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

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Rheault, Mark R., Donna M. Debicki, and Michael J. O’Donnell. Characterization of tetraethylammonium uptake across the basolateral membrane of the *Drosophila* Malpighian (renal) tubule. *Am J Physiol Regul Integr Comp Physiol* 289: R495–R504, 2005. First published April 28, 2005; doi:10.1152/ajpregu.00109.2005.—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 (Vbl) and uptake of [14C]-labeled TEA. TEA uptake was metabolically dependent and saturable (maximal rate of mediated TEA uptake by all potential transport processes, reflecting the total transport capacity of the membrane, 0.87 pmol·tubule⁻¹·min⁻¹; concentration of TEA at 0.5 × maximal rate of TEA uptake value, 24 μM). 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 Vbl. TEA uptake was reduced in response to conditions that depolarized Vbl (high-K⁺ saline, Na⁺-free saline, NaCN) and increased in conditions that hyperpolarized Vbl (low-K⁺ saline). Addition of TEA to the saline bathing Malpighian tubules rapidly depolarized the Vbl, indicating that TEA uptake was electrogenic. Blockade of K⁺ channels with Bu²⁺ did not block effects of TEA on Vbl 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.

*Drosophila melanogaster*; Malpighian tubule; organic cation transport; comparative renal physiology

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 (OCT) system appears to be a normal 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 have led to the classification of OCs into two types (24). Type I OCs, such as tetraethylammonium (TEA) and N⁰-methylnicotinamide (NMN) are typically monovalent with a molecular weight of <400. Type II OCs, such as d-tubocurarine and vecuronium, are typically polyvalent with a molecular weight of >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 (MTs) 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 multialkali 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 MTs 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 that depolarized or hyperpolarized the basolateral membrane potential (Vbl) were used to determine whether TEA uptake is potential dependent. In addition, measurements of Vbl were used to determine whether TEA transport was electrogenic and if whether 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 25°C.

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room temperature (21–25°C) and ambient humidity. Animals 3–7 days postemergence were used in all experiments.

Preparation and isolation of MTs. MTs 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 h; see Ref. 12). The anterior pair of MTs 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 dorsoventral 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 50-μl droplets of bathing saline containing [3H]-TEA (10–100 μM). Higher concentrations of bathing saline TEA were made by combining 100 μM [3H]-TEA and unlabeled TEA. For measurements of TEA uptake over time (10 s to 10 min), tubules were incubated in bathing saline containing 100 μM [3H]-TEA. For uptake vs. concentration experiments, tubule pairs were incubated in TEA (10 μM to 2 mM) for 40 s. The bathing droplets were positioned in depressions in Sylgard on the bottom of a 100 × 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 mM) to displace any remaining surface-bound [3H]-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 min 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 before addition of TEA or at the same time as TEA.

Electrophysiology. The apparatus was mounted on a vibration-damping platform (Technical Manufacturing Corporation, Peabody, MA) within a custom made Faraday cage. V_{bl} was measured in individual principal cells of the lower segment of the Malpighian tubule maintained in a custom-built 0.2- to 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, which was sufficient to exchange the chamber’s volume in ~5 s.

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 M KCl, and one barrel was connected through a chlorided silver wire to the electrometer. Microelectrode resistance was typically 20–40 MΩ. V_{bl} 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 M KCl-4% agar bridge placed in the saline bathing the tubule. Data were recorded using a personal computer-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 (molecular weight of 70,000–150,000, 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 (Narishige, Tokyo, Japan). A sudden shift in potential indicated that the basolateral membrane of a principal cell had been impaled.

Chemicals. [3H]-TEA (55.6 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). All other chemicals were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Chemicals were dissolved in standard bathing saline, ethanol, or DMSO. The final concentration of DMSO or ethanol did not exceed a final concentration of 1%. Previous studies (e.g., Ref. 29) have shown that tubule function is unaffected by these solvents at ≤1%.

Preliminary experiments (data not shown) have demonstrated that TEA uptake is unaffected by these solvents at ≤1%.

Calculations and statistics. Measurements of TEA uptake using [3H]-TEA values are expressed as means ± SE for the indicated number of replicates (N) for number of tubules per replicate (n). Measurements of V_{bl} values are expressed as means ± SE for the indicated number of tubules (n). 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 analyzed by one-way ANOVA. In all cases, 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, Chicago, IL). The iterative procedure allowed estimation of the kinetic parameters V_{max} (maximal rate of mediated TEA uptake by all tubule pairs) and V_{max}/K_{m} for each concentration of [3H]-TEA through a computer-based data acquisition system (Axotape, Axon Instruments, Burlingame, CA).

Table 1. Composition of experimental salines mM solution*

<table>
<thead>
<tr>
<th>Saline</th>
<th>Control saline</th>
<th>K⁺ free</th>
<th>2 K⁺</th>
<th>4 K⁺</th>
<th>100 K⁺</th>
<th>Na⁺ free</th>
<th>PO₄ free</th>
<th>2 K⁺ and PO₄ free</th>
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<td>135.5</td>
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<td>2</td>
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<td>100</td>
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<td>CaCl₂</td>
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<td>MgCl₂</td>
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<td>NaH₂PO₄</td>
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<td>HEPES</td>
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<td>KHCO₃</td>
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<tr>
<td>KH₂PO₄</td>
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<td>NMDG†</td>
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<td>Glucose</td>
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Values are concentrations in mM. NMDG, N-methyl-D-glucamine pH was 7 for all solutions.

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the membrane) and $K_t$ (the concentration of TEA at 0.5 $J_{\text{max}}$) for TEA uptake.

RESULTS

Time-dependent TEA uptake into isolated MTs. The cumulative uptake of [14C]-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 with time and approached a steady state after 2 min. Uptake increased as incubation time was extended beyond 1 min. The 10-min (steady state) tissue accumulation of TEA was 1.85 ± 0.14 pmol/tubule. The addition of 1 mM cimetidine reduced the uptake of [14C]-TEA by ~95% at all time points examined. The $y$-intercept of the uptake vs. time plot in the presence of cimetidine was not significantly different from zero, a finding that is consistent with carrier-mediated, and therefore saturable, uptake of TEA. The data in Fig. 1 indicated that measurements at 40 s were well within the linear portion of the uptake vs. time plot, and this duration was therefore used in subsequent kinetic experiments.

Concentration dependence of TEA uptake into isolated MTs. Figure 2 shows the effect of increasing concentrations of bath TEA on the rate of total TEA uptake in isolated Drosophila MTs. Isolated tubules were exposed to TEA (10–400 μM) for 40 s. 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 nonsaturable (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, 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 ([TEA]) studied. This nonsaturable 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 $J_{\text{max}}$, $K_t$, and $D$ generated by a Michaelis-Menten analysis were 0.87 ± 0.09 pmol-tubule$^{-1}$-min$^{-1}$, 24.3 ± 6.7 μM, and 0.0053 ± 0.0002, respectively ($N = 5$ replicates of $n = 20$ tubules from 10 animals). Line fit to control data was calculated from Eq. 1, and kinetic parameters were derived using a nonlinear regression algorithm (Sigmaplot, SPSS). Lines fit to cimetidine data were calculated from a first-order polynomial equation. Inset: control uptake at high TEA bath concentrations. Where no error bars are apparent, error bars are smaller than the symbol used.
pathway of 0.87 pmol·tubule\(^{-1}\)·min\(^{-1}\) and 24 \(\mu\)M, respectively. These values are identical to those determined in Fig. 2 using nonlinear regression analysis. Additionally, this value of \(J_{\text{max}}\) in this plot is not significantly different from the \(y\)-intercept of the TEA uptake vs. bath concentration line at high bath concentrations (Fig. 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 mM 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 nonmediated 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 \(\mu\)M for control tubules to 77 and 411 \(\mu\)M for 0.1 and 1 mM cimetidine-treated tubules, respectively. This is precisely the pattern expected for competitive inhibition.

**Effects of ion substitution and NaCN on \(V_{\text{bl}}\).** The average steady-state \(V_{\text{bl}}\) in control saline was \(-50.8\) and \(-50.1\) mV for ion substitution and NaCN experiments, respectively (Table 2). These values are very similar to the \(V_{\text{bl}}\) 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-\(K^+\) medium depolarizes. Figure 4A and Table 2 show the effects of changes in bathing saline \(K^+\) concentration on \(V_{\text{bl}}\). A fivefold reduction in the bathing saline \(K^+\) concentration from 20 to 4 \(\mu\)M reversibly hyperpolarized \(V_{\text{bl}}\) by 25 mV in \(<2\) min. A subsequent fivefold increase in bathing saline \(K^+\) concentration from 20 to 100 \(\mu\)M reversibly depolarized \(V_{\text{bl}}\) 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 \(V_{\text{bl}}\), as observed previously (29). The opposite would also hold true for the effects of increasing bathing saline \(K^+\) on \(V_{\text{bl}}\). 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 \(V_{\text{bl}}\) 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 \(V_{\text{bl}}\). 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 mM NaCN resulted in a 45.9-mV depolarization of \(V_{\text{bl}}\) in the lower segment of Drosophila tubules (Table 2). Depolarization due to NaCN was slow, and a new steady state \(V_{\text{bl}}\) was reached within \(7\)–\(12\) min. The effect of NaCN on \(V_{\text{bl}}\) was not reversible.

**Effects of ion substitution and NaCN on TEA uptake isolated Drosophila MTs.** 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/replicate). Figure 5 shows the effects of various bathing media (Table 1) on initial uptake rates of 100 \(\mu\)M \({}^{[14]}\text{C}\)-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-\(K^+\) (\(K^+\) concentration = 100 \(\mu\)M) saline \((N = 5\)

<table>
<thead>
<tr>
<th>Treatment, mM</th>
<th>Change in Membrane Potential, mV</th>
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<tbody>
<tr>
<td>(20 \rightarrow 100 \text{ K}^+)</td>
<td>+32.95 (\pm 0.62) ((14))</td>
</tr>
<tr>
<td>(100 \rightarrow 20 \text{ K}^+)</td>
<td>-30.94 (\pm 1.27) ((14))</td>
</tr>
<tr>
<td>(20 \rightarrow 4 \text{ K}^+)</td>
<td>-19.23 (\pm 1.51) ((9))</td>
</tr>
<tr>
<td>(4 \rightarrow 20 \text{ K}^+)</td>
<td>+20.38 (\pm 1.56) ((9))</td>
</tr>
<tr>
<td>(117.5 \rightarrow 0 \text{ Na}^+)</td>
<td>+13.92 (\pm 2.86) ((3))</td>
</tr>
<tr>
<td>(0 \rightarrow 117.5 \text{ Na}^+)</td>
<td>+13.23 (\pm 2.79) ((3))</td>
</tr>
<tr>
<td>1.0 NaCN</td>
<td>+45.92 (\pm 3.15) ((4))</td>
</tr>
</tbody>
</table>

Data are mean changes \(\pm\) SE with the no. of impalements in parentheses. Positive values indicate depolarization, and negative values indicate hyperpolarization. Potentials in control saline were \(-50.9 \pm 0.6\) mV \((21)\) and \(-50.1 \pm 1.9\) \((4)\) for ion substitution and NaCN experiments, respectively.
replicates of n = 20 tubules/replicate; unpaired t-test, P < 0.05). Reduction in bathing saline K⁺ concentration to 2 mM resulted in a 45% increase in TEA uptake (N = 5 replicates of n = 20 tubules/replicate; unpaired t-test, P < 0.05).

Figure 5 also shows the effects of short-term (40 s) exposure to Na⁺-free media and 30-min preincubation 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/replicate; unpaired t-test, P < 0.05). Again, a treatment that depolarizes Vbl (see above) resulted in a reduction in TEA uptake by Drosophila MTs. The difference between TEA uptake values in short-term Na⁺-free exposures vs. preincubation in Na⁺-free saline will be discussed further below.

Figure 5 also shows the effects of preincubation in 1 mM NaCN on TEA uptake by Drosophila MTs. Preincubation 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/replicate) to 0.09 ± 0.02 pmol·tubule⁻¹·min⁻¹ in NaCN-exposed tubules (N = 3 replicates of n = 20 tubules/replicate). Taken together, the results of Fig. 5 show that TEA uptake decreased when Vbl depolarized and that TEA uptake increased when Vbl hyperpolarized.

Electrogenic effects of TEA on Vbl 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 Vbl. Figure 6 shows a representative recording from an experiment done to test this hypothesis. Addition of 1 mM TEA depolarized the membrane potential by ~15 mV within 30 s, 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 mM TEA caused a mean depolarization of $13.6 \pm 1.1$ mV.

**Electrogenic effects of TEA on Vbl 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 $K^+$ channels in the lower tubule or entry of TEA through these same $K^+$ channels. If the route of TEA entry across the basolateral membrane was not via $K^+$ channels, it would follow that prior addition of Ba$^{2+}$, a known blocker of basolateral $K^+$ channels in *Drosophila* MTs (15), should abolish the hyperpolarization of the $V_{bl}$ in response to a 10-fold reduction in bathing saline $K^+$ concentration but not the depolarization observed by treatment of tubules with 1 mM TEA. Figure 7 is an example of an experiment designed to test this hypothesis. In these experiments, $V_{bl}$ in control saline ($K^+$ concentration = 20 mM) was $-52.4 \pm 1.1$ mV ($n = 8$ tubules). A 10-fold reduction in bath $K^+$ concentration caused a further reversible hyperpolarization of $30.5 \pm 1.7$ mV. Addition of 6 mM Ba$^{2+}$ to the bathing saline had no significant effect on the $V_{bl}$ ($n = 8$ tubules; paired t-test, $P > 0.05$). NaH$_2$PO$_4$ 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 10-fold 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 mM TEA in the presence of Ba$^{2+}$ caused a depolarization of $18.3 \pm 0.6$ mV, consistent with the hypothesis that basolateral TEA uptake does not occur through $K^+$ channels ($n = 8$ tubules). The response of the $V_{bl}$ 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 on return to control saline ($n = 8$ tubules).

**Effects of Ba$^{2+}$ on TEA uptake by isolated tubules.** We examined effect of 6 mM Ba$^{2+}$ on uptake of $100 \mu$M [$^{14}$C]-TEA by isolated *Drosophila* MTs (data not shown). The uptake of TEA by control tubules and tubules exposed to Ba$^{2+}$ were $0.65 \pm 0.16$ and $0.65 \pm 0.12$ pmol-tubule$^{-1}$min$^{-1}$, respectively ($N = 5$ replicates of $n = 20$ tubules/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 tetra-alkylammonium 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 tetra-alkylammonium (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 mM tetramethylammonium, tetrabutylammonium, tetrabutylammonium, and tetrabutylammonium to the bathing saline all in-
hibited basolateral TEA uptake by isolated *Drosophila* MTs. Moreover, it also shows that tetrapentylammonium, the most hydrophobic compound, resulted in greater inhibition of TEA uptake than tetramethylammonium, 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/replicate). All data were corrected for diffusion by subtracting the TEA diffusion component calculated from Eq. 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 IC50 of 33 μM for cimetidine and 6 μM for quinine on the uptake of 100 μM TEA by isolated tubules. These IC50 values were lower than the approximated 50% inhibition of [14C]-TEA uptake by 100 μM cold TEA itself. In contrast, a 10-fold higher concentration of the prototypical type I organic cation NMN 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 1 mM 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 mM 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 (MRP) 1 MK571 (9) inhibited TEA uptake by isolated tubules at all concentrations. However, concentrations of 10 and 100 μM MK571 did not exhibit any difference in the extent of inhibition. An increased inhibition was only observed when a 1 mM concentration of MK571 was used. The addition of 1 mM 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 MTs, 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 MTs 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 support 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 Jmax of 0.87 pmol-tubule −1min−1 and a K1 of 24 μM. Our one-half maximal TEA uptake value is consistent with those previously observed in rabbit (30–75 μM; see Ref. 38), rat (63 μM; see Ref. 3), and snake renal proximal tubules (18 μM; see Ref. 17). We previously determined the Jmax and K1 for transepithelial transport of TEA to be 1.52 pmol-tubule −1min−1 and 180 μM, respectively (32). Using these kinetic values and the kinetic values from our present study, we have calculated that the transport efficiency (Jmax/K1) for basolateral TEA uptake is 4.3-fold higher than the transport efficiency for transepithelial
TEA secretion. Therefore, this higher efficiency for basolateral uptake vs. 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 that 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 Ulrich 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 (Fig. 4, Table 2), led to the inhibition of TEA uptake (Fig. 5). Moreover, we observed that the effects of high K⁺ on V₇50 were very rapid (<30 s) 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 V₇50 occurred much more slowly over the course of ~10 min. Similarly, it could be seen that the inhibition of TEA uptake was enhanced by preincubation 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 (Fig. 4, Table 1), led to an increase in TEA uptake (Fig. 5). In addition, the effects of low-K⁺ saline on V₇50 were very rapid (<30 s) 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 mM) to the bathing saline caused an ~14 mV depolarization of the V₇50 (Fig. 6), consistent with electrogentic uptake of TEA. Previous studies on teleost (33) and snake (17) renal proximal tubules showed depolarizations of the V₇50 of ~7 and ~10 mV, respectively, when 1 mM TEA was added to the bathing medium. In both vertebrate studies, the depolarization occurred slowly over 6–8 min. Kim and Dantzler (17) concluded, on the basis of this slow response, that the depolarization of the V₇50 is due to some slow 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 V₇50 is due to the blockage of K⁺ channels. TEA is an impermeant but potent extracellular and intracellular blocker of K⁺ channels in metazoan neurons and muscle fibers (1, 13). To test whether 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 V₇50 of the lower K⁺-secreting segment of Drosophila tubules in control saline. Ba²⁺ eliminated the response of the V₇50 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 V₇50 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 V₇50. 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 MTs.

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 MTs 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 MTs of 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 MTs. 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 I₅₀ values for cimetidine (33 μM) and quinine (6 μM) are lower than the half-maximal inhibition of [¹⁴C]TEA uptake by cold TEA itself (~100 μM), suggesting that cimetidine and quinine are better substrates for the basolateral organic cation transporter of MTs than TEA. The lack of inhibition of TEA uptake by NMN suggests 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 NNM uptake that cannot be determined by our current data. The functional separation of TEA and NNM 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 characteristic 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 MRPI 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) termed these substrates “bisubstrates” and tested the interaction of a number of these compounds with rat renal basolateral organic cation and organic anion transport pathways. Their 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 noncompetitive manner. Such interaction may explain the effects of the OA pathway substrates PAH, probenecid, and MRPI inhibitor MK571 observed in our study.

It is worth noting that an ortholog of the basolateral OCTs of vertebrate kidney, designated Orct, has been cloned from a 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 vs. vertebrate renal proximal tubules. The depolarizing effect of TEA on V_m 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 MTs. This mechanism appears to be distinct from that present in the basolateral membrane of vertebrate renal tubules.

GRANTS

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REFERENCES

BASOLATERAL TEA UPTAKE IN DROSOPHILA MALPIGHIAN TUBULES


