The Effects of Small Molecule VU573 and Barium on Malpighian Tubule Electrophysiology of the Yellow Fever Mosquito *Aedes aegypti*

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Anthony John Longo

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Supervisor: Dr. Klaus W. Beyenbach

ABSTRACT

VU573 is a synthetic organic molecule weighing 350 daltons, found to block inwardly-rectifying potassium (K_{ir}) channels in high-throughput screening. In order to evaluate VU573 as a novel pesticide capable of inducing mosquito renal failure, electrophysiological analyses were carried out on isolated Malpighian tubules from the female yellow fever mosquito *Aedes aegypti*. Using the Malpighian tubule electrophysiology protocol developed in the laboratory of Klaus W. Beyenbach, exposure to 10µM peritubular VU573 was found to (1) hyperpolarize the basolateral membrane of isolated tubules by an average of 6.76 ± 1.27 mV ($p<0.00012$) and (2) increase cell input resistance by an average of 7.02 ± 1.31 kΩ ($p<0.002$) in conditions of potassium overload (34mM [K^+] peritubular). These results confirm a block of *A. aegypti* $K_{ir}1$ (*Ae*$K_{ir}1$) expressed at the basolateral surface of stellate cells, which seem to be less involved in transport than their counterparts—the principal cells. Additional experiments establish that the 10µM VU573 dosage is equally as effective as the 50µM dosage ($p=0.88$). Furthermore, I-V plots taken of Malpighian tubules demonstrate that different tubules exhibit different degrees of rectification, from no rectification (Ohmic) to very strong rectification.

The barium ion is known to strongly block all K_{ir} channels. With the same protocol used for VU573, 5mM barium was tested and found to (1) hyperpolarize the tubule basolateral membrane by 11.91 ± 3.82 mV ($p<0.008$) and (2) increase cell input resistance by 123.58 ± 12.93 kΩ ($p<0.0003$). The much smaller resistance increase with VU573 compared to barium, coupled with the laboratory of Peter M. Piermarini's finding of *Ae*$K_{ir}1$ on stellate cells, suggests that *Ae*$K_{ir}1$ channels are less involved with potassium transport and likely serve other functions, such as potassium sensing or cellular housekeeping. Taken in all, VU573 is effective at blocking *Ae*$K_{ir}1$ but would need to be refined further to have an even more potent antidiuretic effect.

Keywords: *Aedes aegypti*, Malpighian tubules, VU573, electrophysiology, *Ae*K_{ir}1, barium, potassium, tropical disease, pesticide

INTRODUCTION

Malpighian tubules, the kidney analogues found in mosquitoes, play vital roles in both internal homeostasis and survival. Female mosquitoes require blood meals during their reproductive period mainly to obtain iron, ATP, and other essential nutrients necessary for embryo development.[1] An obvious drawback to this mechanism is that the pregnant female mosquito must quickly engorge from the large volume of fluid that temporarily increases her size and weight. Using simulated studies with the mosquito *Anopheles gambiae*, this increase in body weight and size has been shown to decrease survivability against predation.[2] Appropriately, Malpighian tubules deal with the high demand for expedient diuresis through pathways involving signaling peptides[3-4] coupled with epithelial tissue capable of efficient transport. Mosquitoes such as the yellow fever and malaria vector *Aedes aegypti* and *Anopheles gambiae*, respectively, are so efficient in diuresis that they can excrete urine while simultaneously drawing the blood of their prey.[5]

After a mosquito takes a blood meal, water and solutes pass from the midgut into the hemolymph by osmosis and active transport, respectively.[6-7] Through selective active transport of mainly sodium and potassium and passive transport of chloride and water, Malpighian tubules create a hemolymph-isosmotic primary urine.[8] This primary urine arrives in the rectum, where active reabsorption of sodium, potassium, and chloride occurs.[9-10] In *A. aegypti*, Malpighian tubules are comprised of two different types of cells: the large, cuboidal principal cells and the smaller, squamous stellate cells.[11] Through past tubule electrophysiology and tubule secretion

studies, the main pathway of entrance of potassium ions from the hemolymph of *A. aegypti* into the principal cell was determined to be a combination of (1) inwardly-rectifying potassium (K_{ir}) channels and (2) NaK2Cl cotransporters, both of which are located on the basolateral membrane of the Malpighian tubule.[12, 13, 14] Of interest to this pesticide study is the former, K_{ir}, pathway.

Traditionally, pesticides are modulators of channels in the insect central nervous system. In the mid-1950s, the World Health Organization adopted the strategy of diffusing large quantities of the insect neuronal sodium channel activator dichlorodipentyltrichloroethane (DDT) in malaria-plagued regions of Sub-Saharan Africa and South America with the goal of reducing disease transfer by targeting mosquitoes.[15] While this method was effective in the short-term, many mosquitoes have since then developed resistance to neurotoxins such as DDT and avoidance of the pyrethroid pesticides that were intended to replace DDT.[16] Bewilderingly, a recent study on the malaria mosquito *Anopheles gambiae* in the Ivory Coast demonstrated that the mosquito has resistance to all classes of approved insecticides.[17] Thus physiologically, behaviorally, and environmentally, the traditional neurotoxin strategy has presented major problems in pest management. To further complicate matters, regions of Mexico in recent years have become hyperendemic to dengue fever, also carried by *A. aegypti*. With the possibility of climate change, high-altitude major cities such as New Mexico—currently avoided by *A. aegypti* due to a cooler climate—could see a rise in dengue fever according to another recent study.[18]

The channel blocker VU573 and its molecular family would be the first pesticides developed to induce renal failure in the mosquito. They offer a novel strategy by targeting K_{ir} channels of the mosquito excretory system, rather than channels in the nervous system. VU573 is a proof-of-concept molecule, and the purpose of this study is to demonstrate the *in vitro* efficacy of a synthetic renal K_{ir} block. Theoretically, VU573 would be spiked in a sugar solution placed

in known mosquito breeding sites, and upon ingestion VU573 would inhibit *Ae*K_{ir}1 channels, thereby inducing renal failure. Preliminary studies with VU573 in the laboratory of Peter M. Piermarini show that *A. aegypti* injected with VU573 cannot excrete fluid. These large, swollen mosquitoes are unable to fly and later perish.[19]

Fig. 1. a. The chemical structure of VU573. Crucial to function are the benzyl (top) and aryl-ether (bottom) functional groups. When these key moieties are substituted experimentally, the molecule loses potency as a K_{ir} blocker. **b.** Control adult female *A. aegypti* mosquito injected with vehicle only. **c.** *A. aegypti* injected with VU573, displaying bloating. Mosquito images courtesy of the laboratory of Peter M. Piermarini.

VU573 and its analogues are synthesized in the laboratory of Jerod S. Denton of Vanderbilt University and tested via high-throughput screening. This method is based on the finding that potassium channels are even more permeable to the thallium cation than potassium.[20] Taking advantage of this unusual phenomenon, the Denton laboratory utilizes a custom thallium flux fluorescence bioassay to rapidly screen entire libraries of potential channel ligands. This test, detailed in Niswender *et al.*[21] measures the flux of thallium ions through heteromeric K_{ir}3.1/3.2 channels expressed in human embryonic kidney (HEK) cells in order to screen candidate blocker molecules, of which VU573 was one.[22] VU573 is a proof-of-concept molecule that has not yet been optimized to interact with only mosquito K_{ir} channels. Medicinal chemists at Vanderbilt will need to take additional steps to refine the structure of VU573 or its analogues if it is to be toxic to only mosquito targets and not to other unintended species.

The Malpighian tubule electrophysiology protocol developed in the laboratory of Klaus W. Beyenbach[23] is uniquely suited for a study on VU573, as isolated tubules continue secreting for several hours if left in Ringers solution.[24] For comparison with the VU573 block, the barium ion was selected, as a traditional nonspecific blocker of potassium channels. Barium as a K_{ir} channel blocker was characterized extensively in the 1980s by Brodwick and Eaton, who determined that the external barium K_{ir} block in squid axons operates in a 1:1 stoichiometric ratio and is reversible in high extracellular potassium concentrations.[25] For the latter reason, barium cannot be used as a renal block, as incoming flow from the hemolymph would remove it.

Although VU573 was studied at a concentration of 10µM, barium could not be studied at 10µM because it does not inhibit potassium channels at this concentration, a finding supported by both past and present work in the Beyenbach lab.[26-27] Instead, the concentration of 5mM peritubular Ba^{2+} was chosen based on previous electrophysiological studies in *A. aegypti* Malpighian tubules, showing that 5mM elicits the maximal number of K_{ir} channels to close.[28-29] Pivotal channel inhibition studies in other non-insect species have also utilized this concentration.[30-31] Thus, instead of comparing VU573 to an equal dose of barium, the 5mM barium concentration in this study will demonstrate the effect of a *total* K_{ir} block, to which VU573's block can be compared as a percentage. In this manner, the scope and efficiency of the block can be analyzed. Interestingly, using the nonsynthetic, inorganic barium ion also serves as a noteworthy comparison to the synthetic, organic VU573 molecule.

MATERIALS AND METHODS

Mosquito Preparation and Malpighian Tubule Dissection

Colonies of *A. aegypti* were reared in the lab by the protocol detailed in Pannabecker *et al.*[32] Before each experiment, female mosquitoes that were 3-7 days post-eclosion phase were

collected, cold-anesthetized for two minutes maximally, decapitated, and immediately transferred into Mosquito Ringers solution. While submerged in Mosquito Ringers, the thorax and second-most distal segmentation of the rectum were carefully separated with sharpened Dumont No. 5 forceps (Fine Science Tools, Foster City, CA, USA) to remove the digestive tract containing the five Malpighian tubules. Each tubule was dissected distally and subsequently transferred to the 450μL electrophysiology bath, also containing Mosquito Ringers.

Mosquito Electrophysiological Solutions

All Ringers solutions were prepared on the day of the experiment. Mosquito Ringers solution consists of 150mM NaCl, 25mM HEPES, 3.4mM KCl, 1.7mM $CaCl_2$, 1.8mM $NaHCO_3$, 1.0mM $MgCl_2$, 5mM glucose, and 15mM mannitol. The pH was set to 7.1 using aqueous NaOH. Two Ringers solutions modified for electrophysiological study are referred to as Hi-K^+ Ringers and Barium Ringers. Hi-K^+ Ringers contains a tenfold increase in KCl concentration from Mosquito Ringers, using instead 34mM KCl and 112.6mM NaCl. The sodium ion concentration is reduced to compensate for the potassium increase. Barium Ringers is simply Hi-K^+ Ringers with 5mM $BaCl_2$ in lieu of mannitol. The three solutions could be perfused into the 450μL electrophysiology bath through a valve system of polyethylene tubing.

Two different dosages of VU573 were tested. VU573 was stored at room temperature in a dessicator at 100mM in a 100% DMSO solution. Depending on the dosage, this stock VU573 was diluted appropriately in Mosquito Ringers so that the final in-bath concentrations of the added VU573 were either 10μM VU573 in 0.05% DMSO solvent or 50μM in 0.05% DMSO. For voltage trace barium studies, 50μL of a stock solution of 50mM $BaCl_2 \times 2H_2O$ was added into the 500μL bath, creating a final [Ba^{2+}] of 5mM.

Microelectrode Fabrication and Setup

Borosilicate glass capillaries were pulled on a P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Co., Novato, CA, USA) to form conventional microelectrodes. The capillaries, Kwik-Fil 1B100F-4 model (World Precision Instruments, Inc., Sarasota, FL, USA), have an outer diameter of 1.0mm and an inner diameter of 0.58mm. Each pulled microelectrode was subsequently backfilled with 3.0M KCl. A thin pre-chlorided silver wire was inserted into the back of each microelectrode and secured with a plastic holder, forming the connection to a GeneClamp 500 amplifier (Molecular Devices, Sunnyvale, CA, USA). The amplifier output splits to a MiniDigi 1A digitizer, used for the voltage trace protocol, and a Digidata 1440A digitizer, used for the voltage-clamp protocol (Molecular Devices, Sunnyvale, CA, USA). The connection was grounded with a chloride silver wire into the bath, shielded by a 3% agar Ringers solution. Useable electrode resistances ranged from 20-70 MΩ.

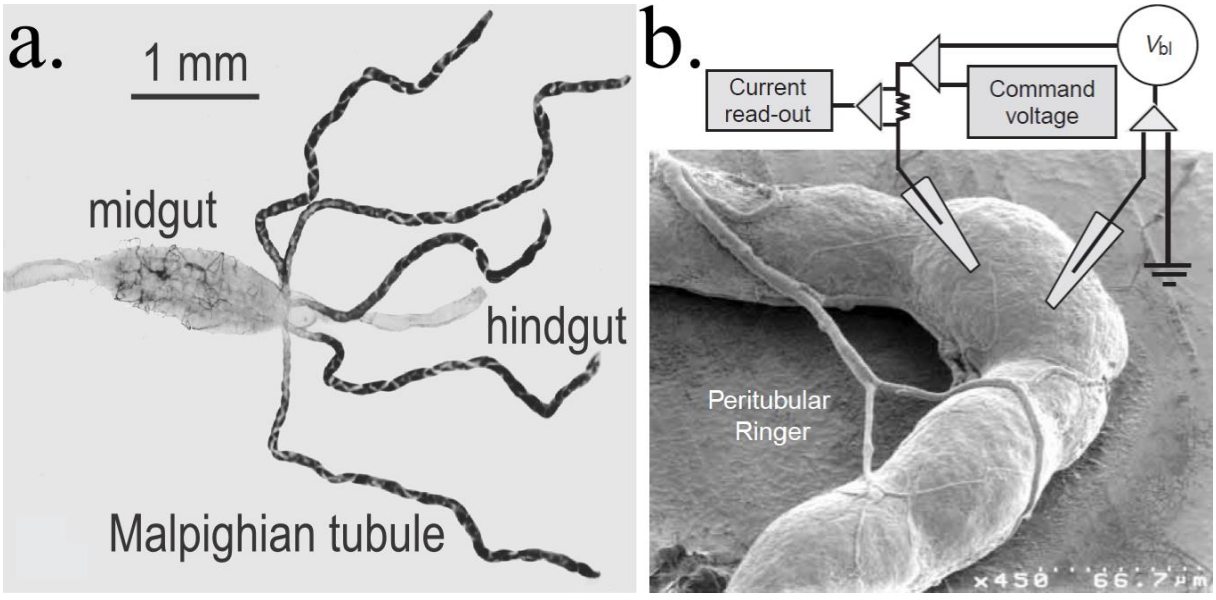

Fig. 2. a. Dissected midgut prior to Malpighian tubule removal for experimentation. **b.** Two-electrode voltage clamp (TEVC) setup and circuit diagram on an isolated Malpighian tubule. A single principal cell is impaled with two electrodes—one voltage sensing (right electrode) and the other current injecting (left electrode). Principal cells and stellate cells are electrically connected via low-resistance gap junctions. This setup can be used to measure basolateral membrane voltage and input resistance.

V*oltage Trace (VT)*

Only one electrode (voltage-sensing) is necessary for voltage trace experiments. The computer program Clampex (Molecular Devices Corp., Sunnyvale, CA, USA) was used for voltage recording. Both VT and TEVC follow the Malpighian tubule electrophysiology protocol developed in the Beyenbach lab. [33] Electrodes were gently lowered into solution by a micromanipulator and guided to a location directly over a principal cell, distinguishable by its engorged appearance and distinctly dark nucleus. A gentle tap on the micromanipulator was then sufficient to nudge the electrode into the cell, upon which the voltage drastically drops in the negative direction. Only immediate, nearly-vertical drops in voltage were taken to be successful impalements. If a stable voltage resulted after the drop, High-K^+ Ringers was perfused into the peritubular bath while Mosquito Ringers was simultaneously drawn out through a suction line. After a baseline voltage is seen in High-K^+ Ringers, 50µL of Ringers solution containing the dissolved molecule of interest is added into the bath from a micropipette—either VU573 or barium. Note that all concentrations listed are final concentrations in the peritubular bath, as the bath dilutes the standard 50µL addition one hundred-fold.

Two-Electrode Voltage Clamp (TEVC)

TEVC follows the same protocol as VT, except that a second (current-injecting) electrode is impaled into the same principal cell as the voltage sensing electrode. The computer program Axoscope (Molecular Devices Corp., Sunnyvale, CA, USA) was used for voltage recording, while Clampex was used to deliver the voltage clamp protocol. VU573 added to form a final concentration of 10µM. Ba^{2+} was tested by perfusion of Barium Ringers, which contains 5mM $BaCl_2 \times 2H_2O$. TEVC measures the amount and directionality of the current needed to stabilize the plasma membrane at pre-defined voltage step changes. Calculating the slope of these points,

with current represented on the y-axis and voltage on the x-axis, yields conductance, the mathematical inverse of resistance.

Data Analysis

All voltage and resistance data from the experiments were collected using the computer program Clampfit (Molecular Devices Corp., Sunnyvale, CA, USA). The resultant raw data were compiled and statistically analyzed with Microsoft Excel 2007 (Microsoft Corp., Redmond, WA, USA). In VT, each tubule served as its own control, as post-addition basolateral membrane voltage is always compared to pre-addition voltage for that particular tubule. Post-addition voltage was taken to be the peak or nadir voltage after addition. Time-to-peak values were also determined. In TEVC, tubules continued to serve as their own control, while peak or nadir resistance values were recorded. The pre-addition resistance value was taken as the average of the two pre-addition I-V plots. The post-addition resistance value was taken as the peak or nadir resistance value out of the 2-3 plots taken. Two-sample paired Student's T-tests were used to evaluate pre- and post-addition voltages and resistances, with a two-tailed α-value of 0.05.

RESULTS

The addition of VU573 elicited significant tubular effects. Peritubular addition of 10µM VU573 in Hi-K^+ Ringers hyperpolarized the Malpighian tubule basolateral membrane voltage (V_{bl}) by an average of 6.76 ± 1.27 mV, with significance at $p<0.00012$. The average control voltage was -45.12 ± 2.23 mV, and the average post-addition voltage was -53.00 ± 2.83 mV. The average time taken for the voltage to reach this trough (most negative) value was 217 seconds, suggesting that, at this concentration, VU573 can act as quickly as just over three-and-a-half minutes when directly exposed to the tubule via diffusion from the hemolymph. Accordingly,

cell input resistance (R_{in}) increases by an average of 7.02 ± 1.31 kΩ, with significance at $p<0.002$. The control input resistance was 227.90 ± 17.05 kΩ, increasing to 234.92 ± 16.39 kΩ with 10μM VU573. The interpretation of this small yet significant resistance increase will be discussed later.

Exposure to 5mM barium more starkly increased both the V_{bl} and R_{in}. Under barium, V_{bl} hyperpolarized from -47.40 ± 3.82 mV to -59.31 ± 3.05 mV, a change of 11.91 ± 2.75 mV with a significance of $p<0.008$. R_{in}, likewise, increased from 199.17 ± 27.26 kΩ to 322.75 ± 28.27 kΩ, with $p<0.0003$. This represents a comparatively enormous input resistance change of 123.58 ± 12.93 kΩ. Another set of trials was undertaken to confirm that VU573 had only a fractional effect of what barium had on the membrane voltage. If VU573 inhibits all K_{ir} channels fully, then adding barium should have no effect on the voltage because all channels are blocked. In this experiment, the control voltage was the baseline voltage seen after adding 10μM VU573, and the experimental voltage was the peak reading taken after adding 5mM barium with the VU573 remaining in the bath. Indeed, barium had its characteristic large effect, hyperpolarizing the basolateral membrane from -44.39 ± 1.87 mV to -62.85 mV ± 2.40 mV, confirming that either VU573 does not block all types of K_{ir} channels or that stellate cells do not contribute majorly to ionic transport.

The dosage dependence of VU573 was evaluated by testing and comparing 10μM and 50μM VU573. These VU573 concentrations indicate the final concentrations after a 50μL stock was added to the 450μL Hi-K^+ Ringers bath containing the isolated tubule. The average V_{bl} response of tubules exposed to 50μM VU573 is a hyperpolarization of 9.75 ± 1.55 mV, compared to the 9.44 ± 1.27 mV hyperpolarization seen with 10μM VU573. Statistically, this difference is not significant ($p=0.88$), suggesting that neither 10μM nor 50μM VU573 is the

minimal concentration to which *A. aegypti* Malpighian tubules will respond. Thus, VU573's target channels appear to reach saturation at the concentrations used in this study.

In all studies, each isolated tubule serves as its own control; the measurements prior to the addition of the molecule(s) of interest were compared to the post-addition measurements and statistically analyzed to determine significance. Since different Malpighian tubules exhibit different initial membrane voltages and input resistances, the average change is taken as the difference of the pre- and post-additions of all of the tubules measured in each study. Two-tailed paired Student's T-tests were used to evaluate the significance of the change. All measurements were taken as the tubule was bathed in Hi-K^+ Ringers, which is a modified Mosquito Ringers solution in which the concentration of potassium is increased tenfold in order to simulate conditions of potassium overload (from 3.4mM to 34mM) expected from a blood meal.

In this study, it was found that the electrophysiological phenomenon of rectification is present more strongly in some tubules than others. I-V plots taken of different tubules thus exhibit varying degrees of rectification or lack of rectification. I-V plots are derived by plotting the amount and directionality of the injected current needed to hold, or clamp, the membrane at particular predefined voltages. The slope of the best fit-line between these points yields the conductance, which is the mathematical inverse of resistance. In *Fig. 6*, the red and light blue data points form Ohmic I-V plots, in which current varies linearly with voltage as predicted by Ohm's Law. In contrast, the dark blue, purple, and green data points display rectifying I-V plots, in which the necessary current peaks and then holds when the cell is experimentally held at positive voltages.

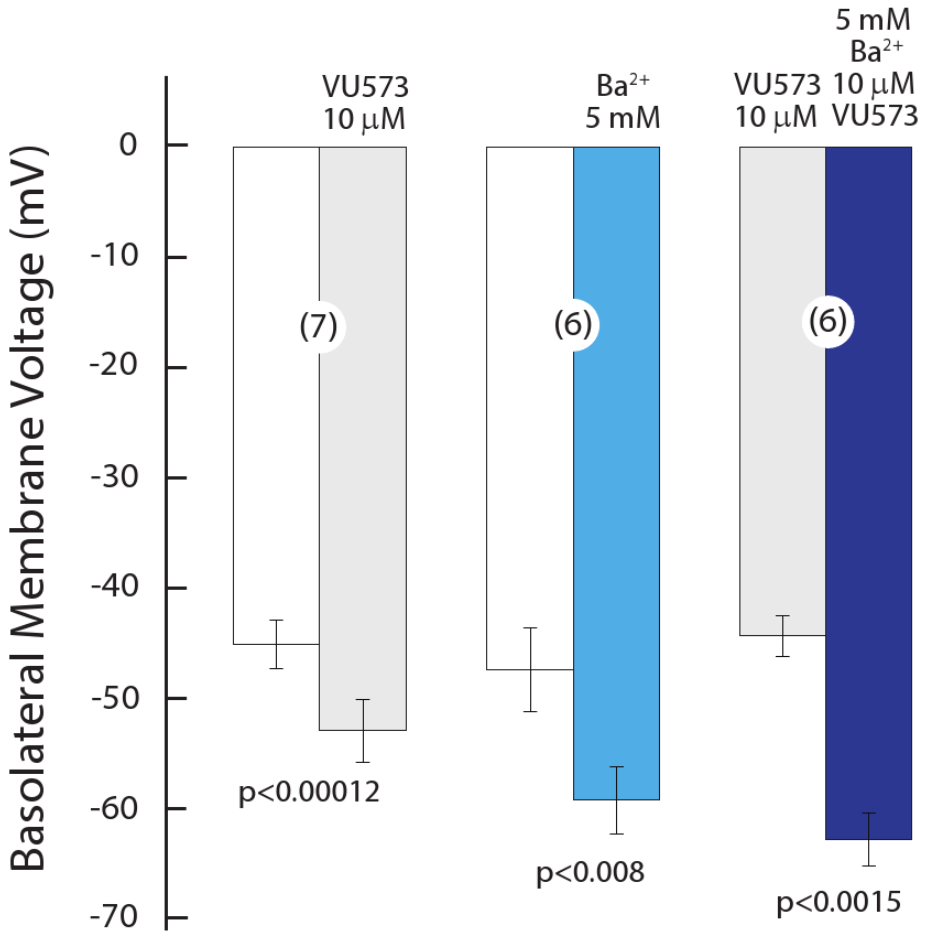

Fig. 3. Average basolateral membrane voltages in different conditions. Data presented as mean ± SE. White bars represent control readings, which are taken before any molecule is added to the Hi-K^+ Ringers bath that bathes the isolated tubule. In the first set of trials (N=7), 10μM VU573 was found to hyperpolarize the basolateral membrane by an average of 7.87 ± 1.31 mV. Likewise, in the second set of trials (N=6), 5mM Ba^{2+} hyperpolarized the membrane with an average of 11.91 ± 3.82 mV. In the third set of trials (N=6), 10μM VU573 was first added and measured, and at the voltage plateau, 5mM Ba^{2+} was added and measured. A hyperpolarization of 18.46 ± 2.93 mV was observed. Although the hyperpolarization in this third trial was larger than that of the Ba^{2+} trial, the difference between the trials is not statistically significant (p=0.13). Thus, VU573 does not appear to interact additively with barium.

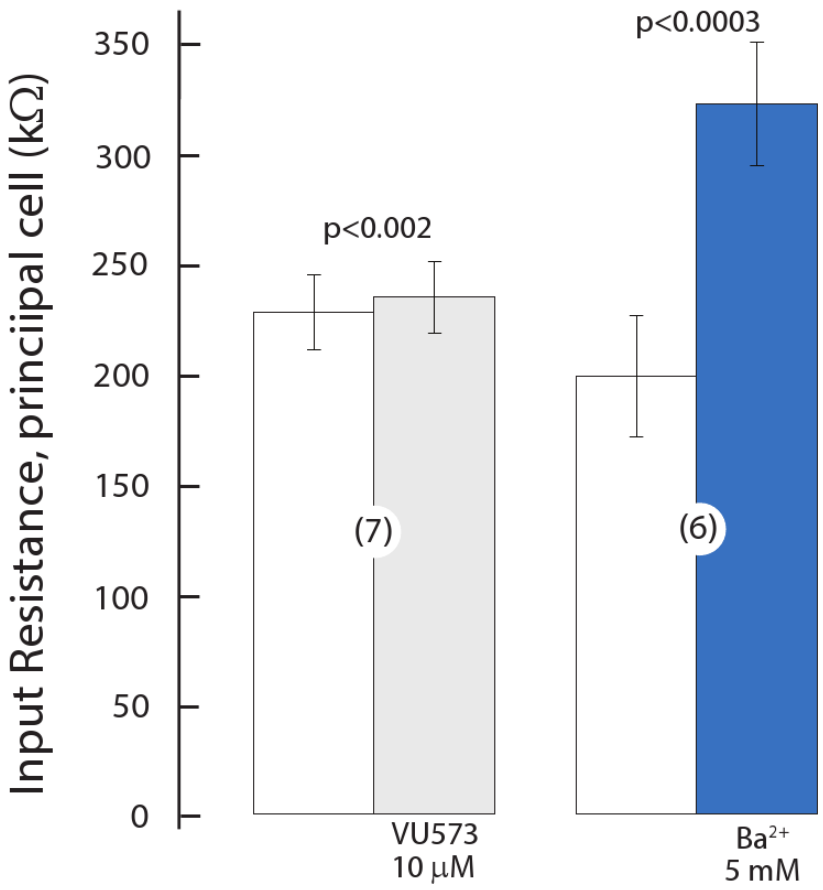

Fig. 4. Average input resistances of principal cells taken with the two-electrode voltage clamp technique in Hi-K^+ Ringers. Data presented as mean ± SE. White bars represent control readings. In the 10μM VU573 set of trials (N=7), a small yet very significant increase in resistance of 7.02 ± 1.31 kΩ was observed. Starkly contrasting this is the second set of trials (N=6), in which 5mM Ba^{2+} elicits an enormous resistance increase of 123.58 ± 12.93 kΩ. From these observations, it can be postulated that VU573 selectively affects a K_{ir} channel that does not contribute majorly to membrane ion traffic.

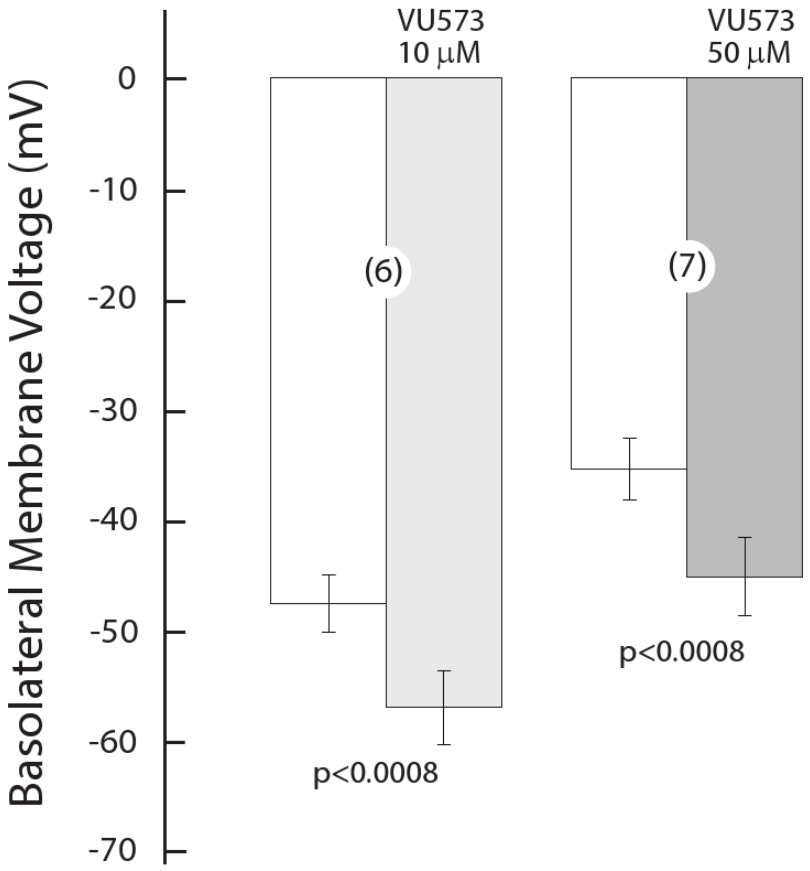

Fig. 5. Dosage response analysis of VU573 in Hi-K^+ Ringers. Data presented as mean ± SE. White bars represent the control voltage, taken before VU573 was added. In this set of trials, 10μM VU573 (N=7) and 50μM VU573 (N=6) hyperpolarize the basolateral membrane voltage by 9.45 ± 1.27 and 9.75 ± 1.55 mV, respectively. The difference between the distributions of these two voltage changes is not statistically significant (p=0.88), leading to the conclusion that both concentrations of VU573 elicit the maximal response.

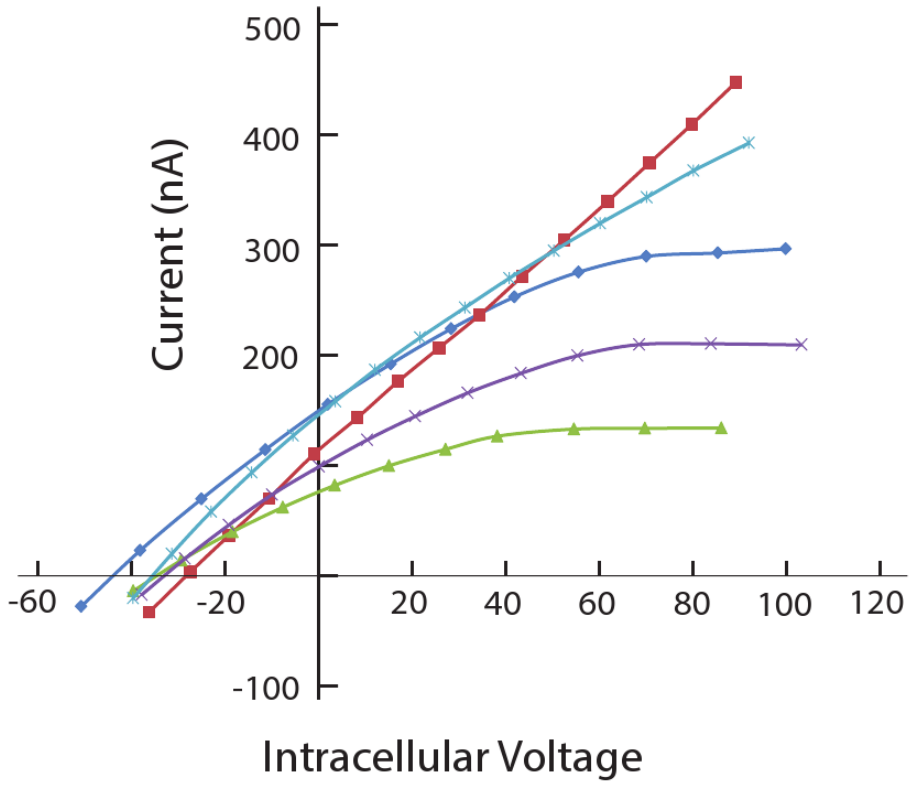

Fig. 6. Comparison of the shape of rectifying and Ohmic I-V plots of assorted Malpighian tubules in Hi-K^+ Ringers. In tubules exhibiting Ohmic I-V plots (red, light blue), current varies linearly with voltage as predicted by Ohm's Law. In tubules exhibiting strong rectification (dark blue, purple, green), the current plateaus at increasingly positive voltages. The source of this behavior is K_{ir} channels, which use an intracellular-originating block at positive voltages to prevent potassium from exiting the cell. In this study, not all Malpighian tubules have been found to exhibit overt rectification, suggesting that the contributions or expression of K_{ir} channels on the basolateral membrane varies from tubule to tubule. In addition, this finding explains why VU573 has such a small effect on membrane resistance; K_{ir} channels seem to contribute little to *direct* transport.

DISCUSSION

One of the five potassium channel families, the inwardly-rectifying potassium family is unique in that it shows a preference for the net inward flow of potassium ions, regardless of membrane voltage.[34] This behavior is due to the ability of endogenous intracellular magnesium ions[35] as well as polyamines—specifically spermine, spermidine, putrescine, and cadeverine[36]—to block the channel on the intracellular side when the cell is depolarized. In depolarization, the positively charged intracellular membrane would normally result in the expulsion of intracellular potassium cations, but magnesium ions and polyamines prevent this. In this manner, inward flow of potassium through K_{ir} channels is always greater than outward flow. In excitable cells, such a loss of potassium ion during depolarization would be especially dangerous to action potential conduction as well as the steady state of transport in the cell. In epithelia such as the Malpighian tubules, K_{ir} channels allow the basolateral membrane voltage to be held near the Nernstian potassium equilibrium potential.[37] I-V Plots taken through voltage clamping demonstrate that the basolateral membrane often exhibits such strong rectification (*Fig. 6*). Therefore, potential targets for this study are the known K_{ir} channels of A. aegypti, *Ae*K_{ir}1, *Ae*K_{ir}2, and *Ae*K_{ir}3.

Seven known subfamilies of K_{ir} channels exist, referred to simply as K_{ir}1 through K_{ir}7.[38] Like all potassium channels, K_{ir} channels allow only single-file progression of potassium ions, but multiple ions can occupy the channel at any one time.[39] Structurally, all K_{ir} channels are tetramers[40] and require the direct binding of PIP_2 for channel activation, though the level in which this cofactor is necessary differs[41]. Because K_{ir} channels lack the S1-S4 voltage sensing domain found in most other potassium family subfamilies, they are less sensitive to voltage than their 'cousins,' the voltage-sensitive potassium channels.

What is left to determine is which specific K_{ir} channel (or channels) VU573 blocks. Fluorescence immunolocalization studies in the lab of Peter Piermarini by Sonja Dunemann demonstrate that *Ae*K_{ir}1 channels are localized to the smaller stellate cells of Malpighian tubules[42] and not the large principal cells that are more heavily involved with transport. *Ae*K_{ir}1 channels likely contribute only minimally to observable potassium transport, since far fewer ions travel through stellate cells compared to principal cells. As per protocol, stellate cells were not impaled directly because their size is insufficient to accommodate microelectrodes. However, stellate cells appear to be electrically-coupled to principal cells through gap junctions, causing membrane voltages to be similar to those of principal cells, which were impaled. As such, the measured membrane voltage includes the contribution of stellate cells. Furthermore, *Ae*K_{ir}3 channels are not likely being targeted because Dunemann has shown that they are localized to principal cell nuclei. Even if VU573 is able to enter the principal cell and reach the nuclear membrane for an *Ae*K_{ir}3 block, it would not account for the observed hyperpolarization and resistance increase of the basolateral membrane.

A block of *Ae*K_{ir}1 channels is consistent with the results of this VU573 electrophysiological study and current model of the *A. aegypti* transport system developed in the Beyenbach lab. By blocking *Ae*K_{ir}1 channels located on the basolateral membrane of Malpighian tubules from the extracellular aspect of the channel, potassium would not be able to enter tubule cells from the hemolymph through these channels. The hemolymph (referred to as the basolateral or peritubular side) is the blood equivalent in arthropods. In mosquito studies, it is approximated using Mosquito Ringers. When VU573 is ingested by a mosquito, it would eventually arrive at the tubules through the hemolymph just as potassium would. Decreasing the influx of positive potassium ions into the cell would cause the basolateral membrane potential (V_{bl}) to become

more negative with time—a hyperpolarization. After application of VU573, a maximum V_{bl} hyperpolarization of 9.44 mV is observed, and this peak is reached in around three minutes post-addition.

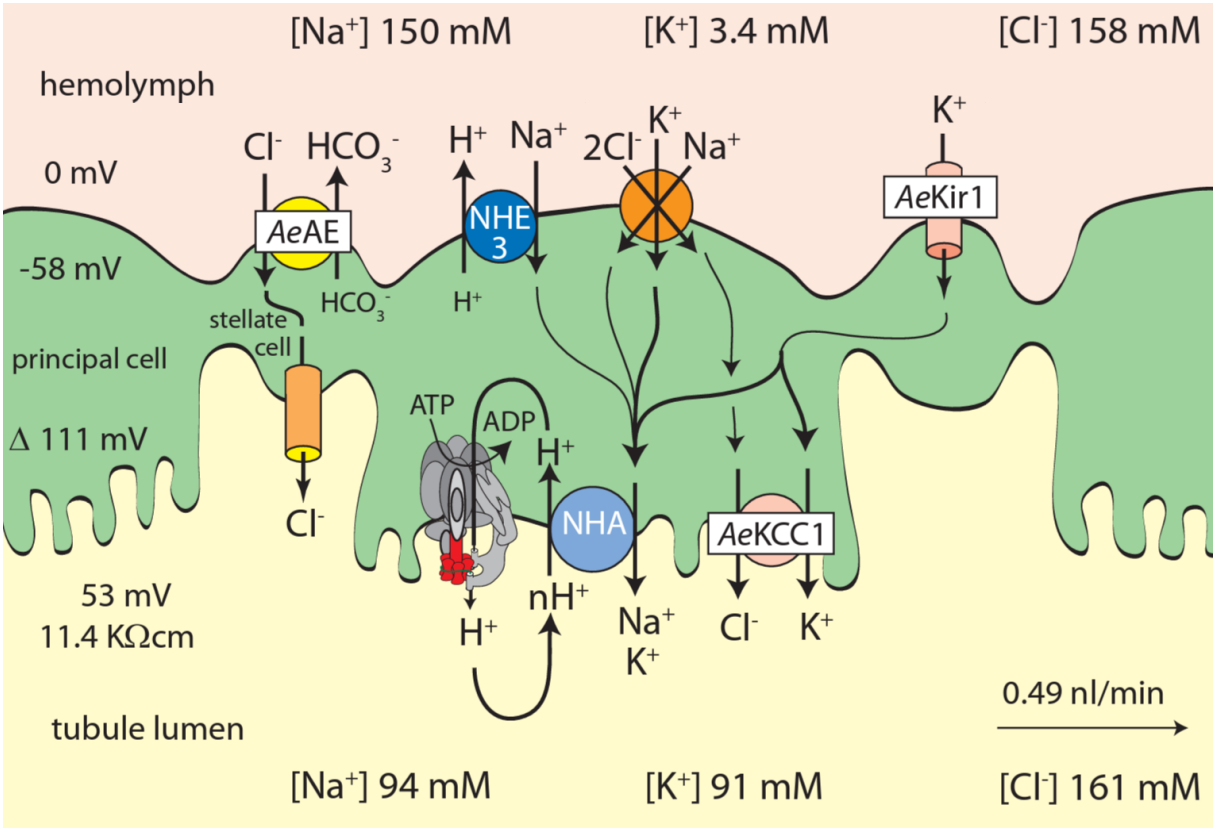

Fig. 7. Revised transport model of the Malpighian tubule, showing the *Ae*K_{ir}1 channel localized to the stellate cells, known from the work of Sonja Dunemann in the laboratory of Peter M. Piermarini (unpublished results). VU573 is theorized to block the AeK_{ir}1 channel at the basolateral aspect of the stellate cell. The relatively small resistance change, when compared to barium, also supports the finding of these channels on the stellate cell. In normal conditions, potassium can enter the cell through the NaK2Cl cotransporter or *Ae*K_{ir}1 channels. In experimental conditions, potassium is impeded from entering the cell through *Ae*K_{ir}1 channels. Low-resistance gap junctions (not shown) between stellate cells and principal cells allow nearly free ionic exchange. Stellate cells seem to be more important to transport than previously thought, with the recent findings of a Cl^-/HCO_3^- exchanger as well as the *Ae*K_{ir}1 channel to be located on the basolateral aspect of these cells.

To understand the meaning of membrane resistance, ionic transport can be likened to an electrical circuit, with membranes and channels functioning as resistors and concentration differences as *emf* sources, all in a series connection. Positive ionic flow constitutes current. When an ion's flow through a particular membrane channel is blocked, the input resistance of the

membrane R_{in} increases. VU573 increases R_{in} by an average of 7.02 kΩ, demonstrating its weak blocking capability. In comparison, the barium ion causes an enormous resistance increase of 123.58 kΩ, constituting a much more thorough block.

Barium is known to block all K_{ir} channels indiscriminately and totally at a concentration of 5mM. Thus, the percentage of K_{ir} channels blocked by VU573 could be estimated by dividing ΔR_{in} in the presence of 10uM VU573 by ΔR_{in} in the presence of 5mM barium. This calculation yields a blockage of 3%, suggesting that the *Ae*K_{ir} channels that VU573 appears to be blocking play a more minor role in potassium equilibrium, such as sensing or cellular 'housekeeping.' This does not go to say that these *Ae*K_{ir} channels do not contribute majorly to secretion in an indirect manner. In different species, potassium channels play a plethora of roles in addition to homeostasis and excitability, from pH and nutrient sensing[43] to volume regulation and even cellular migration,[44] as examples. Such suggestions can only be speculative, but the blocked *Ae*K_{ir} can be concluded as only a minor player in direct, measurable potassium transport because the channels that are blocked only account for only 3% of all potassium flow through K_{ir} channels.

The three studies together allow VU573's mode of action to be discerned. From the voltage study (*Fig. 3*), VU573 elicits a weaker response than barium, and when barium is added to tubules already exposed to VU573, the membrane further hyperpolarizes. These data suggest that VU573 certainly does not block *all* K_{ir} channels expressed on the basolateral membrane. In addition, the dosage comparison study (*Fig. 5*) suggests that VU573 at higher dosages than 10μM has no effect. This datum suggests that saturation has been reached with the concentration of 10μM VU573, and increasing the delivered concentration would not lead to a stronger block.

The resistance study (*Fig. 4*) highlights the very small effect of the VU573 block on input resistance, consistent with the finding of *Ae*K_{ir}1 to be expressed on stellate cells.

Combining the findings from all three studies, the current *A. aegypti* Malpighian tubule transport model, and the immunofluorescence findings of Dunemann of the Piermarini lab, it can be postulated that (1) VU573 selectively inhibits *Ae*K_{ir}1 channels on the basolateral membrane of stellate cells, (2) these *Ae*K_{ir} channels only play a small role in direct, measurable transport, and (3) there are likely more *Ae*K_{ir} channel types on the basolateral membrane not blocked by VU573. For the last two reasons, the VU573 family would need to be further refined by medicinal chemists to target a wider variety of *Ae*K_{ir} channels and induce a more potent antidiuretic effect.

The electrophysiological study of VU573 is only one aspect of a new assay for the study of future small molecule modulators, tying together the disciplines of medicinal chemistry, molecular pharmacology, entomology, and renal electrophysiology. Going through three screens at the 'theoretical' (high-throughput HEK cell screens), *in vitro* (Malpighian tubule electrophysiology), and *in vivo* (whole scale mosquito) levels, VU573 and other similarly-synthesized potassium channel blockers could be discovered, studied, and improved. Being a proof-of-concept, VU573 is only the beginning of a line of molecules that could theoretically be useful in many fields beyond *A. aegypti* and *A. gambiae* mosquitoes, from plant pathology to large-scale agriculture and from delousing to disease control.

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