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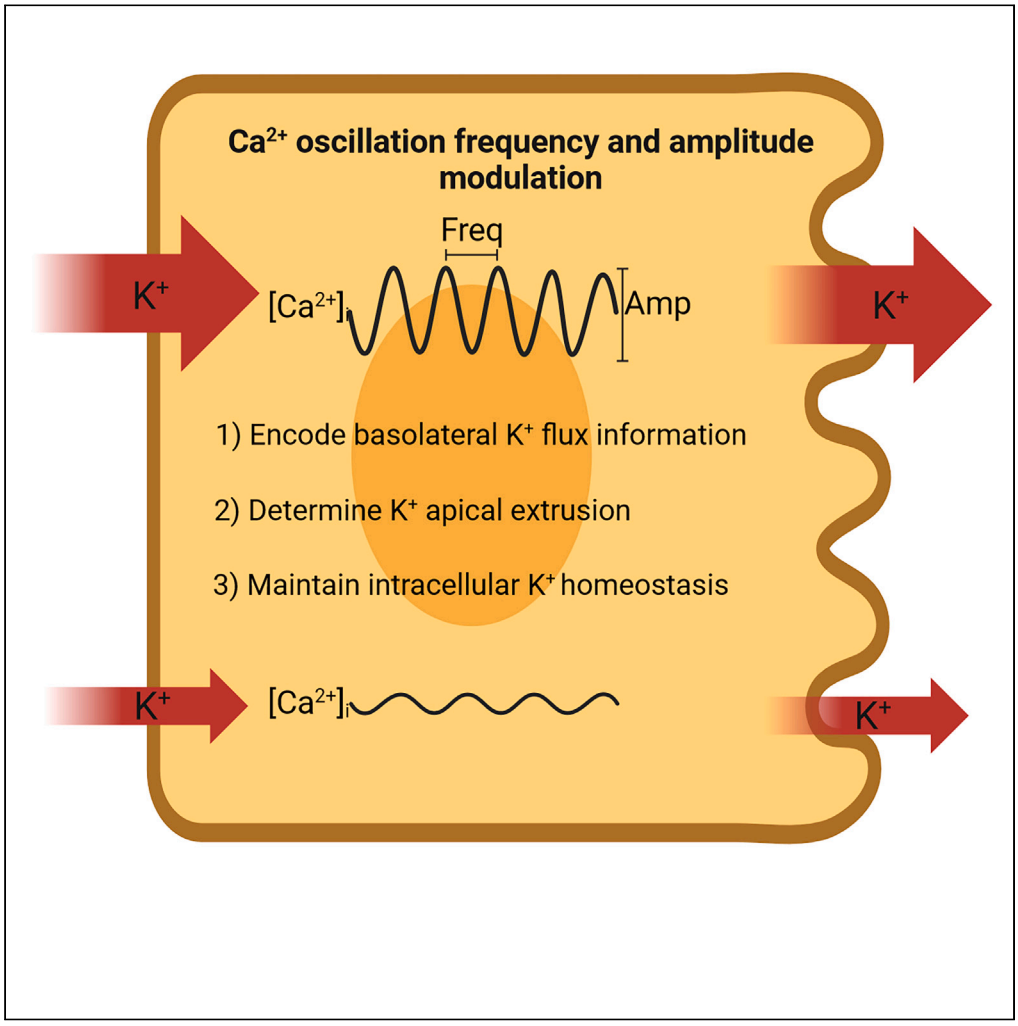

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Highlights

Amplitude and frequency of intracellular Ca^{2+} oscillations correlate with K^+ transport

Inhibiting Ca^{2+} blocks the ability of cells to maintain intracellular homeostasis

Experimentally generated intracellular Ca^{2+} oscillations control K^+ transport

Frequency and amplitude modulation encoded information for apical-basolateral crosstalk

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Intracellular Ca^{2+} oscillation frequency and amplitude modulation mediate epithelial apical and basolateral membranes crosstalk

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SUMMARY

Since the early seminal studies on epithelial solute transport, it has been understood that there must be crosstalk among different members of the transport machinery to coordinate their activity and, thus, generate localized electrochemical gradients that force solute flow in the required direction that would otherwise be thermodynamically unfavorable. However, mechanisms underlying intracellular crosstalk remain unclear. We present evidence that crosstalk between apical and basolateral membrane transporters is mediated by intracellular Ca^{2+} signaling in insect renal epithelia. Ion flux across the basolateral membrane is encoded in the intracellular Ca^{2+} oscillation frequency and amplitude modulation and that information is used by the apical membrane to adjust ion flux accordingly. Moreover, imposing experimentally generated intracellular Ca^{2+} oscillation modulation causes cells to predictably adjust their ion transport properties. Our results suggest that intracellular Ca^{2+} oscillation frequency and amplitude modulation encode information on transmembrane ion flux that is required for crosstalk.

INTRODUCTION

The laws of thermodynamics govern the direction and rate of solute flux across epithelial tissues. The fundamental function of transporting epithelia is to *generate* the electrochemical gradients that will force molecules to move in the required direction that would otherwise be thermodynamically unfavorable. Transporter proteins asymmetrically distributed in the apical and basolateral membranes of polarized epithelial cells achieve the required gradients; ATPases drive the primary active transport of solutes to generate electrochemical gradients that are exploited by channels, cotransporters, and exchangers on the apical and basolateral membranes to produce unidirectional movement of solutes that would otherwise be thermodynamically unfavorable.[1,2,3] This ability to circumvent the laws of thermodynamics through the coordinated action of apical and basolateral membranes is the *raison d'être* of transporting epithelia, in the same way as contraction and relaxation is for muscle cells.

Crosstalk is required to coordinate the activity of the ion transport machinery on the basolateral and apical membranes to ensure generation of the required electrochemical gradients[4–6] while also maintaining homeostasis of intracellular volume and composition.[2,3,7] However, the molecular mechanisms behind the crosstalk between apical and basolateral membranes are not well understood.

There is evidence suggesting that changes in the intracellular concentration of ions may be involved in the crosstalk mechanism,[8] including Cl^-,[9] Cl^- or Na^+,[10,11,12] or K^+ and pH.[13,14] The downstream molecular mechanisms of crosstalk are also unclear, but a promising hypothesis is that intracellular Ca^{2+} signaling may play a role in crosstalk by encoding ion transport information. This hypothesis is supported by some evidence that changes in intracellular Cl^- concentration caused by activation of apical Cl^- channels trigger intracellular Ca^{2+} signaling in rat salivary acinar cells that modulates the activity of the basolateral Na^+:K^+:2Cl^- cotransporter (NKCC), Na^+/H^+ exchangers (NHE) and cation channels.[9,15] Intracellular Ca^{2+} signaling may thus be a mechanism for crosstalk by coordinating the ion transport activities of the apical and basolateral membranes.[9,15]

We studied the contribution of intracellular Ca^{2+} signaling to crosstalk between apical and basolateral membranes using the Malpighian (renal) tubule of the blood-feeding insect *Rhodnius prolixus* as a model (Figures 1A–1C). *R. prolixus* Malpighian tubules are an ideal model for this study as crosstalk can be physiologically activated by simply altering bathing fluid K^+ concentration. Sudden isosmotic change in bath K^+ concentration from 8 to 14.5 mM K^+, an ~80% change in K^+, causes a nearly instantaneous 20% increase in K^+ transepithelial flux[16] without

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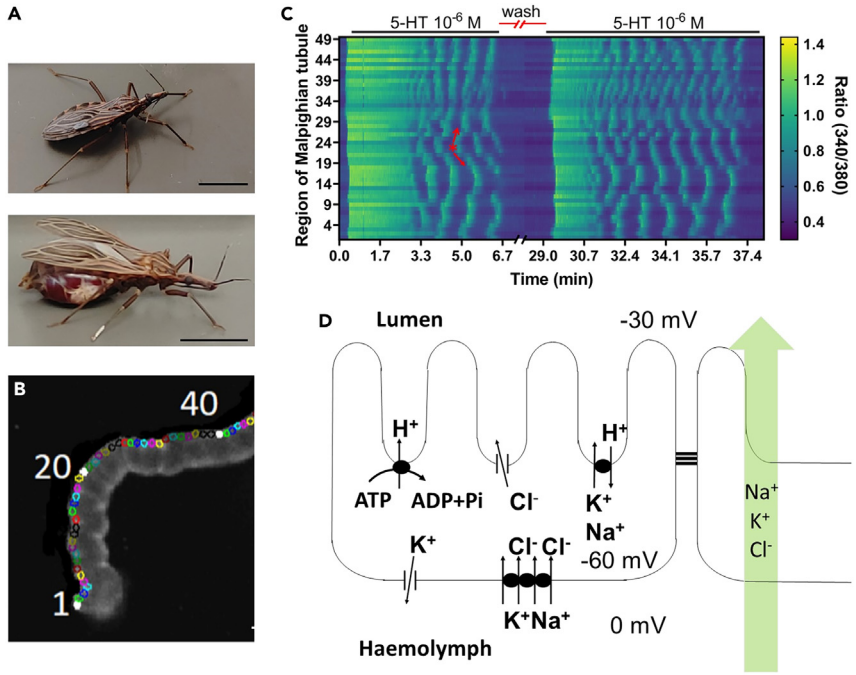

Figure 1. Intracellular Ca^{2+} signaling in Malpighian (renal) tubule cells of the blood-feeding insect *Rhodnius prolixus*

(A) *R. prolixus* ingest large blood meal volumes that can be up to 12 times the unfed (upper panel) body weight (lower panel shows a fed animal, scale bar = 1 cm). While the cellular component of the blood meal is stored in the midgut, the excess water and ions ingested are excreted through the Malpighian tubules. The post-prandial diuretic process is stimulated by the release of diuretic hormones, including serotonin.

(B) Intracellular Ca^{2+} in Malpighian tubules was measured in selected regions of identical size as indicated with circular symbols of different colors along the length of the entire tissue sample. The measuring regions were placed in such as way to avoid including the lumen of the tubule. The numbers 1, 20 and 40 indicate the measuring region from the distal to the proximal side.

(C) Heatmap of the serotonin-triggered intracellular Ca^{2+} oscillations measured along the length of the tubule (y axis) at the sites indicated by the multicolor circles in panel B over time (x axis). The intracellular Ca^{2+} oscillations are required to stimulate transcellular fluid secretion across the Malpighian tubule cells.[26] Oscillations initiate in a cell (red asterisk) and propagate along the length of the tubule (red arrows).

(D) The fluid secreted by the upper segment of Malpighian tubules of *R. prolixus* during diuresis consists of approximately 100 mM NaCl and 80 mM KCl (green arrow).[27] Secretion of ions and osmotically obliged water by the tubules is driven by an apical vacuolar-type H^+-ATPase that generates a H^+ gradient across the apical membrane that energizes the exchange of cytoplasmic K^+ and/or Na^+ for luminal H^+. Na^+, K^+ and Cl^- enter the cell from the serosal side through a basolateral Na^+-K^+-$2Cl^-$ cotransporter (NKCC1).[28] The contribution of paracellular pathways is negligible.

affecting the intracellular K^+ concentration or the prodigious transport rate, with each cell transporting the equivalent of their whole volume every 10–15 s and intracellular content of Na^+ and Cl^- every 5 s (Figure 1D).[17–19] Maintaining intracellular K^+ concentration and the transport rate in these conditions is only possible if there are effective crosstalk mechanisms that allow tubule cells to adjust apical K^+ extrusion to match the sudden changes in basolateral K^+ intake flux.[16] Another feature that makes *R. prolixus* Malpighian tubules an ideal model to study the intracellular mechanism of crosstalk between the two membranes is that ion transport occurs solely through transcellular pathways, thus precluding the contribution of paracellular transport[20] or paracellular electrical coupling.[21,22] Finally, the secretory segment of the Malpighian tubules of *R. prolixus* consists of only one cell type, arranged in a single layer epithelium without contractile cells or neuronal innervation, which simplifies experimentation and data interpretation.[23–25]

We use intracellular Ca^{2+} imaging, fluid secretion and K^+ flux assays, and intracellular Ca^{2+} clamping to study the role of intracellular Ca^{2+} oscillations in crosstalk. Our data suggest that transepithelial ion flux correlates with intracellular calcium oscillation frequency and amplitude. Moreover, Malpighian tubule epithelial cells are capable of decoding imposed experimental intracellular Ca^{2+} oscillations frequency modulation and alter ion transport in response. This is strong evidence that intracellular calcium oscillation frequency and amplitude encodes ion flux information required for crosstalk between the apical and basolateral membranes.

RESULTS

Intracellular Ca^{2+} oscillation frequency and amplitude correlate with basolateral K^+ flux

Crosstalk between the apical and basolateral membranes in Malpighian tubules of *R. prolixus* can be activated by isosmotically changing bathing fluid K^+ concentration (Table 1)[16]: reduced basolateral availability of K^+ triggers a spontaneous replacement of K^+ transport with

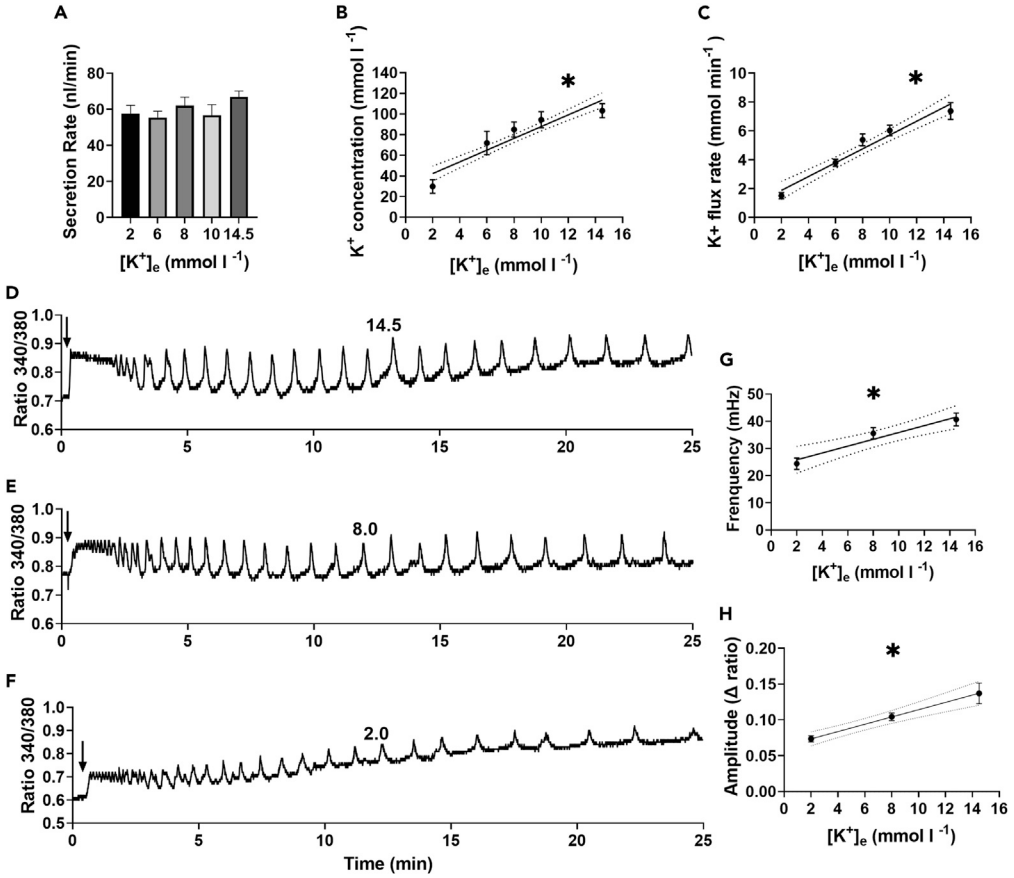

Figure 2. Intracellular Ca^{2+} oscillation frequency and amplitude are proportional to K^+ concentration in the bathing fluid

(A) After stimulation with serotonin the Malpighian tubules secreted fluid at maximum rate when bathed in saline containing 2, 6, 8, 10, and 14.5 Mm K^+ ($[K^+]_e$) while all other ions remain constant ($p = 0.42$, ANOVA $F(4, 32) = 1.003$). However, the secreted fluid (B) K^+ concentration ($p < 0.0001$, linear regression $F(1, 36) = 141.8$) and (C) transepithelial K^+ flux ($p < 0.0001$, linear regression $F(1, 29) = 128.1$) produced by the tubules directly correlated with K^+ concentration in the bathing fluid. Serotonin-stimulated (1 μM, downward arrow) intracellular Ca^{2+} signaling produced by tubules exposed to (D) 14.5, (E) 8, or (F) 2 mM K^+ displayed oscillation with a (G) frequency (first 5 min after serotonin stimulation, $p < 0.0004$, linear regression $F(1, 13) = 22.51$) and (H) amplitude ($p < 0.0001$, linear regression $F(1, 101) = 38.72$) that correlated with the K^+ concentration in the bathing fluid. Asterisks indicate statistical significance.

Na^+ while maintaining constant total transepithelial flux and without affecting intracellular K^+ concentration (Figures 2A–2C).[16] If intracellular Ca^{2+} oscillations encode transmembrane ion flux information required for crosstalk between apical and basolateral membranes, there should be a correlation between bathing fluid K^+ concentration and intracellular Ca^{2+} signaling, and our experiments show that the frequency and amplitude of intracellular Ca^{2+} oscillations in serotonin-stimulated Malpighian tubules correlate directly with bathing fluid K^+ concentration (Figures 2D–2H).

Furthermore, if intracellular Ca^{2+} oscillation encodes information on ion flux, then pharmacologically reducing basolateral ion entry should also result in a modulation of Ca^{2+} oscillation frequency or amplitude, in the same way that different bathing fluid K^+ concentrations do. Indeed, in our experiments, pharmacologically altering ion flux by treating the tubules with the Na^+:K^+:$2Cl^-$ cotransporter blocker bumetanide, which reduces the Malpighian tubule fluid secretion rate (Figure 3A), triggered concentration-dependent changes in intracellular Ca^{2+} oscillation frequency and amplitude (Figures 3B–3F); that is, intracellular Ca^{2+} oscillations correlate to pharmacologically induced changes in basolateral ion flux in a similar way to changes induced by altering K^+ concentration in the bathing saline. Together, these findings are consistent with the hypothesis that intracellular Ca^{2+} oscillation frequency and amplitude modulation encode ion flux information as part of the crosstalk machinery in serotonin-stimulated Malpighian tubules of *R. prolixus*.

Inhibition of intracellular Ca^{2+} oscillations block crosstalk

Crosstalk is hypothesized to ensure that basolateral and apical ion flux are similar, thus maintaining intracellular volume and ion concentration homeostasis as well as maximal transepithelial transport. Thus, if intracellular Ca^{2+} oscillations are part of this crosstalk mechanism, blocking Ca^{2+} oscillations should result in the loss of intracellular ion homeostasis and in an inability to maintain the secretion rate.

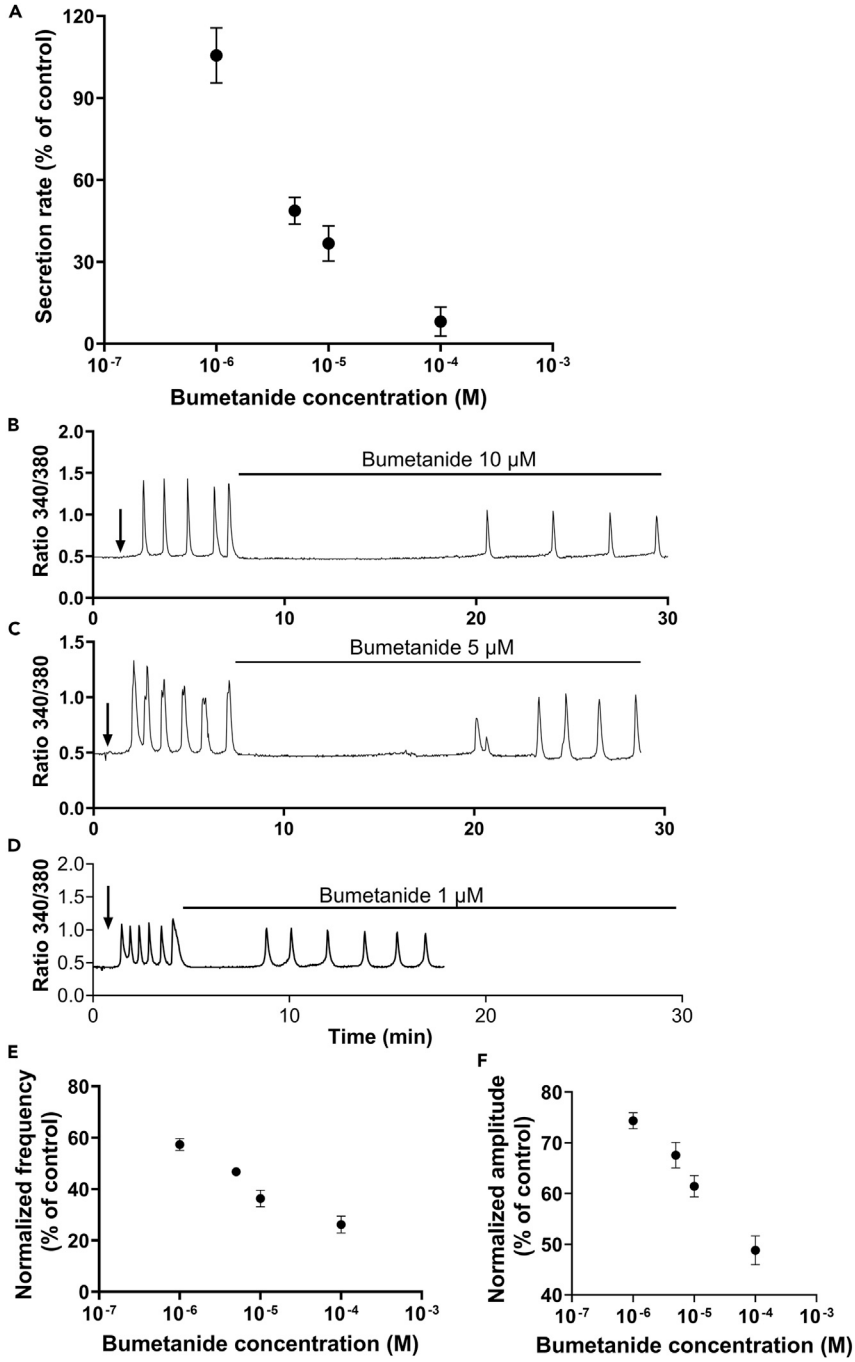

Figure 3. Intracellular Ca^{2+} oscillation frequency and amplitude are proportional to bumetanide-modulated transepithelial ion flux

(A) Na^+:K^+:$2Cl^-$ cotransporter blocker bumetanide reduces Malpighian tubule fluid secretion in a concentration-dependent manner. Sample trace of intracellular Ca^{2+} oscillation from serotonin-stimulated (1 µM, downward arrow) tubules exposed to (B) 10, (C) 5, and (D) 1 µM bumetanide. The intracellular Ca^{2+} oscillation (E) frequency and (F) amplitude.

To test this hypothesis, we altered basolateral K^+ transport by changing bathing fluid K^+ concentration from 14.5 mM to 2, 4, 6, or 8 mM. This treatment triggered changes in the amplitude and frequency of intracellular Ca^{2+} oscillation (Figure 4A) that correlated to the size of the K^+ concentration difference imposed on the tubule (Figures 4B and 4C), indicating that a reduction in bathing fluid K^+ concentration triggers modulation of intracellular Ca^{2+} oscillations, in line with the hypothesis that frequency and amplitude modulation encodes information as part of the crosstalk mechanism.

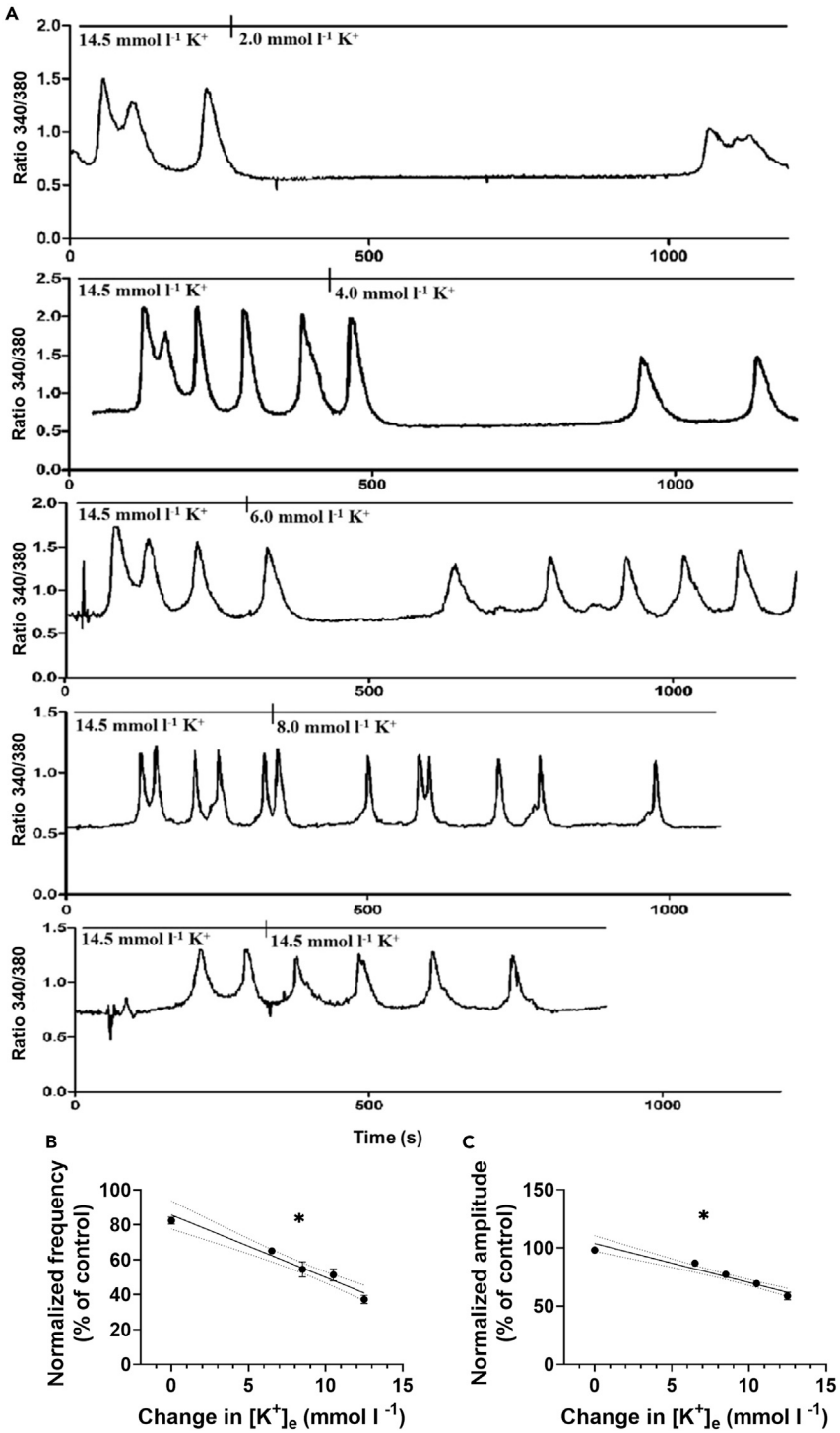

Figure 4. Instantaneous reduction in bathing fluid K^+ concentration ($[K^+]_e$) causes a reduction in intracellular Ca^{2+} oscillation frequency and amplitude

(A) Sample traces of intracellular Ca^{2+} oscillation by Malpighian tubules initially bathed in 14.5 mM K^+ and instantaneously switched to 2, 4, 6, 8, or 14.5 mM K^+.

(B) The intracellular Ca^{2+} oscillation frequency displayed by the Malpighian tubules directly correlates with magnitude of the change in $[K^+]_e$ ($p < 0.0001$, linear regression $F(1, 130) = 67.8$).

(C) The intracellular Ca^{2+} oscillation amplitude displayed by the Malpighian tubules correlates directly with magnitude of the change in $[K^+]_e$ ($p < 0.0001$, linear regression $F(1, 130) = 90.23$). Asterisks indicate statistical significance.

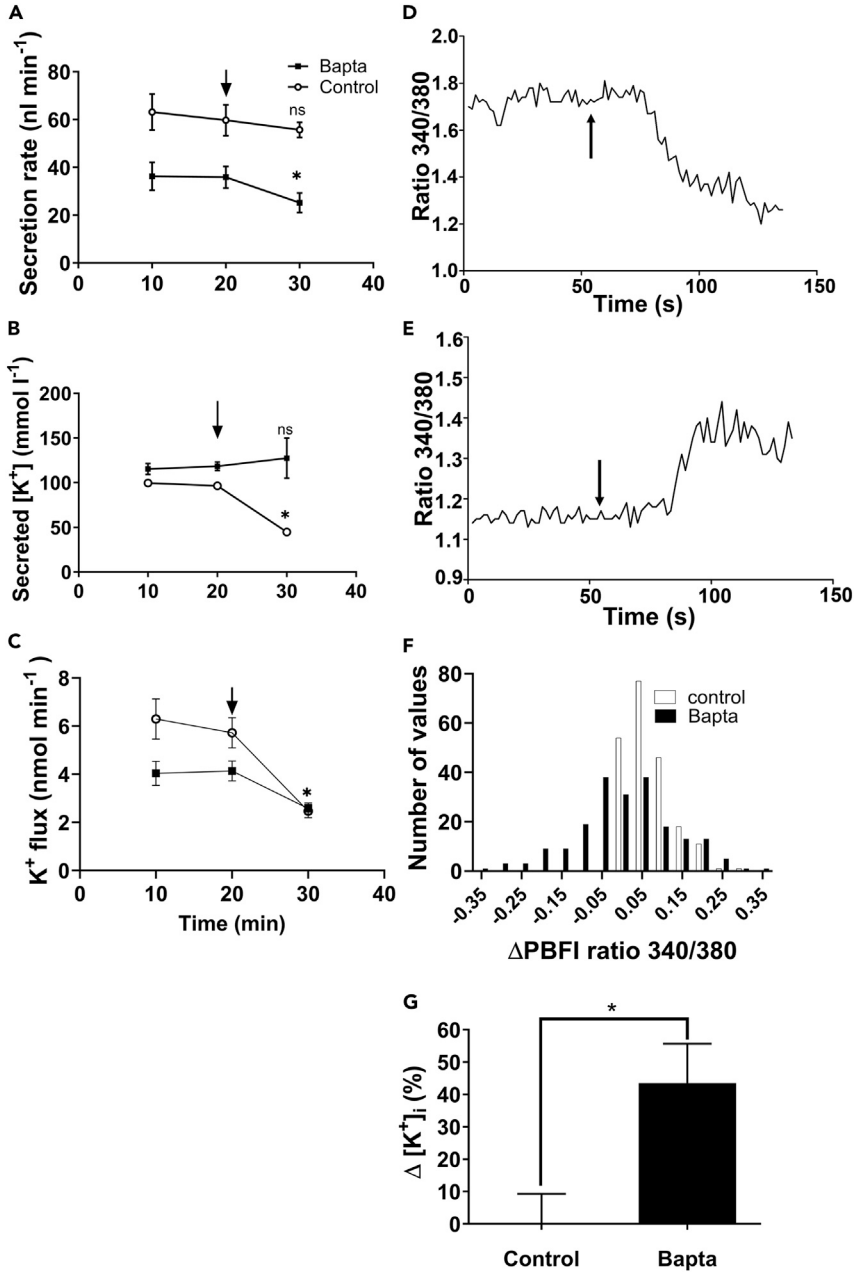

Figure 5. Blocking intracellular Ca^{2+} oscillation with BAPTA-AM inhibits cellular K^+ homeostasis

(A) Secreted fluid produced by Malpighian tubules exposed to an instantaneous change in bath K^+ concentration from 14.5 to 2 mM (downward arrow) in control and BAPTA-AM (300 μM) groups. BAPTA-treated tubules significantly reduced secretion rate after the reduction in bathing fluid K^+ concentration ($p = 0.019$, repeated measures two-way ANOVA, Tukey's multiple comparisons test, $n = 9$) while control preparations maintain constant secretion rate ($p = 0.64$, repeated measures two-way ANOVA, Tukey's multiple comparisons test, $n = 6$).

(B) Secreted fluid K^+ concentration decreased in control preparations ($p = 0.017$, repeated measures two-way ANOVA, Tukey's multiple comparisons test, $n = 7$) while K^+ concentration in secreted fluid remained constant in BAPTA-treated preparations ($p = 0.7$, repeated measures two-way ANOVA, Tukey's multiple comparisons test, $n = 10$).

(C) K^+ flux diminished in both control preparation ($p = 0.024$, repeated measures two-way ANOVA, Tukey's multiple comparisons test, $n = 7$) and BAPTA-treated preparations ($p = 0.034$, repeated measures two-way ANOVA, Tukey's multiple comparisons test, $n = 10$).

(D and E) Sample trace of PBFI-AM-measured intracellular K^+ concentration in the BAPTA-treated tubules before and after (downward arrow) switching bath K^+ from 14.5 to 2 mM. Approximately 50% of tubules show a (D) decrease or (E) increase in intracellular K^+.

Figure 5. *Continued*

(F) Histogram displaying the frequency distribution of changes in intracellular K^+ in response to the change in extracellular K^+. The frequency distribution in BAPTA-treated preparations is significantly different from the control group ($p < 0.0001$, Chi-square test for trend, Chi-square 37.68, df 1).

(G) Normalized change in intracellular K^+ concentration triggered by instantaneous reduction in bath K^+ concentration. There was a significant change in intracellular K^+ concentration in BAPTA-treated tubules but not in control tubules (Student's *t* test, $p = 0.0001$, $n = 7$). Asterisks indicate statistical significance and "ns" indicates no statistically significant difference.

Then, we tested the effect of blocking intracellular Ca^{2+} oscillations on the response of Malpighian tubule cells to changing bathing K^+ concentration from 14.5 to 2 mM. Malpighian tubules exposed to sudden changes in bath K^+ concentration maintained a constant fluid secretion rate (Figure 5A open symbols). Simultaneously, the tubules reduced K^+ concentration in the secreted fluid (Figure 5B) and reduced transepithelial K^+ flux (Figure 5C), likely compensated by an increase in Na^+ flux.[16] In contrast, Malpighian tubules treated with the intracellular Ca^{2+} chelator BAPTA-AM (30 μM), which blocks intracellular Ca^{2+} oscillations,[26] failed to respond effectively to a decrease in the bathing medium K^+ concentration. The secretion rate in the BAPTA-AM-treated Malpighian tubules decreased significantly (Figure 5A), and the tubules failed to reduce K^+ concentration in the secreted fluid; instead, the concentration remained constant (Figure 5B). Interestingly, the transepithelial K^+ flux (Figure 5C) fell to values similar to those displayed by control tubules, but control tubules achieved this K^+ flux reduction while maintaining maximal fluid transport, while BAPTA-AM-treated preparations suffered a drop in fluid transport. These results suggest that blocking intracellular Ca^{2+} oscillations inhibit the ability of Malpighian tubules to modulate cation transport in response to changes in basolateral K^+ influx.

To further test whether intracellular Ca^{2+} oscillations contribute to crosstalk between apical and basolateral membranes, we tested whether blocking intracellular Ca^{2+} signaling interferes with the ability of Malpighian tubule cells to maintain intracellular K^+ concentration homeostasis during sudden changes in bath K^+ concentration. If intracellular Ca^{2+} signaling is involved in crosstalk, blocking Ca^{2+} signaling with the chelator BAPTA-AM should reduce cellular ability to maintain intracellular K^+ concentration homeostasis, as basolateral K^+ uptake and apical extrusion would no longer be coordinated. Using the K^+-sensitive fluorescence dye PBFI-AM, we measured changes in intracellular K^+ in response to sudden decreases in bath K^+ concentration from 14.5 to 2 mM in tubules incubated with BAPTA-AM and in control preparations. We expected to observe reductions in intracellular K^+ in response to reduced basolateral entry (Figure 5D), but instead we found that approximately 50% of both control and BAPTA-AM-treated preparations responded with an increase in intracellular K^+ concentration (Figures 5E and 5F), though the fluctuation in intracellular K^+ concentrations was larger in BAPTA-AM-treated tubules than in control preparations (Figure 5G). That is, the control preparations, not treated with Ca^{2+} chelator and thus with the capacity for intracellular Ca^{2+} signaling intact, were better able to maintain intracellular K^+ concentration at the homeostatic set point than the BAPTA-AM-treated tubules. These findings are consistent with the hypothesis that intracellular Ca^{2+} signaling is a component of the basolateral and apical membrane crosstalk mechanism that maintains both the secretion rate and intracellular ion homeostasis during rapid ion transport in Malpighian tubules.

Experimentally generated intracellular Ca^{2+} oscillations modulate K^+ transport rate

The ultimate experiment to test whether frequency modulation encodes information that the cell can decode to modulate K^+ flux is to generate artificial oscillations where we can control its frequency. If frequency modulation is the "language" the cell uses to transmit information on K^+ transport, we should be able to "talk" to the cells and force them to change K^+ transport by experimentally modulating Ca^{2+} oscillation frequency. Intracellular Ca^{2+} oscillations can be experimentally generated by "clamping" intracellular Ca^{2+} to produce artificial Ca^{2+} oscillations with variable frequencies.[29,30] We optimized intracellular Ca^{2+} clamping methods to modulate the frequency within the range of natural oscillations, i.e., 2.8 mHz, 3.3 mHz, 4.2 mHz, or 5.6 mHz (Figures 6A and 6B).[29,30] Malpighian tubules subjected to Ca^{2+} clamping showed fluid secretion rates (Figure 6) similar to those of control tubules (Figure 2), and there was no difference in the secretion rate among tubules exposed to different oscillation frequencies (Figure 6C), indicating that Ca^{2+} clamped-tubules were healthy and physiologically viable.

Our results show that the transepithelial K^+ flux triggered by the Malpighian tubules depended on the experimentally imposed Ca^{2+} oscillation frequency. In fact, there was a direct correlation between experimentally generated oscillation frequency and the K^+ concentration in the secreted fluid (Figure 6D). That is, tubules exposed to identical bathing K^+ concentration and serotonin stimulation transport K^+ at different rates based on the intracellular Ca^{2+} oscillation frequency.

Having established the effect of different Ca^{2+} oscillation frequencies on K^+ flux, we also tested the effect of imposing changes to the frequency of the experimentally generated intracellular Ca^{2+} oscillations on K^+ flux. Changing the oscillation frequency in a Malpighian tubule from 5.6 mHz to 2.8 mHz triggered large changes in K^+ concentration in the secreted fluid compared to tubules maintained at a constant frequency (e.g., 5.6 mHz–5.6 mHz) (Figures 6E and 6F). Interestingly, approximately 50% of the tubules responded with an increase in secreted fluid K^+ concentration, while the rest responded with a decrease (Figure 6E). These results suggest that the Malpighian tubule cells can decode information from the imposed experimental intracellular Ca^{2+} oscillation frequency and then "decide" on the appropriate K^+ transport rate.

Taken together, our results are consistent with the hypothesis that Ca^{2+} oscillation encodes information on ion flux that is used by the cell to determine its transport properties as part of the crosstalk process.

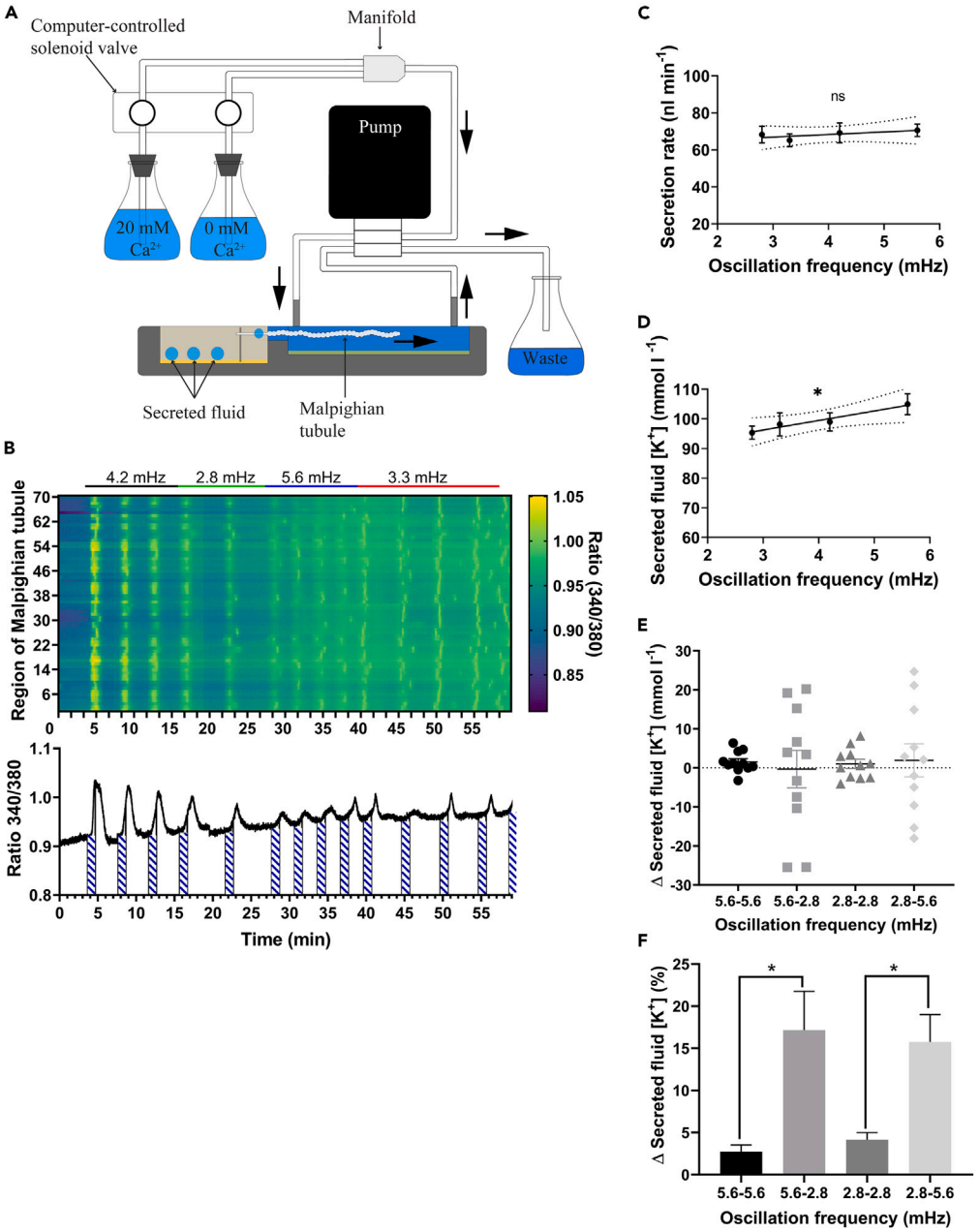

Figure 6. Experimentally generated intracellular Ca^{2+} oscillation modulate transepithelial K^+ flux

(A) Diagram of the calcium clamping apparatus. Flow of Ringer's solution containing 20 and 0 mM Ca^{2+} is controlled with computer-controlled solenoid valves and driven by a peristaltic pump. The Malpighian tubule is placed in a custom-built chamber consisting of a bathing section that allows for superfusion with Ringer's solution (right side) and a secretion-collecting section (left side, arrows indicate direction of fluid flow). The fluid secreted by the Malpighian tubules is collected at the open end of the tubule under paraffin oil and the volume is calculated. The secreted droplets are collected to measure K^+ concentration.

(B) Heatmap of experimentally triggered intracellular Ca^{2+} oscillations measured along the length of the tubule (x axis) over time (y axis) with the frequency indicated in mHz at the top. The lower panel shows that the increase in intracellular Ca^{2+} coincides with the timing of superfusion with Ringer's solution containing 20 mM K^+ (dashed bars).

(C) Calcium-clamped Malpighian tubules (stimulated serotonin 1 μM) secrete fluid at high rates that are similar to control preparations. The rate of transport is independent of the experimentally generated intracellular Ca^{2+} oscillation frequency ($p = 0.67$, linear regression $F(1, 39) = 0.1827$).

(D) The K^+ concentration, and thus K^+ flux, is directly proportional to the experimentally generated intracellular Ca^{2+} oscillation frequency ($p = 0.036$, linear regression $F(1, 42) = 4.679$).

Figure 6. *Continued*

(E) Effects on the K^+ concentration in the secreted fluid of switching the frequency of the experimentally generated intracellular Ca^{2+} oscillation from 5.6 to 2.8 mHz or from 2.8 to 5.6 mHz (n = 11).

(F) Percentage absolute change of K^+ concentration in the secreted fluid triggered by switching the frequency of the experimentally generated intracellular Ca^{2+} oscillation from 5.6 to 2.8 mHz (p = 0.0017, ANOVA, Sidak's multiple comparisons test, n = 11) or from 2.8 to 5.6 mHz (p = 0.012, ANOVA, Sidak's multiple comparisons test, n = 11). Asterisks indicate statistical significance and "ns" indicates no statistically significant difference.

DISCUSSION

Our results are consistent with the hypothesis that modulation of the frequency and amplitude of intracellular Ca^{2+} oscillations encodes information about ion flux and plays a role in the crosstalk between the apical and basolateral membranes during fluid secretion.

We found a direct correlation between the rate of transepithelial K^+ flux and the frequency and amplitude of intracellular Ca^{2+} oscillations, whether the flux rate was modified by altering bath K^+ concentration or by inhibiting the basolateral Na^+:K^+:2Cl^- cotransporter with bumetanide (Figures 2 and 3). Moreover, changing the bath K^+ concentration triggered a modulation in oscillation frequency and amplitude that correlated directly with the K^+ flux change (Figure 4). These results provide compelling evidence that Malpighian tubule cells from *R. prolixus* can detect the rate of basolateral K^+ entry and modulate the frequency and amplitude of intracellular Ca^{2+} oscillations accordingly. A similar role for intracellular Ca^{2+} oscillation has been observed in hepatocytes, where the intensity of stimulation with glucogenic hormones is encoded by frequency modulation of intracellular Ca^{2+} oscillation and intercellular calcium waves integrate hormonal control of glucose output by the liver.[31–36]

Sudden isotonic reduction in K^+ concentration in the bathing fluid triggers a reduction in K^+ transport (Figure 5) while maintaining the fluid secretion rate (Figure 5A) and intracellular K^+ concentration homeostasis (Figure 5F). These results are consistent with our previous report showing that the Malpighian tubule cells undergo a minimal (~2%) change in intracellular K^+ concentration in response to an 80% reduction in basolateral K^+ concentration (from 8 to 14.5 mM).[16] Maintaining this intracellular homeostasis is only possible if basolateral and apical ion transport is coordinated in such a way that reductions in basolateral K^+ entry triggers an equivalent reduction in K^+ apical extrusion. We found that blocking intracellular Ca^{2+} oscillation with BAPTA-AM inhibits these homeostatic mechanisms, causing a severe reduction in transepithelial fluid transport and the loss of intracellular K^+ concentration homeostasis, as expected when apical and basolateral transports are decoupled due to the loss of crosstalk (Figure 5). The frequency and amplitude of Ca^{2+} oscillation thus appears to encode information about ion flux that is important for basolateral and apical crosstalk.

Finally, our results from using experimentally generated intracellular Ca^{2+} oscillations also support the hypothesis that Ca^{2+} frequency modulation encodes information about ion flux needed for basolateral and apical crosstalk. Malpighian tubule cells under identical conditions altered the K^+ transport rate and concentration in the secreted fluid in direct correlation to the frequency of the experimentally generated Ca^{2+} oscillations (Figure 6). These Ca^{2+}-clamping tests have never been done before in epithelial cells and provide the most compelling evidence to date of the role of Ca^{2+} signaling in basolateral and apical crosstalk in epithelial tissues.

We demonstrate that tubules under Ca^{2+}-clamping conditions (Figure 6) secrete fluid at rates comparable to control tubules during the entirety of the experimental time (30 min), indicating that they are fully functional and healthy. However, natural, i.e., not experimentally produced, Ca^{2+} oscillation seemed to simultaneously modulate both frequency and amplitude in response to changes in K^+ flux. Unfortunately, we were not able to produce amplitude modulation in our experimentally imposed Ca^{2+} signals, thus we could not test the effect of amplitude modulation, but the effect of imposed experimental Ca^{2+} oscillations on K^+ flux may have been even larger if we could have simultaneously controlled both frequency and amplitude of the signal.

Table 1. List of Ringer's solutions and their composition (mmol L^{-1})

	Control 14.5 K^+	8.0 K^+	6.0 K^+	4.0 K^+	2.0 K^+
NaCl	122.6	122.6	122.6	122.6	122.6
KCl	14.5	8	6	4	2
$CaCl_2$	2	2	2	2	2
$MgCl_2$	8.5	8.5	8.5	8.5	8.5
Glucose	20	20	20	20	20
$NaHCO_3$	10.2	10.2	10.2	10.2	10.2
NaH_2PO_4	4.3	4.3	4.3	4.3	4.3
HEPES	8.6	8.6	8.6	8.6	8.6
NMDG[a]	–	6.7	8.7	10.7	12.7

[a]NMDG: *N*-methyl-D-glucamine.

These innovative experiments have produced the most compelling evidence to date that suggest a likely mechanism for crosstalk between basolateral and apical membranes: information about basolateral ion transport flux is encoded in the amplitude and frequency modulation of intracellular Ca^{2+} oscillations, and these oscillations communicate information about transport flux to the apical membrane. The molecular mechanisms behind the detection of membrane ion transport rate are not clear, however an exciting possibility is that Na^+/K^+-ATPase may act as a signaling transducer.[37–39] The intracellular Ca^{2+} oscillation frequency or amplitude modulation signal would then be decoded by frequency-decoding Ca^{2+}-binding protein(s) that would activate kinases and phosphatases, modulating the activity of apical ion transporters.[36,38] In *Drosophila melanogaster* Malpighian tubules there is evidence that ion transport may be modulated by a calcium binding protein analogous to calcium binding protein 39 (Cab39, also called Mo25), which controls the activity of With No Lysine (WNK) kinases to regulate apical and basolateral ion transporters.[40–42]

Similar intracellular Ca^{2+} oscillations have been described in several epithelial tissues, including *D. melanogaster* Malpighian tubules,[43] gastric parietal cells,[44,45] mammary epithelial cells,[46] lacrimal acinar cells,[46–48] and parotid acinar cells.[46,49–52] Thus, it is possible that the Ca^{2+} oscillation frequency and amplitude modulation contribution to crosstalk may be a mechanism common to other epithelial tissues in vertebrate and invertebrates.

Limitations of the study

Our results do not allow us to determine if intracellular Ca^{2+} frequency and amplitude modulation play a role in crosstalk in vertebrate or mammalian epithelial cells. It is possible that the mechanisms of crosstalk we describe in *R. prolixus* Malpighian tubules may be a singular adaptation of this species to its specific ecological niche. Further research is required to determine the role of Ca^{2+} in crosstalk in vertebrates, mammals, and human epithelial tissues.

STAR★METHODS

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AUTHOR CONTRIBUTIONS

N.H., B.G.M., S.J., and R.T.: developing experimental techniques, conducting experiments, acquiring data, analyzing data, and writing the manuscript. G.S.K. and J.P.I.: design of research studies, design and development of experimental techniques, interpretation of the data, and writing the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Fura2-AM	Life Technologies	F1221
Probenecid	Sigma	P8761-100G
Pluronic F-127	Sigma	P2443-250G
PBFI-AM	Life Technologies	P1267MP
Potassium ionophore I, cocktail B	Sigma	99373-0.1ML-F
Thapsigargin	Sigma	T9033-5MG
BAPTA-AM	Sigma	196419-25MG
N-Methyl-D-glucamine (NMDG)	Sigma	M2004-500G
Experimental models: Organisms/strains		
Rhodnius prolixus (Stål)	In-house colony	N/A
Software and algorithms		
Prism 10	GraphPad	https://www.graphpad.com/
BioRender	BioRender.com	https://www.biorender.com/

RESOURCE AVAILABILITY

Lead contacts

Further information and requests for resources should be directed to and will be fulfilled by the lead contacts, Juan P. Ianowski (juan.ianowski@usask.ca) or George S. Katselis (george.katselis@usask.ca).

Materials availability

This study did not generate new materials.

Data and code availability

(1) Data: All data reported in this paper will be shared by the lead contact upon request.
(2) Code: This paper does not report original code.
(3) Other items: No other new unique reagent was generated. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animal and tissue dissection

Rhodnius prolixus were obtained from a colony maintained at 25-26°C with 60% relative humidity located at the Department of Anatomy Physiology and Pharmacology, College of Medicine, University of Saskatchewan. All experiments were carried out at room temperature (20-23°C) with female and male 3rd and 5th nymphal stages, 3–4 weeks after molting. The fluid secreting distal segment of the Malpighian tubules, which comprises the two-thirds most distal segment of the tubule, was dissected from the animals under control saline solution (Table 1) with the aid of a dissecting microscope. The distal tubules are one cell thick and are made up of only 2 to 3 cells that encircle the lumen.[23,25] All experiments conform to the relevant regulatory standards.

METHOD DETAILS

Calcium imaging

An isolated upper Malpighian tubule from a 3rd instar insect was attached to the bottom of a custom-built superfusion chamber pre-coated with poly-L-lysine to facilitate adherence of the tubules under saline. The tubules were submerged in control saline solution (Table 1) containing 5 μmol L^{-1} Fura2-AM dye (Life Technologies, Burlington, Ontario, Canada) for 60 min with 1 mmol L^{-1} probenecid (Sigma, St. Louis, MO,

USA) and 0.1% pluronic F-127 (Sigma, St. Louis, MO, USA) to facilitate dye loading.[26] After incubation, the Fura2-AM solution was washed out with control saline solution containing probenecid (1 mmol L^{-1}). Intracellular Ca^{2+} was visualized with an upright fluorescent microscope (BX61WI, Olympus, Tokyo, Japan) using a 20× water dipping objective with a field of view of 800× 700 μm (UMPLFLN 20×W, Olympus, Tokyo, Japan). The light source for excitation was an arc lamp (Prior Lumen 200PRO, Prior Scientific, Cambridge, UK) filtered with 340 nm or 380 nm excitation filters (BrightLine, Semrock, Rochester, NY, USA). Fluorescent light filtered with a 510 nm emission filter (BrightLine, Semrock, Rochester, NY, USA) was collected every 0.5 s with an EMCCD camera (QImaging Rolera EM-C2, QImaging designs, Surrey, BC, Canada). The 340 nm/380 nm ratio was calculated using Metafluor version 7.0 software (Molecular Devices, Burlington, ON, Canada) (Figure 1C).

Intracellular Ca^{2+} oscillation amplitude was calculated from the baseline to the peak of each oscillation, and frequency was measured from peak to peak (Figure 1C). Normalized values as percentage of control amplitude or frequency were calculated as:

$$Normalized\ amplitude\ or\ frequency\ (\%\ of\ Control) = \left(\frac{Treatment}{Control}\right) \times 100$$

Each tubule was its own control, where the Ca^{2+} oscillation before treatment (i.e., adding bumetanide in Figure 3 and changing K^+ concentration in Figure 4) was the control condition.

Potassium imaging

Changes in intracellular K^+ were studied using the K^+-sensitive fluorescence dye, potassium-binding benzofuran isophthalate (PBFI-AM, Life Technologies, Burlington, Ontario, Canada).[53] Tubules were prepared as described above for Ca^{2+} imaging but were incubated instead with 5 μmol L^{-1} PBFI-AM, as well as 1 mmol L^{-1} probenecid and 0.1% pluronic F-127 for 60 min. Then the tubules were washed and submerged in saline solution containing 1 mmol L^{-1} probenecid. The preparation was analyzed under a 20× water dipping objective with the fluorescent microscope. The light source for excitation was an arc lamp (Prior Lumen 200PRO, Prior Scientific, Cambridge, UK) filtered with 340 nm or 380 nm excitation filters (BrightLine, Semrock, Rochester, NY, USA). Fluorescent light filtered with a 510 nm emission filter (BrightLine, Semrock, Rochester, NY, USA) was collected every 0.5 s with an EMCCD camera (QImaging Rolera EM-C2, QImaging designs, Surrey, BC, Canada). The 340 nm/380 nm ratio was calculated using Metafluor version 7.0 software (Molecular Devices, Burlington, ON, Canada). We studied the ability of the Malpighian tubules to maintain intracellular K^+ concentration homeostasis after experimentally changing bathing solution K^+ concentration from 14.5 to 2 mmol L^{-1}. Preliminary measurements using PBFI-AM showed that Malpighian tubules can maintain intracellular K^+ concentration within a range around the initial homeostatic set point value, with some cells displaying an increase in intracellular K^+ concentration and others a decrease. Hence, we assessed how effectively the cells regulate intracellular K^+ homeostasis around the initial set point by analyzing the size of the changes in K^+ concentration, regardless of whether it was an increase or decrease. We report the module of the value of intracellular K^+ fluctuation:

$$\sqrt{\left(\frac{340}{380} ratio\right)^2}$$

We then calculated the percent change of the square root of intracellular PBFI ratio for each cell and normalized to control using the formula:

$$\Delta PBFI\ ratio(\%) = \left(\frac{\left(\sqrt{Ratio^2} - \sqrt{Ratio^2}\ Control\ Average\right)}{\sqrt{Ratio^2}\ Control\ Average}\right) x\ 100$$

Secretion assays

Malpighian tubules were isolated from 5th instar *R. prolixus* and placed in 100 μL droplets of control saline solution under paraffin oil in a Petri dish lined with sylgard (Sigma, Oakville, ON, Canada).[54,55] The proximal (cut) end of the tubule was pulled out of the saline droplet and wrapped around a fine steel pin placed in sylgard base. The distal part of the tubule in the control saline droplet was stimulated with 1 μmol L^{-1} serotonin. The secreted fluid was collected every 10 min for 40–60 min. The secreted droplets were photographed using a microscope camera for analysis (MiniVid, LW Scientific, Lawrenceville, GA, USA). To calculate the secretion rate, the diameter (d) of the spherical droplets under oil was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and volume (V) was calculated using the sphere volume equation[54,55]:

$$V = \frac{4}{3}\pi r^3$$

Droplet volume was then divided by the total time of collection (10 min) to determine the secretion rate.

Potassium concentration measurement in secreted fluid

K^+ concentration was measured in the fluid secreted by the Malpighian tubules using ion-selective electrodes.[55] Non-filamented borosilicate glass capillaries (World Precision Instruments, Sarasota, FL, USA) were pulled using a vertical micropipette puller (PE-2, Narishige, Japan). To

ensure retention of the hydrophobic ionophore cocktail the electrodes were silanized with dichlorodimethylsilane (Sigma, Oakville, ON, Canada) and baked on a hot plate for at least 1 h at 250°C. K^+ selective electrodes were based on potassium ionophore I, cocktail B (Sigma, Oakville, ON, Canada), and backfilled with 1 mol L^{-1} potassium chloride (KCl). The reference electrode was filled at the tip with 1 mol L^{-1} Na^+ acetate and the rest with 1 mol L^{-1} KCl. The K^+-selective electrode was calibrated in solutions of 15 KCl:135 mmol L^{-1} NaCl', and 150 mmol L^{-1} KCl. Only electrodes that displayed a change of $\geq$50 mV per 10-fold change in K^+ concentration of the calibration solution were used. The electrodes were connected to an amplifier (FD223A, World Precision Instrument, Sarasota, FL, USA) and data acquisition system (PowerLab, AD Instruments, Colorado Springs, CO, USA). All measurements were performed in a Faraday cage. Ion concentration in fluid droplets was calculated using the formula:

$$C^d = C^c \times 10^{\Delta V/s}$$

where C^d is the ion concentration in the secreted droplet, C^c is the ion concentration in one of the calibrating solutions, ΔV is the difference in voltage measured between the secreted droplet and the same calibrating solution, and s is the slope of the electrode (i.e., the V measured in response to a 10-fold change in ion activity during calibration).

Calcium clamping

Intracellular calcium clamping allows intracellular Ca^{2+} concentration to be controlled. Calcium clamping is achieved by pharmacologically opening the store-operated Ca^{2+} channels in the plasma membrane (CRAC), thus gaining the ability to control intracellular Ca^{2+} concentration.[29,30,56] Treating the Malpighian tubule cells with the inhibitor of endoplasmic reticulum Ca^{2+}-ATPases thapsigargin irreversibly depletes intracellular Ca^{2+} stores and activates store-operated Ca^{2+} channels (CRAC) on the plasma membrane.[29,30,56] Intracellular Ca^{2+} can then be controlled by changing the bathing solution Ca^{2+} concentration and, thus, generating artificial intracellular Ca^{2+} oscillations.[29,30] Increasing extracellular Ca^{2+} elevates intracellular Ca^{2+} due to influx through CRAC channels, while removing extracellular Ca^{2+} allows intracellular Ca^{2+} to return intracellular concentration to baseline levels.[29,30] It is therefore possible to experimentally generate intracellular Ca^{2+} oscillations by rapidly changing the concentration of extracellular Ca^{2+} concentration.[29,30]

Malpighian tubules were treated with 1 µmol L^{-1} thapsigargin at room temperature for 10 min and placed in the superfusing section of a custom-built chamber (Figure 6A). The cut end of the tubule was passed through an opening to a separate chamber filled with paraffin oil and it was wrapped around a pin to anchor it (Figure 6A). We used computer-controlled solenoid valves (VC-66CS, Harvard Apparatus Canada, Saint-Laurent, QC, Canada) to rapidly switch the superfusing bathing solutions from saline containing either 20 mM or 0 mM Ca^{2+}. The solution was superfused using a peristaltic pump at 5 mL min^{-1} (P-70, Harvard Apparatus Canada, Saint-Laurent, QC, Canada), sufficient to exchange the volume of the experimental chamber in 12 s (Figure 6A).

The Malpighian tubule was then superfused with solution containing 0 mM Ca^{2+} (in $mmol l^{-1}$): 108.9 NaCl, 6 KCl, 8.5 $MgCl_2$, 0 $CaCl_2$, 10.2 $NaHCO_3$, 8.6 HEPES, 20 glucose, and 28.5 NMDG; pH 7. Or 20 mM Ca^{2+} (in $mmol l^{-1}$): 108.9 NaCl, 6 KCl, 8.5 $MgCl_2$, 20 $CaCl_2$, 10.2 $NaHCO_3$, 8.6 HEPES, 20 glucose, and 8.5 NMDG; pH 7 equilibrated with air.

Drugs

All chemicals were purchased from Sigma (Oakville, ON, Canada) except for bumetanide and BAPTA-AM (Tocris, Bioscience, Bristol, UK). Drugs were dissolved in control Ringer's solution (Table 1) or DMSO (max DMSO concentration <0.1%).

QUANTIFICATION AND STATISTICAL ANALYSIS

Results are presented as mean ± SEM and analyzed using Student's *t* test or ANOVA using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).