrate renal proximal tubules. In summary, our application of conventional radio tracer and electrophysiological techniques provides the first physiological evidence for an electrogenic carrier-mediated mechanism for transport of organic cations across the basolateral membrane of insect Malpighian tubules. This mechanism appears to be distinct from that present in the basolateral membrane of vertebrate renal tubules.

**Grants**

This work was supported by National Science and Engineering Research Council Grants (NSERC) Canada to M.J. O’Donnell.
References


25. **Miller DS and Holohan PD.** Organic cation secretion in flounder renal tissue. 


Figure Legends

Fig. 1. Time course of 100 μmol l⁻¹ tetraethylammonium, (TEA) uptake by isolated Malpighian tubules (MTs). Uptake in the absence (filled circles) and presence (open circles) of 1 mmol l⁻¹ cimetidine is shown. Each point represents mean ± SEM value for tubules from N = 5 replicates of n = 20 tubules from 10 animals. Where no error bars are apparent, error bars are smaller than the symbol used.

Fig. 2. Effect of increasing TEA concentration on the rate of TEA uptake into isolated Drosophila MTs. Initial rates were estimated from 40 second incubations. Uptake in control (●), and after the addition of 0.1 mmol l⁻¹ (▲) or, 1 mmol l⁻¹ cimetidine(○) is shown. Each point represents mean ± SEM value of N = 5 replicates of n = 20 tubules from 10 animals. Line fit to control data was calculated from Eq. 1 (text) and kinetic parameters were derived using a nonlinear regression algorithm (Sigmaplot, SPSS Inc.) Lines fit to cimetidine data were calculated from a first order polynomial equation. Inset shows control uptake at high TEA bath concentrations. Where no error bars are apparent, error bars are smaller than the symbol used.

Fig. 3. Double reciprocal plot for mediated TEA uptake. Values were calculated from Fig 2 as described in the text. The x-intercept of each line equals -1/Kt and the slope equals Kt/Jmax.
Fig 4. Sample recording showing the effects of changes in (A) extracellular [K\(^+\)] and (B) extracellular [Na\(^+\)] on the basolateral membrane potential of the lower segment of a *Drosophila* Malpighian tubule. In both A and B the upper panel indicates the changes in bathing saline [K\(^+\)] or [Na\(^+\)] concentration during the recording. Bottom panels show the corresponding changes in basolateral membrane potential. In this and subsequent figures, impalement is indicated by arrows pointing downward and the removal of the electrode is indicated by arrows pointing upward.

Fig 5. Effects of Na\(^+\)-free saline, changes in saline [K\(^+\)] concentration, and NaCN on 100 µmol l\(^{-1}\) tetraethylammonium (TEA) uptake by *Drosophila* Malpighian tubules expressed as percent of control uptake. Uptake was determined over a 40 second time period for all tubules. Tubules used in the Na\(^+\)-free and NaCN preincubation experiments were bathed in Na\(^+\)-free saline or 1 mmol l\(^{-1}\) NaCN, respectively, for 30 minutes prior to their 40 second TEA uptake exposure. Each bar represents mean ± SEM value for tubules from N = 3-5 replicates of 20 tubules. Control levels of TEA uptake are indicated by the dashed line. Significant difference from control: * P<0.05.

Fig 6. Recording showing the effect on the basolateral membrane potential (V\(_{bl}\)) of the lower Malpighian tubules of *Drosophila* before and after exposure to tetraethylammonium (TEA). The tubules were exposed to the control saline and to saline containing 1 mmol l\(^{-1}\) TEA for the periods indicated by the horizontal bars.
Fig 7. Recording showing the effects of 6 mmol l\(^{-1}\) Ba\(^{2+}\) on basolateral membrane potential (Vbl) in control saline ([K\(^+\)] = 20 mmol l\(^{-1}\)), low K\(^+\) saline (2 mmol l\(^{-1}\)) and control saline containing 1 mmol l\(^{-1}\) TEA. The duration of exposure to each saline is indicated by the horizontal bars.

Fig 8. Effects of 1 mmol l\(^{-1}\) \(n\)-tetraalkylammonium (TAA) compounds on uptake of 100 µmol l\(^{-1}\) TEA by isolated Drosophila Malpighian tubules. Data are expressed as a percent of control uptake. Each bar represents mean ± SEM value for tubules from N = 5 replicates of n = 20 tubules per replicate. TMA, tetramethylammonium; TPrA, tetrapropylammonium; TBA, tetrabutylammonium; TPeA, tetrapentylammonium.

Fig 9. Effects of various OCT, OAT, p-glycoprotein and MRP1 inhibitors on 100 µmol l\(^{-1}\) TEA uptake by isolated Drosophila Malpighian tubules. Data are expressed as mean ± SEM of TEA uptake for N = 3-18 replicates of n = 20 tubules per replicate. Asterisks indicate significant differences from the controls. (one-way ANOVA, P<0.05, Tukey-Kramer Multiple Comparisons Test). TEA, tetraethylammonium; CIM, cimetidine; QUIN, quinine; VER, verapamil; PAH, p-aminohippurate; PROB, probenecid; MK571, \((E)-3-[[[3-[2-(7-Chloro-2-quinolinyl)ethenyl]phenyl]-[[3-dimethylamino]-3-oxopropyl]thio]methyl]thio]-propanoic Acid; NIC, nicotine; NMN, N\(^{1}\)-methylnicotinamide; L-CARN, L-carnitine.
### TABLE 1. Composition of experimental salines (concentrations in mmol l⁻¹)

<table>
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<td></td>
<td>Control Saline</td>
<td>K⁺-Free</td>
<td>2 K⁺</td>
<td>4 K⁺</td>
<td>100 K⁺</td>
<td>Na⁺-Free</td>
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</tbody>
</table>

*pH was 7 for all solutions

† N-methyl-D-Glucamine
Table 2. Response of basolateral membrane potential of the Drosophila lower Malpighian tubule to altered bathing saline composition.

<table>
<thead>
<tr>
<th>Treatment, mmol l⁻¹</th>
<th>Change in Membrane Potential, mV</th>
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<tr>
<td>20 → 100 K⁺</td>
<td>+ 32.95 ± 0.62 (14)</td>
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<tr>
<td>100 → 20 K⁺</td>
<td>− 30.94 ± 1.27 (14)</td>
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<tr>
<td>20 → 4 K⁺</td>
<td>− 19.23 ± 1.51 (9)</td>
</tr>
<tr>
<td>4 → 20 K⁺</td>
<td>+ 20.38 ± 1.56 (9)</td>
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<tr>
<td>117.5 → 0 Na⁺</td>
<td>+13.92 ± 2.86 (3)</td>
</tr>
<tr>
<td>0 → 117.5 Na⁺</td>
<td>+13.23 ± 2.79 (3)</td>
</tr>
<tr>
<td>1.0 NaCN</td>
<td>+45.92 ± 3.15 (4)</td>
</tr>
</tbody>
</table>

Data are expressed as mean changes ± s.e.m. for the number of impalements in parentheses. Positive values indicate depolarization and negative values indicate hyperpolarization. Potentials in control saline were -50.9 ± 0.6 mV (21) and -50.1 ± 1.9 (4) for ion substitution and NaCN experiments, respectively.
Fig 2

[Graph showing the relationship between [TEA]Bath (µmol l⁻¹) and TEA uptake (pmol tubule⁻¹ min⁻¹). The graph includes multiple curves, each representing a different condition or group. The axes are labeled as follows:

- Y-axis: TEA uptake (pmol tubule⁻¹ min⁻¹)
- X-axis: [TEA]Bath (mmol l⁻¹) for the upper graph. For the lower graph, the X-axis is labeled as [TEA]Bath (µmol l⁻¹).]
Fig 3.
Fig 4.
Fig 5.

% Control TEA Uptake

- Na⁺-Free
- Na⁺-Free Preincubation
- 2 mM [K⁺]
- 100 mM [K⁺]
- 1 mM NaCN
Fig 6.

![Graph showing voltage changes over time with labeled control and TEA conditions.

- Vb (mV)
- Time: 2 min
- Conditions: Control, TEA, TEA, TEA, TEA, Control]
Fig 7.
Fig 8.
Fig 9.