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Characterization of transepithelial potential oscillations in the *Drosophila* Malpighian tubule

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Summary

The Malpighian tubule of *Drosophila melanogaster* is a useful model system for studying the regulation of epithelial ion transport. In acutely isolated tubules, the transepithelial potential (TEP) undergoes large oscillations in amplitude with a period of approximately 30 s. The TEP oscillations are diminished by reductions in the peritubular chloride concentration in a manner consistent with their being caused by fluctuations in chloride conductance. The oscillations are eliminated by pretreating tubules with the calcium chelator BAPTA-AM, although removal of peritubular calcium has no effect, suggesting that the oscillations are a result of either the release of calcium from intracellular stores or the entry of calcium from the tubule lumen. Transcripts encoding two calcium-release channels, the ryanodine receptor and the inositol trisphosphate receptor, are detectable in the tubule by reverse transcription–polymerase chain reaction. To identify the cell type responsible for the oscillations, tubules were treated with diuretic hormones known to alter calcium levels in each of the two cell types. Leucokinin-IV, which increases calcium levels in the stellate cells, suppressed the oscillations, whereas cardioacceleratory peptide 2b (CAP_{2b}), which increases calcium levels in the principal cells, had no effect. These data are consistent with a model in which rhythmic changes in transepithelial chloride conductance, regulated by intracellular calcium levels in the stellate cells, cause the TEP oscillations.

Key words: *Drosophila melanogaster*, Malpighian tubule, calcium, transepithelial potential, leucokinin, CAP_{2b}.

Introduction

The insect Malpighian (renal) tubule has been a productive system for studying epithelial ion transport (Phillips, 1981; Beyenbach, 1995; Dow et al., 1998). In the fruit fly *Drosophila melanogaster*, the Malpighian tubules secrete an iso-osmotic, potassium-rich urine into the digestive tract (Dow et al., 1994b; O'Donnell and Maddrell, 1995). The main segment of the tubule, in which the secretion of primary urine occurs, contains two morphologically defined cell types: principal cells (type I) and stellate cells (type II) (Wessing and Eichelberg, 1978; Sözen et al., 1997). In the tubules of *Drosophila*, as in other insects, cations are actively transported into the lumen by the principal cells (Dow et al., 1994b; Beyenbach, 1995; O'Donnell et al., 1996; Linton and O'Donnell, 1999). This transport is driven by a vacuolar-type proton-ATPase in the apical membrane; protons are then transported back into the cell and potassium secreted by an amiloride-sensitive alkali metal/proton exchanger (Nicolson, 1993; Beyenbach, 1995). Potassium enters the principal cells by way of a K^+/Cl^- cotransporter in the basolateral membrane, although a substantial potassium conductance is also present in this membrane; the fate of the cotransported chloride is unclear (O'Donnell et al., 1996; Linton and O'Donnell, 1999). Chloride moves passively into the lumen down its electrochemical gradient through a pathway that lies outside of the principal cells (Pannabecker et al., 1993; O'Donnell et al., 1996). The anatomical site of this chloride-shunt conductance has been debated; in *Drosophila* it is believed to be transcellular through the stellate cells, based primarily on studies of the increase in chloride permeability caused by the diuretic hormone leucokinin (O'Donnell et al., 1996; O'Donnell et al., 1998). Studies in the mosquito *Aedes aegypti*, however, support a paracellular route for chloride flux, based on an increased permeability of the tubule to sucrose and inulin following treatment with leucokinin (Wang et al., 1996) and on the behavior of the leucokinin-induced chloride conductance pathway as a single diffusion barrier (Pannabecker et al., 1993). This latter finding is also consistent with certain models of transcellular transport, and recent papers have reported chloride channels in the apical membranes of stellate cells both in *Aedes* and *Drosophila* (O'Donnell et al., 1998; O'Conner and Beyenbach, 2001).

The net effect of ion transport in the *Drosophila* tubule is the establishment of a lumen-positive transepithelial potential (TEP) with an amplitude of approximately 50–60 mV. This

TEP is not constant but undergoes large oscillations in amplitude (Davies et al., 1995). No function has yet been determined for these oscillations, but similar oscillations are seen in the tubules of other insect species, raising the possibility that they may be important for the regulation of normal ion transport (Pilcher, 1970; Morgan and Mordue, 1981; Williams and Beyenbach, 1984). A recent study in *Aedes* demonstrated that, in that species, the oscillations are the result of fluctuations in the conductance of the chloride-selective shunt (Beyenbach et al., 2000). The experiments in this paper were designed to determine whether the oscillations in the *Drosophila* tubule arise from a similar mechanism and to identify the cell type responsible for their generation.

Materials and methods

Drosophila *maintenance*

Drosophila melanogaster (Canton-S) were maintained on a 12 h:12 h L:D cycle at 25 °C using standard procedures (Ashburner and Roote, 2000).

Tubule isolation

Posterior Malpighian tubules were dissected under dissecting saline from adults of both sexes 6–8 days post-eclosion, placed in a tissue culture dish in which a 100 µl drop of 0.125 mg ml^{-1} poly-L-lysine had been dried in order to promote adhesion of the tubule to the dish, and the solution replaced with recording medium (O'Donnell et al., 1996). The dissecting saline contained (in mmol l^{-1}): 85 NaCl, 20 KCl, 3 $CaCl_2$, 12 $MgSO_4$, 7.5 $NaHCO_3$, 4 NaH_2PO_4, 15 glucose, 10 Hepes, pH 6.75 [osmolality=255–270 mmol kg^{-1} as measured with a vapor pressure osmometer (Wescor, Logan, UT, USA)]. In most experiments, the recording medium consisted of a 1:1 mixture of Schneider's *Drosophila* Medium (Life Technologies, Rockville, MD, USA) and a 'diluting saline' containing 36 NaCl, 21 KCl, 15 $MgCl_2$, 5 $CaCl_2$, 4.8 $NaHCO_3$, 2 NaH_2PO_4, 11.1 glucose, 15 Hepes, pH 6.75. The osmolality of this solution was 255–270 mmol kg^{-1}. For the experiment involving removal of external calcium, the recording medium consisted of dissecting saline (either with or without $CaCl_2$) containing 0.5 mmol l^{-1} L-tyrosine. Tyrosine is required in the recording solution for TEP oscillations (E. Blumenthal, manuscript in preparation); it is present in Schneider's medium, but must be added when recordings are conducted in saline alone. For the barium experiment, the recording medium consisted of dissecting saline (including 0.5 mmol l^{-1} L-tyrosine) in which the $MgSO_4$ was replaced with $MgCl_2$. For the chloride replacement experiment, the recording media consisted of various mixtures of dissecting saline and a saline containing 85 mmol l^{-1} sodium isethionate, 10 mmol l^{-1} K_2SO_4 and 3 mmol l^{-1} $CaSO_4$ instead of NaCl, KCl and $CaCl_2$, respectively. All of the resulting low-chloride salines also contained 0.5 mmol l^{-1} L-tyrosine. Experiments were conducted within 2 h of tubule dissection.

For BAPTA loading, tubules were incubated for 1 h at room temperature in standard recording medium containing 100 µmol l^{-1} BAPTA-AM (Molecular Probes, Eugene, OR, USA), 0.04 % Pluronic F-127 (Molecular Probes) and 0.2 % dimethyl sulfoxide (DMSO). Control tubules were incubated in medium containing Pluronic F-127 and DMSO only. Pluronic F-127 was included to increase the solubility of the BAPTA-AM.

Recording

The tubule lumen and principal cells were impaled with a sharp electrode (R>35 MΩ) pulled from theta-glass (Sutter Instruments, Novato, CA, USA) and filled with 3 mol l^{-1} KCl. Potentials were amplified (Axopatch 200B, Axon Instruments, Foster City, CA, USA), digitized at 100 Hz and stored online. Recording and analysis was conducted using pClamp and Axoscope software (Axon Instruments). The peritubular bath was continuously perfused during recording.

Drugs were applied to and removed from the tubules during recording by switching perfusion lines; drugs used were niflumic acid (NA) (Sigma, St. Louis, MO, USA), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (Calbiochem, La Jolla, CA, USA), 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) (Calbiochem), diphenylamine-2-carboxylic acid (DPC) (Fluka, Milwaukee, WI, USA), dantrolene (Calbiochem), 2-aminoethoxy-diphenylborate (2-APB) (Calbiochem), Xestospongin C (Calbiochem), 3,4,5-trimethyloxybenzoic acid 8-(diethylamino)octyl ester (TMB-8) (Calbiochem), ryanodine (Calbiochem), leucokinin IV (Bachem, King of Prussia, PA, USA) and CAP_{2b} (gift of Dr Nathan Tublitz, University of Oregon).

Data analysis

Because of the complex waveform of most of the TEP records, it is difficult to measure the amplitude of individual oscillations. As an alternative, I used the coefficient of variation of the TEP as a measure of overall variability. The TEP coefficient of variation was calculated as the standard deviation of the TEP divided by the mean. In most cases, the coefficient of variation was calculated for the entire record. For the BAPTA-loading experiment, each trace was divided into non-overlapping 20 s segments and the coefficient of variation was calculated for each segment. These values were averaged to give a value for the entire trace.

For period analysis, individual TEP records were decimated tenfold and subjected to fast Fourier transform non-linear least-squares analysis (Straume et al., 1991). This analysis reduces each record to a series of rhythmic components and returns an amplitude, period and relative uncertainty of the amplitude for each component. For each record, components with an amplitude of <1 mV were discarded, as were those components with periods longer than half of the length of the record. The average period of each record was then calculated by taking a weighted average of the periods of the remaining components, using the inverse square of the amplitude uncertainty as the weighting factor.

Reverse transcriptase–polymerase chain reaction

For whole fly cDNA, RNA was isolated from adult female flies using Trizol reagent (Life Technologies, Rockville, MD, USA) and cDNA was synthesized using the Superscript Preamplification System (Life Technologies). For tubule-specific cDNA, anterior and posterior tubules were dissected from young adult female flies, and RNA was isolated using the RNAqueous-4PCR kit (Ambion, Austin, TX, USA). The RNA was resuspended in 20 μl of water, divided between two tubes, and cDNA synthesized as above. Reverse transcriptase (RT) was added to only one of the tubes, with the other serving as a control for genomic DNA contamination. Gene-specific fragments were amplified from approximately one fly's worth of cDNA by 35 cycles of polymerase chain reaction (PCR) using standard procedures and the components of the Advantage cDNA PCR kit (Clontech, Palo Alto, CA, USA). Primer sequences were: for the ryanodine receptor, TCACTGAAGTCATCCAGGGTCC and AGGAGCACA-GCCAGGTTGAAAC, and for the inositol trisphophate receptor, CACTGCCCAAATAGAGATTGTTCG and TT-GCTCCTCAAAGTCCACGCTG.

Results

I used sharp electrode recording to measure the TEP of Malpighian tubules acutely isolated from adult *Drosophila*. As has been reported by others (Davies et al., 1995), the tubule exhibits a lumen-positive TEP that undergoes dramatic oscillations in amplitude (Fig. 1). These oscillations are present in almost all tubules and persist for the duration of a recording, which can last for tens of minutes. In some recordings, the oscillations are nearly sinusoidal, while in others they are much more complex and appear to be composed of many components. The average period of the oscillations was 36±3 s (N=12 tubules, mean ± S.E.M.), as determined by fast Fourier transform analysis (see Materials and methods).

Although oscillations were seen in the TEP of virtually all tubules tested, intracellular recordings from principal cells did not reveal any oscillations in the basolateral membrane potential (V_{bl}) of these cells, in contrast to the clear V_{bl} oscillations seen in *Aedes* (Beyenbach et al., 2000). This observation would appear to violate Kirchhoff's laws; a voltage oscillation across the entire tubule wall should affect the potential drop across each individual membrane. Such a contradiction has also been raised with regard to the response of the *Drosophila* tubule to leucokinin, as the large depolarization in the TEP is not accompanied by a change in V_{bl} of the principal cells (Beyenbach et al., 2000). One possible explanation for these observations is that the fractional resistance of the principal cell basolateral membrane (fR_b) could be extremely low as a result of a low basolateral input resistance and a high apical input resistance. This situation would result in the voltage response to a transepithelial current occurring predominantly at the apical membrane (see Pannabecker et al., 1992; Weltens et al., 1992, for a more detailed discussion of this topic). To test this possibility, tubules were treated with barium, which has been shown to block a potassium conductance in the principal cell basolateral membrane and should therefore increase fR_b (O'Donnell et al., 1996). As shown in Fig. 2, application of 3 mmol l^{-1} $BaCl_2$ rapidly hyperpolarizes V_{bl} (from –48.3±1.5 mV to –63.3±1.0 mV, P<0.05 by paired t-test, N=3 tubules) and causes the membrane potential to oscillate with a pattern similar to that seen in the TEP (the coefficient of variation increased from 0.016±0.002 to 0.037±0.003, P<0.01 by paired t-test, N=3 tubules). A barium-induced hyperpolarization of V_{bl} has been reported in both *Aedes* and the forest ant *Formica polyctena* and is the result of an increased polarizing effect of the apical proton pump on V_{bl} (Weltens et al., 1992; Masia et al., 2000). The behavior of the *Formica* tubule in particular is similar to *Drosophila*: in both species, V_{bl} is dominated by a high potassium conductance and is at or very near the Nernst equilibrium potential for potassium, E_K (Weltens et al., 1992; O'Donnell et al., 1996). The addition of barium increases fR_b and makes V_{bl} more sensitive to electrical events at the apical membrane, most notably the hyperpolarizing drive of the

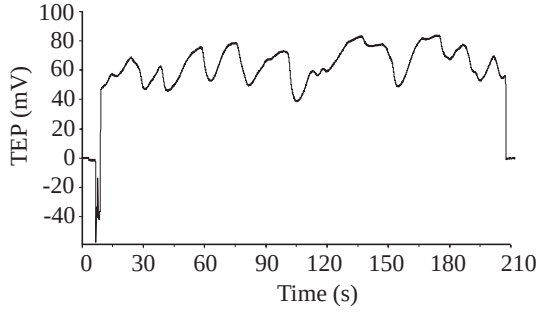

Fig. 1. Representative recording from a Malpighian tubule showing transepithelial potential (TEP) oscillations. At the beginning of the trace, the electrode is in the bath. The negative deflection reflects the potential across the basolateral membrane of a principal cell as the electrode enters the cell; as the electrode is advanced into the lumen of the tubule, the recorded potential becomes positive. At the end of the trace, the electrode is withdrawn into the bath again.

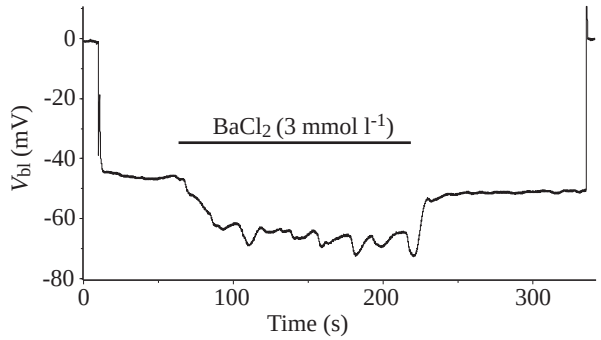

Fig. 2. Effect of Ba^{2+} on principal cell basolateral membrane potential. The electrode is positioned inside a principal cell. Application of 3 mmol l^{-1} $BaCl_2$ for the duration indicated by the bar results in a hyperpolarization of V_{bl} and the appearance of voltage oscillations. Similar results were seen in two additional tubules.

ATPase, resulting in a hyperpolarization of V_{bl}. The increase in fR_b also explains the appearance of the oscillations in V_{bl}, which is no longer dominated by the potassium diffusion potential and will now reflect oscillations in the total transepithelial current.

The TEP oscillations in *Aedes* have been shown to result from oscillations in chloride conductance (Beyenbach et al., 2000). To determine if the same is true in *Drosophila*, I examined the dependence of the oscillations on the peritubular chloride concentration ($[Cl^-]_p$). Oscillations were observed in seven of eight tubules tested, and in all seven of these tubules, reducing $[Cl^-]_p$ from the control concentration of 111 mmol l^{-1} by replacement with isethionate affected the magnitude of the oscillations. In one tubule (Fig. 3A), the oscillations disappeared upon reduction of $[Cl^-]_p$ to 55.5 mmol l^{-1} and then resumed upon further reduction; at low $[Cl^-]_p$, the polarity of the oscillations appears to have reversed from depolarizing to hyperpolarizing, although this is difficult to assess quantitatively due to their complex waveform. In the six remaining tubules, the magnitude of the oscillations decreased monotonically as $[Cl^-]_p$ was reduced from 111 mmol l^{-1} to 11.1 mmol l^{-1} but did not reverse (Fig. 3B,C). The dependence of the TEP oscillations on $[Cl^-]_p$ is similar to that seen in *Aedes* and is the expected result if they are caused by fluctuations in chloride conductance. Because chloride transport across the tubule is passive, the change in TEP resulting from a given increase in transepithelial chloride conductance, all else being equal, will depend only on the driving force for chloride, which is the difference between the TEP and the transepithelial chloride equilibrium potential (E_{Cl}). E_{Cl}, in turn, depends on both $[Cl^-]_p$ and the luminal chloride concentration $[Cl^-]_l$. As $[Cl^-]_p$ is decreased, E_{Cl} should become more positive relative to the peritubular bath, reducing the driving force for chloride diffusion and thus the magnitude of the voltage oscillations, as is observed. The heterogeneity in the response among different tubules is not unexpected given the many uncontrolled factors that determine the value of $[Cl^-]_p$ at which a chloride-selective current would be expected to reverse. The TEP varies substantially among tubules; indeed, the tubule shown in Fig. 3A had the lowest TEP of the seven tested. In addition, $[Cl^-]_l$ would be expected to decrease along with $[Cl^-]_p$ as less chloride is available to enter the lumen (Beyenbach et al., 2000); the precise dependence of $[Cl^-]_l$ on $[Cl^-]_p$ is likely to be complex and determined by the magnitude and selectivity of the total anion permeability of each particular tubule.

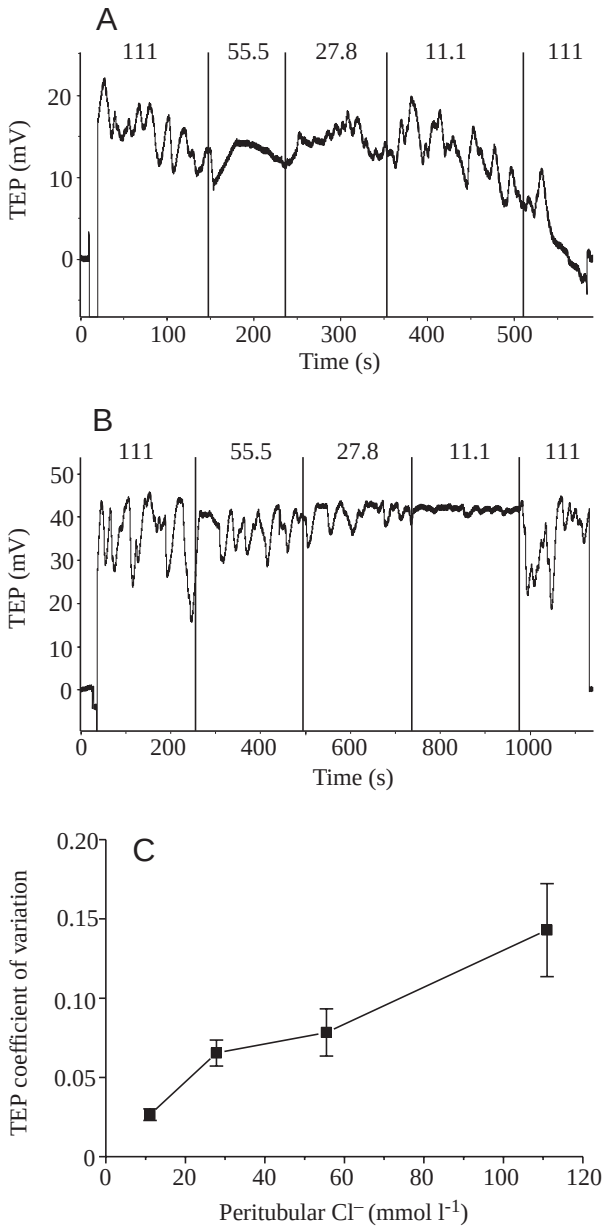

Fig. 3. Dependence of TEP oscillations on $[Cl^-]_p$. Eight tubules were exposed to varying concentrations of Cl^- (in mmol l^{-1}; indicated at top of panels) in the peritubular bath; seven of these tubules exhibited TEP oscillations. In one tubule (A), the oscillations disappeared at 55.5 mmol l^{-1} Cl^- and reappeared as $[Cl^-]_p$ was reduced further. In the six other tubules, the amplitude of the oscillations decreased as $[Cl^-]_p$ decreased (B). (C) Averaged data from the latter six tubules only. Values are means ± S.E.M.

It has previously been reported that the treatment of *Drosophila* tubules with chloride channel blockers eliminates fluid secretion, suggesting that ion channels are responsible for the movement of all of the chloride across the *Drosophila* tubule (O'Donnell et al., 1998). Given this result, one would expect that these blockers would also eliminate the TEP oscillations. As shown in Fig. 4, application of the chloride channel antagonist DPC rapidly and reversibly depolarizes the TEP. A similar depolarization was seen with two additional drugs (Table 1), although the speed of the depolarization and the degree of reversibility differed slightly among the various agents. The concentrations of channel blockers used in this study were the same as or lower than those previously shown to block fluid secretion (O'Donnell et al., 1998). Lower concentrations of each drug either had no effect or caused a less severe depolarization; at no concentration did any of these blockers eliminate the TEP oscillations without also depolarizing the tubule (data not shown). A fourth, structurally unrelated channel blocker, DIDS, caused a slow depolarization

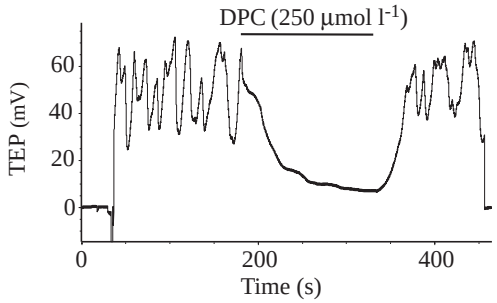

Fig. 4. Effect of DPC on tubule electrophysiology. Application of 250 μmol l^{-1} DPC (bar) rapidly depolarizes the TEP, which recovers upon washout of the drug. Similar results were seen in four additional tubules (see Table 1).

of the TEP. Short-term (6–8 min) treatment of tubules with 300 μmol l^{-1} DIDS had no consistent effect on the TEP (*N*=4, data not shown). However, tubules that had been incubated in 100 μmol l^{-1} DIDS for 50–84 min were profoundly depolarized. Of nine tubules tested, three did not show a lumen-positive TEP in either branch of the tubule, three showed a positive TEP in only one branch, and three showed a positive TEP in both branches. The average TEP for the nine branches showing a positive TEP was only 12.6±2.2 mV. Long-term exposure to lower concentrations of DIDS had no consistent effect on the TEP (data not shown). The depolarization caused by all of these channel antagonists is not consistent with a simple blockade of chloride channels, which would be expected to hyperpolarize the TEP, and most likely results from the inhibition of some other transporter. Indeed, a sodium-dependent anion exchanger has recently been cloned from *Drosophila* and shown to be inhibited by carboxylate chloride channel blockers and DIDS (Romero et al., 2000).

Membrane potential oscillations with a period similar to those seen in the tubule have been reported in several different types of non-excitable cell. In many cases, these oscillations have been shown to result from oscillations in intracellular free calcium levels that affect the membrane potential through the rhythmic activation of calcium-dependent ion channels (Yada et al., 1986; Westphale et al., 1992; Ritter et al., 1993). To test the role of intracellular calcium in the TEP oscillations, tubules were loaded with the calcium chelator BAPTA-AM, which will buffer any increases in intracellular calcium and has been shown previously to block calcium-dependent signaling in this preparation (O'Donnell et al., 1996). As shown in Fig. 5, there is a clear difference between the electrical behavior of control and BAPTA-loaded tubules. The oscillations seen in all of the control traces are not present in the BAPTA-treated tubules; although the treated tubules do sometimes exhibit large drops in TEP amplitude, this is probably due to an increased susceptibility of these tubules to damage from electrode impalement and does not resemble the oscillations seen in controls. The effect of BAPTA on the TEP is most clearly seen by dividing each trace into 20-second increments and calculating the coefficient of variation of these segments (Fig. 5C). The elimination of oscillations is not due to general toxicity as the average TEP amplitude is not reduced by BAPTA treatment (control TEP: 42.4±4.8 mV, *N*=6 tubules; BAPTA-treated TEP: 38.0±2.9 mV, *N*=6, *P*>0.45 by unpaired *t*-test). These results suggest that a rise in intracellular calcium levels is necessary for the TEP oscillations but do not identify the cell type in which such changes occur.

Although changes in intracellular calcium appear to be necessary for TEP oscillations, peritubular calcium is not required. Fig. 6 illustrates that acute treatment of tubules with nominally calcium-free medium does not affect their electrical behavior. Removal of peritubular calcium did not affect the average amplitude of the TEP (from 33.6±3.0 mV in the presence of calcium to 32.5±2.7 mV in 0 calcium, *N*=8 tubules, *P*>0.7 by paired *t*-test), the coefficient of variation (from 0.17±0.02 to 0.14±0.01, *N*=8, *P*>0.05), or the average period of the oscillations (from 38.8±4.6 s to 43.8±2.9 s, *N*=5, *P*>0.10). Because the TEP oscillations persist in the absence of peritubular calcium, the necessary increase in intracellular

Table 1. *Effect of chloride channel blockers on the TEP*

			During drug administration			Washout		
Drug (μmol l^{-1})	*N*	Pre-drug TEP (mV)	TEP (mV)	Time (s)	*P*	TEP (mV)	Time (s)	*P*
DPC, 250	5	39.9±5.3	7.7±2.0	90–300	<0.001	38.9±6.5	35–75	NS
NA, 100	6	39.6±5.4	4.4±2.5	70–330	<0.002	28.3±6.4	100–320	<0.05
NPPB, 100	6	40.5±5.3	0.1±1.4	60–120	<0.001	44.2±5.8	140–235	NS

The average TEP was measured before drug application, in the presence of the drug, and after washout. The second measurement was taken after the TEP had reached a steady state, and the third after no further recovery of the TEP was seen (although the TEP was oscillating and so was not at steady state).

Each drug was dissolved in DMSO such that the final concentration of solvent applied to the tubule was 0.1%; at this concentration, DMSO does not affect the TEP (see Fig. 5).

DPC, diphenylamine-2-carboxylic acid; NA, niflumic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid.

The kinetics of depolarization and recovery varied among tubules; the times given represent the range of times (from tubule to tubule) following drug application or washout at which the TEP was measured.

P-values represent paired *t*-tests comparing each value with the pre-drug TEP measurement; NS, not significant.

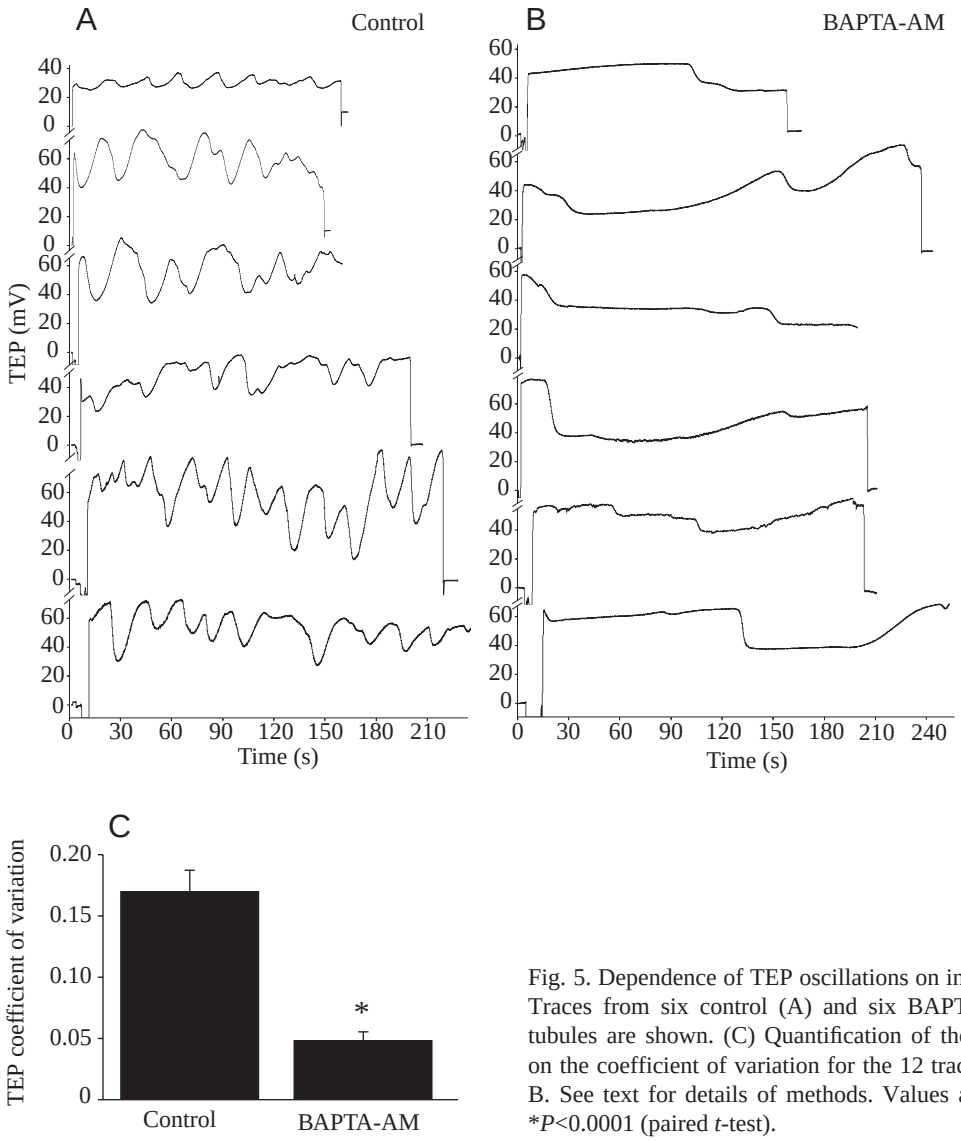

Fig. 5. Dependence of TEP oscillations on intracellular calcium. Traces from six control (A) and six BAPTA-AM loaded (B) tubules are shown. (C) Quantification of the effect of BAPTA on the coefficient of variation for the 12 traces shown in A and B. See text for details of methods. Values are means ± S.E.M., **P*<0.0001 (paired *t*-test).

calcium must result either from a release of calcium from intracellular stores or from the entry of calcium from the tubule lumen. In most cases calcium oscillations require both intracellular calcium stores and the entry of extracellular calcium in order to refill the stores (Berridge, 1990; Fewtrell, 1993).

Intracellular calcium stores can be separated into two pharmacologically distinct types: those sensitive to inositol trisphosphate (IP_3), and those sensitive to ryanodine (Tsien and Tsien, 1990). Release from these stores is governed by the IP_3 receptor (IP_3R) and the ryanodine receptor (RyR), respectively. As the *Drosophila* genome contains only single copies of genes encoding each of these release channels (Hasan and Rosbash, 1992; Yoshikawa et al., 1992; Takeshima et al., 1994; Littleton and Ganetzky, 2000), I sought to determine whether either or both of them are expressed in the tubules. As shown in Fig. 7, RT-PCR of tubule-specific mRNA amplified fragments corresponding to both the IP_3R (lane 3) and the RyR (lane 7). These amplification products are unlikely to result from contamination by genomic DNA; controls lacking reverse transcriptase did not show any product (lanes 4,8), and the primers were designed such that genomic DNA would have produced either a larger product, due to the inclusion on intronic DNA (RyR), or no product at all, due to the primer spanning an exon–intron boundary (IP_3R). Thus both calcium-release channels are expressed in the tubule.

Treatment of tubules with a number of pharmacological agents known to block release of calcium from intracellular stores gave inconclusive results. Two antagonists of the IP_3R, Xestospongin C (Gafni et al., 1997) and 2-APB (Ascher-Landsberg et al., 1999) failed to block the oscillations specifically. Xestospongin had no effect (5–20 $\mu mol\,l^{-1}$, nine tubules), while 2-APB caused an overall reduction in the TEP, possibly due to general toxicity (25–100 $\mu mol\,l^{-1}$, three tubules). Neither of these agents has been reported to be effective in insect cells. Two antagonists of the RyR, which have been used in insect cells (Arnon et al., 1997; Heine and Wicher, 1998), also had no consistent effect: dantrolene

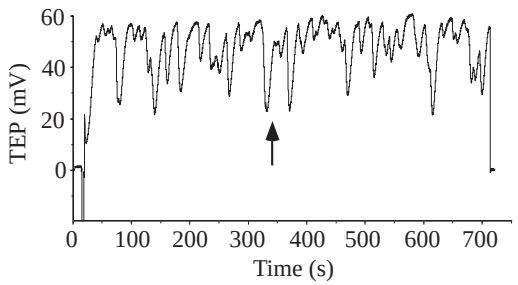

Fig. 6. Lack of dependence of TEP oscillations on peritubular calcium. In the trace shown, the tubule is bathed in a normal, calcium-containing saline. At the arrow, the bathing solution is switched to a nominally calcium-free saline. Similar results were obtained from seven other tubules.

(10 μmol l^{-1}, five tubules) and ryanodine (5 μmol l^{-1}, three tubules). Finally, TMB-8, a less specific antagonist with demonstrated effects in insects (Van Marrewijk et al., 1993; Wegener and Nassel, 2000), had no effect on TEP oscillations (100–500 μmol l^{-1}, three tubules). The failure of these agents to block the oscillations could indicate that the intracellular calcium-release channels are not involved; however, it is also possible that the tubule's ability to transport small organic solutes prevents these drugs from accumulating in the cytoplasm at sufficient concentrations to block their targets.

The finding that a rise in intracellular calcium levels is necessary for the TEP oscillations raises the question of the cellular localization of the calcium increases. Unfortunately, traditional calcium imaging is not possible in the tubule due to the rapid excretion of the imaging dyes (Rosay et al., 1997; E. Blumenthal, unpublished results). An alternative method for measuring calcium levels in tubules cells has been developed, employing the selective expression of aequorin in different classes of tubule cells (Rosay et al., 1997). The bioluminescence output from these tubules, however, is not sufficient to image individual cells with the temporal resolution necessary to observe calcium oscillations. For this study, therefore, I took advantage of the availability of agents that increase intracellular calcium levels specifically in each of the two cell types. Fig. 8 illustrates the response of a tubule to an application of the diuretic hormone leucokinin IV. As has been shown by others, leucokinin causes a rapid collapse of the TEP due to an increase in chloride conductance mediated by a specific rise in intracellular calcium levels in the stellate cells (O'Donnell et al., 1996; O'Donnell et al., 1998). Following washout of the drug, the TEP amplitude recovers quite rapidly; however, the TEP oscillations are completely suppressed for several minutes after removal of leucokinin, even after the TEP amplitude has completely recovered. A lower concentration of leucokinin (1 μmol l^{-1}) causes a more transient suppression of the oscillations (data not shown). Thus an increase in stellate cell calcium levels causes a relatively long-lasting disruption of the TEP oscillations.

In contrast to leucokinin, a second diuretic hormone, CAP_{2b}, has no effect on the TEP oscillations. Application of CAP_{2b} has been shown to stimulate the activity of the apical proton pump in the principal cells by activating the NO/cGMP

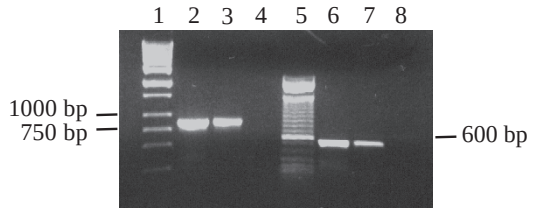

Fig. 7. Expression of genes encoding intracellular calcium-release channels in the tubule. The gel shows the result of RT-PCR from whole-fly mRNA (lanes 2,6) and tubule-specific mRNA (lanes 3,4,7,8) using primers specific to the IP_3 receptor (IP_3R) (lanes 2–4) and the ryanodine receptor (RyR) (lanes 6–8). In lanes 4 and 8, reverse transcriptase was omitted from the cDNA synthesis reaction to control for possible genomic DNA contamination. The PCR reactions give products of the expected sizes: 547 base pairs (bp) for RyR, compared to 678 bp from genomic DNA, due to the inclusion of two short introns (Adams et al., 2000) and 843 bp for IP_3R (Sinha and Hasan, 1999). Because the 5′ IP_3R primer spans an exon–intron junction, genomic DNA should not give any PCR product. Lane 1: 1 kb ladder (Stratagene, La Jolla, CA, USA); Lane 5: 100 bp ladder (Life Technologies).

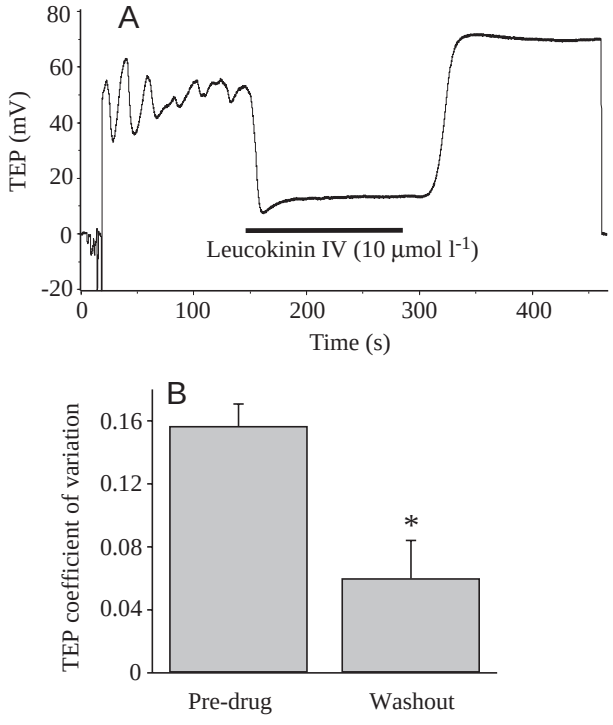

Fig. 8. Effect of leucokinin on TEP oscillations. (A) Addition of leucokinin IV (10 μmol l^{-1}) rapidly depolarizes the TEP. After drug washout, the TEP recovers amplitude while the oscillations remain inhibited. (B) Average results from six tubules show long-lasting inhibition of oscillations after leucokinin washout. Values are means ± S.E.M., *P<0.01 (paired t-test). Inhibition of oscillations was measured beginning 60 s after washout following an application of 10 μmol l^{-1} leucokinin lasting 25–140 s.

signaling pathway Dow et al., 1994a; Davies et al., 1995; Davies et al., 1997). At the concentration used in this study, CAP_{2b} has been reported to cause a rapid increase in intracellular calcium levels in the principal cells while having no effect on calcium levels in the stellate cells (Rosay et al., 1997). As shown in Fig. 9, the TEP oscillations are not altered either upon initial exposure to CAP_{2b}, at which time calcium levels should be increased in the principal cells, or after several minutes of hormone treatment, when the amplitude of the TEP has increased due to stimulation of the proton pump. Taken together, therefore, my data strongly suggest that intracellular calcium levels in the stellate cells play a central role in controlling the TEP oscillations.

Discussion

The TEP of the *Drosophila* Malpighian tubule oscillates, and these oscillations appear to result from rhythmic changes in the chloride conductance across the tubule. In this way, the *Drosophila* tubule behaves very similarly to the *Aedes* tubule. Using measurements of transepithelial resistance in the latter system, Beyenbach and coworkers concluded that the oscillating chloride conductance was located outside of the principal cells (Beyenbach et al., 2000), leaving either a paracellular pathway or a transcellular pathway through the stellate cells as the possible routes of chloride flux. The small size of the *Drosophila* tubule makes such resistance measurements unfeasible; however given the similarity in the oscillations between the two systems and the published evidence against chloride transport through the principal cells in *Drosophila* (O'Donnell et al., 1996), it seems likely that the oscillations described in this paper also result from a chloride conductance pathway that resides outside of the principal cells.

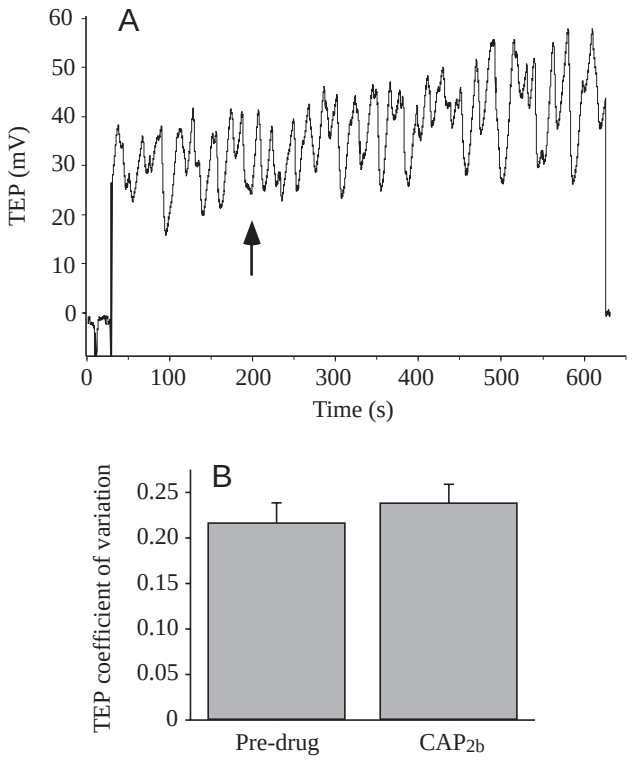

Fig. 9. CAP_{2b} does not alter the oscillations. (A) Trace from a tubule exposed to 100 nmol l^{-1} CAP_{2b} starting at the arrow. Note the lack of any immediate effect of the drug on the TEP. Similar results were seen in five additional tubules. (B) Graph showing no effect of CAP_{2b} on TEP variability. The TEP coefficient of variation was measured before drug application and during exposure to 100 nmol l^{-1} CAP_{2b} lasting 5.5–11 min. Values are means ± S.E.M., N=6 tubules, P>0.14 (paired t-test).

As mentioned in the Introduction, the location of the chloride-selective shunt pathway, transcellular *versus* paracellular, is a matter of some controversy. Evidence for a transcellular route in *Drosophila* includes vibrating probe measurements of current 'hot spots' above stellate cells, the identification of small patches of chloride channels in the luminal membrane of the tubule, and the extremely rapid increase in chloride conductance in response to leucokinin (O'Donnell et al., 1996; O'Donnell et al., 1998). The argument that *all* chloride flux in *Drosophila* is transcellular is based on the abolition of urine secretion by several different chloride channel blockers (O'Donnell et al., 1998). However, the electrophysiological data presented in this paper clearly indicate that, at the concentrations used in the previous study, these blockers cause a depolarization of the TEP, an action that is inconsistent with chloride channel blockade but that could account for the inhibition of urine secretion. It therefore remains possible that at least some of the chloride flux in the *Drosophila* tubule is paracellular.

Previous work in the leucokinin-stimulated *Drosophila* tubule has shown a link between intracellular calcium levels in the stellate cells and peritubular chloride conductance (O'Donnell et al., 1996; O'Donnell et al., 1998). Given this work and my demonstration that the fluctuations in chloride permeability underlying the TEP oscillations require an increase in intracellular calcium levels, the stellate cells would appear to be likely candidates for the site of such a calcium increase. The suppression of oscillations by leucokinin shown in this paper provides further evidence for the role of the stellate cells. If one assumes that the TEP oscillations result from oscillations in stellate cell calcium levels, the aforementioned suppression can be explained by a long-lasting desensitization of calcium release following the large leucokinin-induced increase in stellate cell calcium. Such a calcium-dependent desensitization has been reported in mammalian cells (Oancea and Meyer, 1996). Furthermore, the lack of any effect of CAP_{2b} on the oscillations shows that calcium levels in the principal cells do not acutely affect the TEP. The data presented here are most consistent with a central role for the stellate cells in the control of the TEP oscillations. This paper extends the link between stellate cells and chloride permeability, previously shown in leucokinin-treated tubules, to unstimulated tubules. Importantly, I do not claim to have demonstrated that the oscillations are the result of chloride passing through the stellate cells, only that they are regulated by the stellate cells.

Many questions remain unanswered regarding tubular chloride transport, most notably the relative contributions of transcellular and paracellular pathways, which, given the paucity of specific pharmacological tools, could prove difficult to determine. In addition, the description of oscillations in chloride transport in the tubules of multiple insect species begs the question of why such oscillations exist in the first place. It is possible that they could provide a mechanism for the regulation of urine secretion by some unknown environmental factor. Perhaps the molecular and genetic approaches available in *Drosophila* will prove fruitful in answering these questions.

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